Diverse protocols for correlative super-resolution fluorescence imaging and electron microscopy of cells and tissue

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Abstract
The nanometer-scale localization of target proteins/organelles/cells within tissue is critical to biology. Many factors determine optimal experimental design, including attainable localization precision, ultrastructural preservation of the sub-cellular environment, manageable fields of view, compatibility with three-dimensional (3D) samples, and ease of implementation. The advent of super-resolution fluorescence microscopy has greatly increased the precision with which emitters can be localized. Meanwhile, electron microscopy remains the gold standard for capturing details of the sub-cellular milieu, such as membranes, organelles and vesicles. Our groups have recently developed related approaches for super-resolution imaging within endogenous cellular environments using correlative light and electron microscopy (CLEM). Here we discuss the advantages of each method and provide detailed protocols. These protocols can be completed in 2-7 days and are broadly applicable across biology.

Introduction
Since the advent of microscopy, scientists have sought ways to provide contrast to tissues, cells, and subcellular structures in order to enhance visualization. At first, stains that delineated specific cellular populations within tissues were developed (e.g., Golgi stain for neurons), followed by methods to visualize sub-cellular objects (e.g., hematoxylin for nucleic acids). Concomitant improvements in sample processing and preservation were additionally developed to maximize utility for biological specimens. The invention of electron microscopy (EM) provided unprecedented nanometer-level resolution of cellular structures; on the other hand, it has suffered from low throughput and the limitations of current methods for providing protein-specific information. Therefore, light and electron microscopy largely diverged as separate modalities, each with distinct staining, sample processing, and imaging methods utilized.

The discovery and optimization of fluorescent proteins (FPs) like green fluorescent protein (GFP)\(^1,2\) reinvigorated light microscopy owing to the superb specificity of the label (i.e. genetic encoding: easy targeting of specific proteins, organelles, cell populations, and diverse organisms)\(^3\). A broad palette of FPs is available across the visible spectrum, and FPs routinely provide high contrast over specimen background autofluorescence. The main drawback to fluorescence microscopy compared to EM has historically been the resolution limit, i.e. the size of the smallest discernible structures in the sample. Using conventional microscopy under ideal conditions, fluorescence signals can only be localized to within ~200 nm, compared to the sub-nanometer resolution of electron microscopy.

Recent advances both in labels (i.e. the discovery and engineering of photoactivatable/photoswitchable FPs (paFPs)\(^4\)) and in microscope design and hardware have given birth to a new generation of fluorescence microscopy approaches with dramatically improved spatial resolution (<50 nm, and as low as ~10 nm under ideal conditions), collectively dubbed “super-resolution imaging”\(^5-10\). A major super-resolution imaging modality is “localization microscopy”, which relies on the serial determination of the location of individual emitting molecules. Localization microscopy techniques include Photoactivatable Localization Microscopy (PALM)\(^5\) and Fluorescence PALM (FPALM)\(^6\), which rely on paFP labels, and Stochastic Optical Reconstruction Microscopy (STORM)\(^7\) and direct STORM (dSTORM)\(^11\), which use photoswitchable small molecule dyes brought into vicinity of the target protein/structure via affinity reagents such as antibodies. Localization microscopy is slow relative to conventional microscopy. This typically necessitates the use of fixed tissue,
precluding studying live-cell dynamic processes, but is ideal for combination with EM, another fixed-sample technique.

The ability to capture and correlate complementary datasets from fluorescence and electron microscopy channels has been possible for decades. However, until the advent of super-resolution imaging, the resolutions of light and electron microscopy data were not well matched (~1 nm for EM compared to ~200 nm for light), making such endeavors mostly useful only for general feature identification. Additionally, traditional CLEM approaches can suffer from degraded sample/image quality in either one or both imaging modalities. Examples include: weak tissue preservation protocols resulting in poor ultrastructure, e.g. mitochondrial cristae membranes not resolved; strong tissue preservation protocols that destroy target fluorescence or create unacceptable autofluorescence; or protocols that are only applicable to very small samples, precluding critical experiments such as those on neurons and whole organs.

In the last several years, with advances in FPs, microscopes and preservation protocols, these failure modes are beginning to be systematically addressed. In this article, we provide protocols of several successful methods developed by our groups over the last several years for super-resolution fluorescence CLEM. The better match between the resolution of super-resolution fluorescence microscopy (~10 – 50 nm) and that of EM permits quantitative studies that go beyond general feature identification to the elucidation of sub-cellular and macromolecular complexes. Methods to develop correlative super-resolution fluorescence with cryo-electron tomography (CET) have also recently been described; in general, these experiments focus on very small samples.

To guide researchers to the super-resolution fluorescence CLEM technique most suitable for their specific question, we first describe some criteria through which the biological question may be filtered, keeping in mind that the most suitable technique may be one, none, or a combination of these techniques. We have left out of our discussion here a variety of other promising labeling techniques, such as electron density deposition by oxidases, immunolabeling EM (e.g. with hyper-antigenic tags), fusion to electron dense proteins, self-labeling enzymes such as Halo tag and SNAP tag, and interaction-dependent fluorogenesis, e.g. Point Accumulation for Imaging in Nanoscale Topography (PAINT).

Overview of the protocols

The protocols detailed here were developed independently to accommodate various biological questions but share many steps. In all cases the samples were initially fixed with aldehydes, to preserve sample integrity and prevent molecular movement. After this, the protocols diverge depending on the specific targets and questions. Super-resolution images were acquired in similar ways, the exception being if dye-labeled antibodies were used, in which case a reducing buffer was required to induce blinking (as in STORM/dSTORM). PALM/STORM image processing was the same in each case. Protocols for electron microscopy sample preparation and imaging diverge depending on the system. Registration of the PALM/STORM and EM datasets was then performed similarly.

Protocol 1. Tokuyasu cryosectioning

The original PALM paper used Tokuyasu cryosectioning but fine ultrastructural details were not observable. We improved the original protocol, in both sample handling and ultrastructural preservation, through several modifications. First, we began by increasing the amount of glutaraldehyde in the initial fixation step from 0.1% to 2%. The high level of
glutaraldehyde had no discernable effect on the fluorescent proteins or dyes used. However, the use of glutaraldehyde creates a prohibitive level of autofluorescence that can be mitigated by treating the samples with 0.5% sodium borohydride (an aldehyde “quencher”) prior to fluorescence imaging. After primary fixation with aldehydes, the sample is prepared using a standard procedure for Tokuyasu cryosectioning, including embedding in gelatin, infiltration with sucrose, sectioning the frozen sample, and retrieving the sample with a sucrose/methylcellulose solution. Sample sections are placed on 25 mm glass coverslips that: 1) have gold nanoparticles deposited, and 2) are coated with indium tin oxide (ITO) (Box 1). The gold nanoparticles (which are both fluorescent and electron-dense) are used to register fluorescence and EM data sets, and the ITO coating is required for charge conductance in scanning EM (SEM). Placing the sections on these glass coverslips allows optical and electron microscopy to be performed without transferring the sections to another substrate (i.e., EM grid), thus reducing sample handling damage and distortions. Using this method, we were able to correlate super-resolution fluorescence and electron microscopy images of diverse targets.

For our experiments, we used the fluorescent proteins mEos2 and photoswitchable cyan fluorescent protein 2 (PS-CFP2). Both of these proteins worked well for our studies, but additional photoactivatable protein derivatives have been developed since our initial work that may work better. We also found that caged small molecule dyes attached to the actin-labeling molecule phalloidin. A quick and easy test, and necessary control, to determine if your fluorescent label withstands primary fixation is to grow and label the cells in chambered coverslips. Examining the labeled cells by fluorescence microscopy, both pre- and post-fixation, provides insight as to how the fluorescent label withstands primary fixation.

Tokuyasu cryosectioning requires a specialized ultramicrotome with a cryogen attachment. Cryosections are picked up with a droplet of methylcellulose/sucrose solution and placed on a glass coverslip. The gelatinous methylcellulose/sucrose solution prevents the samples from drying out (for time periods less than 24 hours) but must be washed away with buffer prior to imaging. From this point forward until the final drying steps it is imperative that the sections remain in an aqueous environment. Incubating inverted coverslips on drops of 0.5% sodium borohydride for 15 minutes minimizes autofluorescence from glutaraldehyde and may also improve blinking properties of pFPs.

Three-dimensional iPALM uses dual objectives requiring that another coverslip be placed over the cryosection containing coverslip and sealed with epoxy and Vaseline. A thin layer of buffer is trapped between the two coverslips to ensure the sample remains hydrated. After PALM image acquisition, the coverslip sandwich is separated and the sample is placed in a small dish with buffer. At this point, staining depends on whether 2D or 3D electron microscopy will be performed.

If thicker sections (>250 nm) were used for 3D PALM imaging then serial focused ion beam milling/SEM (FIB-SEM) can be used to obtain 3D EM data from the sample. The preparation of thick Tokuyasu cryosections for FIB-SEM is relatively straightforward. Drops of methylcellulose containing 0.5% uranyl acetate (UA) are placed on a piece of Parafilm attached to an aluminum plate sitting on ice. The coverslip is inverted such that the section is down and placed on the methylcellulose drops. After an incubation of 15 minutes, the coverslip is dried by dragging the edge of the coverslip across filter paper. The coverslip may be held on its edge in contact with the filter paper until completely dry. Prior to FIB-SEM, the addition of cyanoacrylate directly on top of the sections aids ion beam milling. This is followed by carbon coating to avoid charging. FIB-SEM operates in a two-step cycle where a focused beam of Ga+ ions is used to mill a section.
ions mills a few-nanometer-thick layer to expose a new layer inside a sample, which is then imaged by SEM\textsuperscript{33}. This procedure is repeated thousands of times to form a 3D EM image stack. This stack is then registered to a 3D fluorescent image stack using Au nanoparticles, which are localized with high precision in both EM and fluorescent images (Figure 2).

Methylcellulose interferes with electron microscopy for thinner sections, causing the image to appear hazy. This is not an issue with FIB-SEM since the methylcellulose layer is cut through and the section is imaged from the side. Thus, for non-FIB-SEM, more extensive staining is needed to provide greater electron-dense contrast. After PALM imaging, the coverslip is placed in a small Petri dish in which all subsequent staining is performed. The sample is treated with 2\% osmium tetroxide (OsO\textsubscript{4}) reduced with 1.6\% potassium ferrocyanide (K\textsubscript{4}[Fe(CN)\textsubscript{6}]) for 15 minutes. Next, the sample is incubated with 0.6\% UA in polyvinyl alcohol for 15 minutes. Finally, the sample is incubated with 0.0075\% Sato’s triple lead (citrate, acetate and nitrate salts of Pb\textsuperscript{2+}) in polyvinyl alcohol. The coverslip is placed on a spinning slide drier and dried for 10 seconds at 5000 rpm. Polyvinyl alcohol acts in a similar manner to methylcellulose, by reducing surface tension effects during sample drying that may result in artifacts\textsuperscript{44}. Samples dried in the presence of polyvinyl alcohol do not have the haziness observed when methylcellulose is used.

**Protocol 2. Whole-cell mount**

The whole-cell mount correlative imaging protocol provides a relatively simple and convenient method for obtaining images of membrane surface topologies, while providing molecular specificity for surface molecular assemblies. This protocol was inspired by previous work using standard diffraction-limited fluorescence microscopy and correlative SEM imaging\textsuperscript{45}. This protocol does not require mechanical sectioning of the specimen and as such does not require any special preparation of the sample prior to standard fluorescence microscopy imaging. As this protocol also does not necessitate strong fixation of the specimen prior to fluorescence imaging, many standard fluorescent proteins used for PALM can be utilized without significant loss of fluorescent signal. This protocol also requires very little sample preparation for SEM following fluorescence image acquisition. Briefly, the cellular specimen is placed on coverslips, allowed to express the probes of interest, and fixed using paraformaldehyde and a low concentration of glutaraldehyde. The specimen is then imaged using fluorescence super-resolution protocols. Upon acquisition of super-resolution datasets, the specimen is post-fixed with glutaraldehyde and osmium. Optionally, the sample can be further fixed with successive rounds of OsO\textsubscript{4} and thiocarbohydrazide (“OTOTO”) to improve membrane contrast and stability\textsuperscript{46}. The specimen is dehydrated using an ethanol dehydration series and critical point drying (CPD)\textsuperscript{47}. The surface topology of the specimen is then visualized using SEM. It is important to note, however, that SEM only provides surface information, precluding visualization of intracellular membranes and structures using the whole-cell mount procedure. This technique typically provides only 2D information about surface specimen features. An important consideration for implementation of this protocol comes from the observation that large cellular specimens (high mass objects) can be displaced due to the drying procedure for SEM observation. Care must be taken to meticulously follow each dehydration step in the protocol to ensure movement of the specimen does not occur. The optional OTOTO protocol, however, can often mitigate specimen movement. This protocol describes the use of cellular (tissue culture) specimens for interrogating surface topologies and associated molecular assemblies involved in membrane remodeling.
Protocol 3. Platinum replica TEM/unroofing

Platinum replica TEM of unroofed cells is a high-contrast method well suited for observing the topography of the inner surface of the plasma membrane. In these images the spatial organization of membrane events (e.g. endocytosis and exocytosis) can be viewed in an en face view with high resolution. Unfortunately, using immunogold methods to locate proteins in these replicas is difficult due to the inherently high contrast of the platinum (Pt) coating, which thus necessitates the use of sterically bulky 15 nm gold particles to achieve visibility over the Pt coating. To overcome these issues, we developed a correlative super-resolution localization microscopy technique to provide an alternative to immunogold labeling in platinum replicas. This was important in our studies of clathrin-mediated endocytosis, where the clathrin meshwork and associated proteins likely produce a steric impediment to large immunogold probes. Furthermore, the accessibility of the unroofed sample means that in addition to FP fusions, added antibodies and self-labeling enzyme substrates typically provide high label density.

In this method, we grow cells on gold nanorod embedded coverslips, fix with paraformaldehyde (PFA), and gently sonicate the specimen to remove the top of the cells (unroofing). These thin membrane sheets have exposed and buffer-accessible cytoplasmic faces. Super-resolution microscopy can then easily be performed on these samples with nearly any fluorescent label that is compatible with the user’s biological system. Alexa Fluor 647, Alexa Fluor 750, mEos3/mEos4, and PS-CFP are our labels of choice due to high sampling and photon outputs. After fluorescence imaging, the sample is further stabilized with glutaraldehyde, tannic acid, and UA prior to ethanol dehydration and CPD. The dry sample is coated with Pt and carbon to make a rigid replica of the sample that can be transferred to a TEM grid for imaging. This method provides highly reproducible correlation with 20 nm accuracy across the 20 µm-wide landscape, which can be directly observed in many cases if using immunofluorescence because the antibody assemblies are often large enough to be visible in the EM micrograph. This robust correlation owes to the tight adherence of the thin sample to the coverslip during the dehydration and CPD of the sample in preparation for EM and makes it an especially trusted method for finding unknown positions of single proteins. However, this method requires physical disruption of the cell by sonication during fixation, which disrupts the cytoskeleton and washes away cytoplasmic components. Therefore, this method is specific to membrane-bound systems or other thin systems tightly adhered to a coverslip.

Protocol 4. Resin embedding

Because thick volumetric samples are incompatible with cryosectioning, embedding into an easily sectioned plastic resin remains the best option for these samples. Many standard resin options exist from water-incompatible Epon epoxy resins to hydrophilic resins such as glycol methacrylate (GMA), LR Gold/White, and Lowicryl resins (e.g. K4M and HM20). Epon is typically preferred for EM because of its superior ultrastructure preservation and sectioning properties. Epon, however, requires complete sample dehydration and epoxy polymerization, which can extinguish fluorescence and render antibodies unable to bind antigens. Of note, it is possible that self-labeling enzymes such as Halo/SNAP tag might still function in Epon-embedded tissue if the enzymes remain active. In contrast, hydrophilic resins generally preserve the function of proteins including FPs and antigens better than hydrophobic resins but lack the strong cross-linking of Epon. This results in weaker samples that are more sensitive to electron beam damage.
Correlative super-resolution/EM imaging in plastic sections was first shown with PALM and stimulated emission depletion (STED) microscopy in both GMA and LR White/Gold resins. In this study, GMA was selected as the optimal resin due to its retention of FP fluorescence and homogeneous polymerization. However, due to the weak fixation conditions used (0.1% potassium permanganate and 0.001% OsO₄, no aldehydes) necessary to retain fluorescence, ultrastructure preservation was poor compared to that achievable with much higher OsO₄ concentrations.

Other protocols have advanced the use of the acrylic resins. In this example, Lowicryl HM-20 infiltrated samples could be sectioned and antibody labeled for dSTORM-type super-resolution imaging followed by UA staining for SEM. A related protocol retains switching of fluorescent proteins. Another protocol discusses STORM dyes in various resins.

We set out to optimize the protocol of Watanabe et al., both through the systematic exploration of fixation cocktails and through protein engineering of mEos2 to better resist fixatives, in particular OsO₄, while retaining fluorescence and photoconvertibility. We first began by decreasing the amount of water used in the GMA resin mix, which slightly improved ultrastructure preservation without affecting FP fluorescence. Second, we systematically explored combinations of primary fixatives, optimizing for EM ultrastructure, preservation of mEos2 fluorescence properties and low autofluorescence. This led to the selection of 4% PFA + 0.2% glutaraldehyde as primary fixatives before secondary fixation and embedding. Use of aldehyde quenchers such as borohydride before imaging decreased the background fluorescence attributable to glutaraldehyde polymerization. Finally, we mutated surface residues on mEos2 to remove nucleophilic groups, which are involved in cross-linking with aldehydes and OsO₄. This resulted in the selection of two mutants, mEos4a and mEos4b, each with significantly improved resistance to OsO₄ fixation and fluorescence properties unchanged from the starting scaffold mEos2.

These proteins facilitated the development of two protocols: 1) a “consecutive-section” approach where adjacent ultra-thin sections cut from resin are separately split between PALM imaging and EM fixation and imaging; and 2) a “same-section” approach, where a single resin-cut section is subjected to both PALM and TEM/SEM. In both cases, plastic resin embedding dramatically decreases tissue distortion from dehydration and secondary fixation, and additionally improves performance of the specimen under the electron beam.

Choice of protocol
The choice of protocol depends both on the biological question and available equipment. There are hundreds of linear imaging pipelines that can be combined from preparation and imaging options for super-resolution optical and electron microscopy. Here, we present a small but diverse sampling of optimized and tested protocols that illustrate the relative advantages and limitations of these techniques. The protocols described here vary in sample preparation and the data they allow one to collect (summarized in Table 1).

At one limit, the biology may present itself on the cellular surface and two protocols cover this situation well. One protocol is suited to image the outer morphology of the surface of a whole cultured cell. This approach of PALM-SEM is relatively simple to implement and yields qualitative assessment of membrane curvature as a function of probe localization. The other protocol images the rich morphology of a mechanically exposed surface of the inner cell membrane. This correlative 2D PALM-TEM of a platinum replica of the plasma membrane can
be extended to 3D with iPalm-Electron Tomography (ET). Thus, it is a powerful tool for studies of endocytosis or exocytosis (and potentially other processes) on membranes. The membrane environment of isolated organelles such as nuclei and mitochondria could also be investigated.

If the biological target is not already or cannot be exposed on an open surface then some form of mechanical sectioning is needed to expose and image the interior of a cell or tissue by EM and localization microscopy techniques operating within the total internal reflection fluorescence (TIRF) regime (~200 nm). The two main alternatives are Tokuyasu cryosections and plasticized sections. The former is faster and simpler to implement (Tokuyasu protocol can be performed in 1-2 d), but the ultrastructure preservation may be not as good as with plasticized sections. The latter is more time-consuming but may offer better ultra-structure preservation, and is also compatible with serial sectioning. Another very important consideration when deciding between these protocols is the labeling strategy. The Tokuyasu technique generally permits milder fixation and thus allows a wider choice of fluorescent labels. In order to have good ultrastructure preservation in plasticized sections, staining with heavy metals such as OsO₄ is desirable, which significantly limits the choice of labels that can preserve fluorescence under such stringent fixation. The choice of resin used for embedding may also be limited by how well fluorescence is preserved. Acrylic resins, such as LR White, GMA, and Lowicryl have been successfully used for CLEM purposes, while epoxy resins, such as Epon, remain challenging.

Two protocols are based on Tokuyasu cryosectioning. One focuses on simplicity and speed of implementation, while retaining 2D correlative images with PALM-SEM. The other, with a significant extension of equipment sophistication, time and effort, achieves 3D correlative imaging on thick cryosections. The final protocol illustrates how high-quality PALM imaging can be extended to sections of plastic embedded specimens, showing excellent EM preservation. This was enabled by developing a customized photoactivatable fluorescent protein that survives these harsher preparation conditions.

In deciding which protocol to use or develop there are several considerations:

*x-y resolution.* This protocol describes methods for super-resolution localization (~50 nm), although users may substitute typical confocal, epifluorescence or TIRF microscopy if lower resolution is sufficient. Additionally, if very precise registration between light and EM modes is required, then the same sample section should be used in both light and electron microscopy, ideally with fluorescent, EM-dense gold particles added, and with minimal manipulation (e.g. secondary fixation, staining) between light and EM imaging. If lower registration precision is acceptable then non-identical (i.e. consecutively cut, adjacent) sections can be used, or LM can be performed before samples are further processed for EM, in which case samples should be processed in situ.

*z-resolution.* Many biological problems can be investigated in thin sections, reducing CLEM to 2D. This is attractive because one could use simpler and more widely available equipment. Also, sample distortion is minimized. If high z-resolution is required in 3D samples such as thick sections, then iPalm or other 3D-super-resolution fluorescence methods can be combined with electron tomography, FIB-SEM, or serial sectioning of resin-embedded specimens (plasticized samples can also be imaged under FIB-SEM). iPalm provides the highest z-resolution (~10 nm) of current super-resolution fluorescence methods. Electron tomography and FIB-SEM provide high z-resolution (5-10 nm) in electron microscopy. The z-
resolution of serial sectioning is dependent on the thickness of the sections (generally limited to >30 nm).

**Sample size.** Sample sectioning allows for greater fields of view than FIB milling, which is generally limited to 300 µm x 100 µm. Imaging tissues with a large z-depth (mm) is best performed through a serial sectioning procedure using resin-embedded specimens where one can cut reliable sections over a large volume. Here an array tomography-type approach can be taken whereby sections are placed on a coverslip, imaged in fluorescence mode, and then imaged by SEM (or possibly TEM). Commercial solutions to automate LM/EM image acquisition are becoming available, such as the Zeiss Shuttle & Find SEM system. Serial-sectioning could theoretically be used to image very large samples such as brain regions. Loss of material can occur upon mechanical sectioning, however, which can yield imperfect sampling along volumes of a specimen.

**Degree of ultrastructural preservation.** Although all of the protocols here provide adequate ultrastructural preservation for most applications, the researcher must weigh whether it is sufficient for their specific application. Some targets with high-labeling density may withstand harsher fixatives and stains that better preserve ultrastructure, but low-copy number targets may require gentler methods to preserve fluorescence while retaining an appropriate level of ultrastructure.

**Location of a target within cells or tissues.** If targets are located at the outer surface of the plasma membrane, then using whole cells grown and imaged on coverslips may be the best option. If the target is on the inner-leaflet of the plasma membrane, then a specialized technique such as “unroofing,” which removes the body of the cell, may be the best option. Purified nuclei or mitochondria, for instance, could also be immobilized and observed at the tight coverslip interface. Visualizing internal sub-cellular components more broadly requires some form of sectioning.

**Processing time.** The Tokuyasu cryosectioning and whole-cell mount protocol achieve the fastest turnaround time (~2 days) of the methods discussed here.

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**CONSTRUCT AVAILABILITY**

Constructs encoding mEos4a and mEos4b, as well as a variety of fusions, are available at Addgene (addgene.org).

**SOFTWARE AVAILABILITY**

Software is commercially available.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing interests.

UNCLASSIFIED
MATERIALS

REAGENTS

- 16% Paraformaldehyde (PFA), aqueous (Electron Microscopy Sciences, cat. no. 15700)
  !CAUTION Paraformaldehyde is a tissue fixative. Handle it with appropriate protective clothing, gloves, and goggles. Work in an appropriate fume hood.
- 50% Glutaraldehyde (GA), aqueous (Electron Microscopy Sciences, cat. no. 16320)
- 8% Glutaraldehyde (Electron Microscopy Sciences, cat. no. 16000)
  !CAUTION Glutaraldehyde is a strong tissue fixative. Handle it with appropriate protective clothing, gloves, and goggles. Work in an appropriate fume hood.
- Sodium borohydride (Sigma-Aldrich, cat. no. 480886)
- 0.2 M Sodium cacodylate buffer, pH 7.2 (Electron Microscopy Sciences, cat. no. 11652)
- Agarose (Sigma-Aldrich, cat. no. A9539)
- Bovine serum albumin (BSA), lyophilized powder (Sigma-Aldrich, cat. no. A2153)
- 4% Osmium tetroxide, aqueous (Electron Microscopy Sciences, cat. no. 19150)
  !CAUTION Fumes of osmium tetroxide are toxic; open the reagent only in an appropriate fume hood
- Osmium tetroxide, crystalline (Electron Microscopy Sciences, cat. no. 19110)
  !CAUTION Fumes of osmium tetroxide are toxic; open the reagent only in an appropriate fume hood
- Ethanol, 200 proof (Electron Microscopy Sciences, cat. no. 15055)
- Acetone (Electron Microscopy Sciences, cat. no. 10012)
- Glycol methacrylate resin (SPI, cat. no. 02626-AB)
- Butyl methacrylate resin (SPI, cat. no. 02822-BA)
- Benzyol peroxide catalyst (SPI, cat. no. 02825-CA)
- N,N-Dimethyl-p-toluidine (Sigma-Aldrich, cat. no. D189006-100ml)
- Uranyl acetate (Electron Microscopy Sciences, cat. no. 22400)
  !CAUTION Uranyl acetate is an alpha emitter. Uranyl acetate is a cumulative toxin. Wear protective clothing, gloves, and goggles.
- Hexadecene (Sigma-Aldrich, cat. no. H2131)
- Gold 80 nm nano-spheres (Corpuscular, cat. no. 790120-010)
- Gold bare nanorods 25 x 57 nm (Nanopartz, cat. no. A12-25-600) or 25 x 75 nm (Nanopartz, cat. no. A12-25-700)
- Pioloform (Ted Pella cat no., 19244)
- 1,2-Dichloroethane (Sigma-Aldrich, cat. no. 284505)
- Lead citrate (Electron Microscopy Sciences, cat. no. 17810)
  !CAUTION Lead citrate is toxic; use the reagent in a hood and wear gloves while handling.
- Lead acetate (Electron Microscopy Sciences, cat. no. 17600)
  !CAUTION Lead acetate is toxic; use the reagent in a hood and wear gloves while handling.
- Lead nitrate (Electron Microscopy Sciences, cat. no. 17900)
  !CAUTION Lead nitrate is toxic; use the reagent in a hood and wear gloves while handling.
- Sodium citrate (Electron Microscopy Sciences, cat. no. 21140)
- 49 % Hydrofluoric Acid (Fisher Scientific, cat. no. A147-1LB)
  \textbf{CAUTION}. Hydrofluoric acid is toxic. Use in chemical hood with gloves. Contact with skin requires immediate medical attention.
- Sodium hydroxide (Sigma-Aldrich, cat. no. S8045)
- Poly-L-lysine hydrochloride (Sigma-Aldrich, cat. no. P2658-100MG)
- Hydrogen peroxide, 50% (Fisher Scientific cat. no. H341-500)
- Ammonium hydroxide, 29% wt/wt (Fisher Scientific cat. no. A669-500)
  \textbf{CAUTION} ammonium hydroxide is toxic; use the reagent in a hood and wear gloves and goggles while handling.
- Sucrose (Sigma-Aldrich, cat. no. S7903)
- Glycerine (Sigma-Aldrich, cat. no. 50046)
- Gelatin from porcine skin, Bloom 90-110 (Sigma-Aldrich, cat. no. G6144)
- Phosphate buffer, pH 7.4
- Phosphate buffered saline, pH 7.4
- Potassium hexacyanoferrate(II) trihydrate, \textit{a.k.a.} potassium ferrocyanide (Sigma-Aldrich, cat. no. P3289)
- Methylcellulose, 25 centipoises (Sigma-Aldrich, cat. no. M6385)
- Polyvinyl alcohol (Sigma-Aldrich, cat. no. S8045)
- Tannic acid (Mallinckrodt cat. no. 1764)
- Carbon dioxide with syphon tube (United Oxygen Company cat. no. CO250ST)
- 10 nm gold if making a 3D EM tomogram. We use a 1 in 5 dilution of 10 nm gold anti-rabbit conjugate (Cytodiagnostics cat. no. AC-10-01).
- Molecular sieve (Sigma-Aldrich, cat. no. 208574)
- Hexamethyldisilazane (EMS cat. no 16710; optional)
- Potassium chloride (Sigma cat. no. P9541)
- HEPES (Sigma cat. no. H3375)
- Magnesium chloride (Sigma cat. no. M8266)
- KOH (Sigma cat. no. H3375)
- EGTA (Sigma cat. no. E3889)
- Glucose oxidase (Fisher Scientific cat. no. ICN19519650)
- Catalase (Fisher Scientific cat. no. S25239A)
- 2-mercaptoethanol (Sigma Aldrich cat. no. 63689-25ML-F)
  \textbf{CAUTION}. 2-mercaptoethanol is toxic. Use in chemical hood with gloves and seal before removal.

**EQUIPMENT**

- Bench-top micro-centrifuge
- Rotating platform (Electron Microscopy Sciences, cat. no. 61050-10)
- Wohlwend HPF Compact 01 high-pressure freezer (Techno Trade)
- HPF specimen carrier (TechnoTrade, Type A 0.1/0.2 mm, Type B flat)
- Freeze substitution unit (Leica, EMAFS2)
- 25mm #1.5 coverslips (Werner Instruments)
- Heating plate with magnetic stirrer
- Sputter–deposition system (Denton Explorer 14, Denton Vacuum)
- Glow discharge unit (Ted Pella, cat. no. 91000 or Pelco EasiGlow)
• Synaptek slot grid (Ted Pella, cat. no. 4514)
• Ultra-microtome (Leica, EM UC6)
• Cryo-chamber for ultramicrotome (Leica, EM FC7)
• 120-200 kV transmission electron microscope (FEI, Tecnai 20)
• Jeol 1400 transmission electron microscope equipped with a high tilt specimen retainer (JEOL EM-21311HTR) and Serial EM Freeware
• Zeiss Merlin or Zeiss Sigma field-emission scanning electron microscope fitted with a focused ion beam gun (FEI).
• Biosafety cabinet
• Chemical Fume hood
• Diamond trimming knife (Diatome, TrimTool 45, cat. no. TT-45)

**CAUTION** Diamond knives are extremely sharp and fragile. Care must be taken not to touch the knife-edge to protect both user and the knife.

• Cryo immuno diamond knife (Diatome, cat. no. DCIMM3530)

**CAUTION** Diamond knives are extremely sharp and fragile. Care must be taken not to touch the knife-edge to protect both user and the knife.

• Perfect loop (Electron Microscopy Sciences, cat. no. 70944)
• Vacuum slide spinner
• StatSpin™ DiffSpin™ 2 Slide Spinner (Fisher Sci. cat no. 22-314143)
• Aluminum cryo specimen pins (Electron Microscopy Sciences, cat. no. 70446)
• Cell scraper (Research Products International Corp., cat. no. 162423)
• Cryogenic vials (Sigma-Aldrich, cat. no. CLS431416)
• Sonicator: We use a Branson Sonifier 450 (VWR International 47727-492) with a 1/8" tapered microtip (VWR International cat. no. 33996-163)
• Critical point dryer (Tousimis Samdri cat. no. 795)
• Freeze fracturer (Jeol JFD-V)
• Glow discharger (Pelco EasiGlow)
• Diamond objective marker m25 thread (Leica 11505059) ^CRITICAL. You will most likely need to make an adaptor or spacer to fit this objective marker to your microscope. We have machined a 14 mm spacer tapped with m25 to receive the marker and m25 threading to fit into our Nikon NSTORM turret.
• Attofluor cell chamber (Life Technologies cat. no. A-7816), if using a commercial microscope stage. This is only for STORM/dSTORM. You may use any other coverslip chamber or slide but we find that this chamber has a useful upper lip where an additional coverslip can sit and seal in the STORM blinking buffer.
• Platinum 4 mm Inoculation loop (Electron Microscopy Sciences cat. no. 62433-04)
• Diamond scriber (Electron Microscopy Sciences cat. no. 62108-ST)
• Single edge razor blade (Fisher Scientific cat. no. 12-640)
• Formvar/carbon coated 75 mesh copper TEM grids (Ted Pella cat. no. 01802-F)
• Filter paper (Whatman cat. no. 1001-042)
• Critical point drying sample holder. We machined our own coverslip holder that looks very similar to Tousimis cat. no. 8767 but has an extended diameter to accommodate the 25 mm coverslip. Spare wavy washers (Tousimis cat. no. 8767-01) should be used as coverslip spacers.
• Two 100 ml beakers with a metal mesh shelf that will accommodate the critical point drying sample holder on top and a stir rod on the bottom
• Magnetic stir plate
• 12-well plate (Corning cat. no. 3512)
- Plastic bulb transfer pipettes (Fisher cat. no. 13-711-7M)
- IMOD freeware software
- Super-resolution fluorescence microscope. Can be custom-built or one of several commercially available models such as ELYRA (Zeiss), NSTORM (Nikon), Leica GSD (Leica) Vutara 352 (Bruker). Commercially available models all have differences in their abilities, including 3D capabilities.
- Upright light microscope equipped with 10x phase objective and camera. We use a Zeiss Axioplan 2 with a Zeiss A-Plan 10x / 0.25 NA Ph1 objective.

REAGENT SETUP

**CLEM fixative.** 4% PFA, 0.2% GA in 0.1 M phosphate buffer (PB), pH 7.2.

**2% agarose.** Mix 2 grams of agarose in 100 ml 0.1 M PB, and microwave to a clear liquid.

**1% BSA.** 1% BSA (wt/vol) in 0.1 M PB.

**1% Osmium tetroxide.** Dilute 4% osmium tetroxide to 1% osmium tetroxide in water.

**1% Uranyl acetate (100 ml).** 1 g uranyl acetate in 100 ml water, mix well and filter through 0.2 μm filter.

**Sato’s triple lead.** A mixture of 1% lead nitrate, 1% lead citrate, 1% lead acetate and 2% sodium citrate in water. Shake the mixture vigorously for 5 min, and sonicate for 30 seconds, then add in 20% total volume of freshly made 4% sodium hydroxide.

**GMA resin (20 ml).** Add 14 ml glycol methacrylate, 6 ml butyl methacrylate and 0.12 g benzoyl peroxide. Use glass pipet and glass vial. **CRITICAL** Sonicate mixture to clear liquid.

**30% GMA.** 30% (vol/vol) GMA in 95% ethanol.

**70% GMA.** 70% (vol/vol) GMA in 95% ethanol.

**Freeze substitution medium.** 96% acetone, 3% water, 0.1% UA, 1% MeOH. Optionally include 0.5% OsO₄ for improved ultrastructural preservation for OsO₄-resistant probes such as mEos4.

**1% pioloform.** 1% (vol/vol) pioloform in 1,2-dichloroethane.

**Basic Piranha (aka RCA clean).** 5:1:1 solution of deionized H₂O: 50% H₂O₂: 29% wt/vol NH₄OH.

**0.1% (wt/vol) Poly-L-lysine.** For consistent results always make fresh 0.1% wt/vol solution of dry stock poly-L-lysine hydrochloride in deionized water.

**2% osmium reduced with potassium hexacyanoferrate (a.k.a. Potassium ferrocyanide).** Working in a hood, combine 0.32 ml distilled water, 0.4 ml of 1 M phosphate buffer pH 7.4, 1.28 ml of 5% potassium ferrocyanide, and 2 ml of 4% osmium tetroxide. **CRITICAL** Solution must be made just prior to use.

**0.6% uranyl acetate in polyvinyl alcohol.** Mix 1 ml of 3% uranyl acetate with 4 ml of 2% polyvinyl alcohol. Filter with a 0.2 μm filter prior to use.

**0.0075% Sato’s lead in polyvinyl alcohol.** Mix 10 μl 3% Sato’s lead with 3.99 ml of polyvinyl alcohol.

**2% Methylcellulose.** Resuspend the methylcellulose powder in 60°C distilled water with mixing. Move the solution to 4°C for 3 days. Centrifuge at 40,000 xg for 1 hour at 4°C to pellet debris and undissolved methylcellulose.

**2.3 M Sucrose in phosphate buffer.** Measure 100 ml water using a graduated cylinder and pour into 250 ml beaker. Mark meniscus using tape or another marker then pour out water. Add stir bar and then 80 g of sucrose to beaker. Add 10 ml of 1 M phosphate buffer pH 7.4 to beaker then fill to mark on beaker with distilled water. Stir until sucrose is dissolved then filter-sterilize using 0.22 μm vacuum filtration unit.

**0.5% uranyl acetate in 1.8% methylcellulose.** Combine 0.2 ml of 5% uranyl acetate solution with 1.8 ml of a 2% methylcellulose solution.

**10% gelatin.** Combine 5g of gelatin and 5 ml of 1 M phosphate buffer pH 7.4 and fill to 50 ml with distilled water in a 50 ml conical tube. Heat in a microwave until ~60°C (avoid boiling).
Screw on cap and vortex until gelatin is dissolved. Centrifuge at 2000 xg for 5 min at room temperature and transfer to a clean container. Store at 4°C.

20% (w/v) sucrose. Dissolve 20 g of sucrose in distilled water for a final volume of 100 ml.

Aldehyde fixative for Tokuyasu cryosectioning. Combine 3 ml distilled water, 2 ml 1 M phosphate buffer pH 7.4, 5 ml of 20% sucrose, 5 ml of 8% glutaraldehyde, and 5 ml of 16% paraformaldehyde. !CRITICAL Solution must be used the same day of preparation.

Unroofing stabilization buffer. 70 mM KCl, 30 mM HEPES brought to pH 7.4 with KOH, 5 mM MgCl₂, 3 mM EGTA.

dSTORM blinking buffer. 10% w/v glucose in PBS, 0.8 mg/ml Glucose oxidase from 80 mg/ml frozen stock, 0.04 mg/ml Catalase from 10 mg/ml frozen stock, 100 mM 2-mercaptoethanol ! CAUTION. 2-mercaptoethanol is toxic. Use in chemical hood with gloves and seal before removal. !CRITICAL Must be made fresh within minutes of using.
PROCEDURE

1) **Sample Preparation.** Prior to choosing your sample preparation method refer to the Introduction and Table 1. You will need to determine the best protocol for your system depending on your sample, desired imaging target, and equipment availability. It is also suggested that you familiarize yourself with the original articles on which the following protocols were based before proceeding.

A) Tokuyasu cryosectioning • TIMING 2 d

- **i)** Tissue culture cells should be prepared based on your particular study (*i.e.* transfected with an appropriate plasmid or subjected to a particular treatment).
- **ii)** Wash cells with PBS warmed to 37 °C (or cell-line appropriate temperature).
- **iii)** Add primary aldehyde fixative to cells and incubate at 37 °C for 15 min. **CRITICAL** If performing small molecule or antibody labeling, or when using a sensitive fluorescent protein, a lower concentration of glutaraldehyde may be used.
- **iv)** Aspirate fixative solution, add fresh fixative, and incubate at room temperature for 1 hr.
- **v)** Wash twice for 5 mins each with PBS. Washes should be performed on platform shaker with gentle agitation.
- **vi)** Aspirate PBS and add 50 mM glycine in PBS. Incubate for 15 min at room temperature.
  (1) **If performing labeling with small molecule caged dyes,** incubate the cells with 0.1% sodium borohydride solution in PBS for 7 min.
  (2) **Aspirate the sodium borohydride solution and permeabilize the cells with 0.1% saponin and 3% BSA in PBS for 15 min. **CRITICAL.** We found that using Triton X-100 detergent for permeabilization resulted in worse ultrastructure compared to saponin.
  (3) **Perform small molecule or antibody labeling according to desired protocol.
- **vii)** Aspirate previous solution and add 1% BSA solution in PBS. Using a Teflon cell scraper, scrape the cells from the tissue culture dish and place in a 50 ml conical tube or other appropriately sized tube.
- **viii)** Pellet the cells by centrifugation at 2000 g for 10 mins at room temperature.
- **ix)** Remove most of the supernatant, leaving behind ~0.5 ml covering the pellet.
  Resuspend the pelleted cells in the BSA solution and transfer to a 1.5 ml microcentrifuge tube.
- **x)** Centrifuge at 2000 g for 5 mins at room temperature.
- **xi)** Put some solidified 10% gelatin into a microcentrifuge tube (can transfer chunks using a metal spatula). Warm gelatin in microcentrifuge tube until melted in a water bath set to 37°C, ~20 mins. Gelatin can also be melted in a microwave and kept molten in a 37°C water bath.
- **xii)** Also, warm the microcentrifuge tube containing the pellet in the water bath set to 37°C. This will prevent the gelatin from solidifying prematurely when added to the sample.
- **xiii)** When the gelatin has melted, remove the supernatant covering the pellet and gently wash the pellet twice with the warm gelatin.

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xiv) Add 1 ml of warm gelatin to the pellet containing microcentrifuge tube, and keeping the microcentrifuge tube in the warm water bath, resuspend the pellet in the gelatin. Mixing can be done using a syringe needle.

xv) Once the pellet is resuspended in the gelatin, centrifuge at 16,000 g for 3 mins at room temperature.

xvi) Put the microcentrifuge tube on ice for 20 mins until the gelatin solidifies.

xvii) Use a razor blade to cut off the bottom of the tube just above the pellet and gently remove the gelatin containing the pellet.

xviii) Place 1 ml of 2.3 M sucrose in 0.1 M phosphate buffer, pH 7.4 into a 1.5 ml microcentrifuge tube.

xix) Place an aluminum plate on ice under a stereomicroscope. Place the gelatin embedded pellet on the plate and use a scalpel to cut away excess gelatin from the cell pellet (discard non-pellet-containing gelatin). Using a scalpel, cut the cell pellet into pyramidal pieces less than 1 mm³.

xx) Using a metal spatula, transfer the small pieces of sample into the microcentrifuge tube containing the 2.3 M sucrose solution.

xxi) Place the microcentrifuge tube(s) on a rotator inside a 4°C refrigerator or cold room. Infiltrate the samples with sucrose by rotating overnight (12-16 hours).

xxii) The following day or after the incubation: Remove the sample pieces using a wooden applicator cut to have an angled flat edge, leaving a small amount of sucrose solution in contact with the sample.

xxiii) Place a sample piece onto the center of an aluminum pin holder and freeze by plunging it into liquid nitrogen. The pins can then be placed in cryovials and stored until sectioning. PAUSE POINT Frozen samples can be stored indefinitely in liquid nitrogen until sectioning. Note: having a hole in the cryovial helps ensure the pins remain frozen in liquid nitrogen. It also reduces the probability of a cryovial exploding due to liquid nitrogen expansion.

Cryosections of various thicknesses can be cut based on the application and imaging mode. For two-dimensional imaging, sections with thicknesses of 100 nm give good results. For three-dimensional imaging, sections of 500 – 1000 nm may be used. For an in-depth protocol for cryosectioning see 56 TROUBLESHOOTING

xxiv) Place cryosections directly on the center of prepared coverslips (Box 1). The cryosection will be covered with a drop of methylcellulose/sucrose solution that will prevent dehydration. The sample should be stored at 4°C until just before imaging.

xxv) Just prior to imaging, place three large drops (~250 µl each) of PBS on a piece of Parafilm lying flat on a benchtop. Invert the coverslip, section side down onto the first drop and incubate for 2 mins to wash away residual methylcellulose/sucrose solution. Repeat for the other two PBS drops.

xxvi) Place the inverted coverslip onto a drop of freshly prepared 0.5% sodium borohydride solution for 15 mins.

xxvii) Wash twice for 2 mins on drops of PBS to remove sodium borohydride solution.

xxviii) Proceed to fluorescence imaging (Section 2).
**B) Whole cell mount • TIMING 2 - 3 d**

i) Mark 18-25 mm coverslips at two locations to spatially distinguish a coordinate system for general referencing of the location of cellular specimens. Typically, a “north” and “east” direction marking is sufficient to locate cells of interest (Box 2).

**TROUBLESHOOTING**

ii) Seed tissue culture cells to marked 18-25 mm coverslips using standard practices.

iii) Transfect cells with plasmid DNA containing photoswitchable/photoactivatable probe fused to protein of interest (Optional). Incubate cell specimens for 18-36 hours to allow for protein fusion expression. Transfection should be performed using conditions and reagents suitable and optimized for your specific cell line and protein of interest.

iv) Rinse samples in PBS and fix using 4% paraformaldehyde and 0.2% glutaraldehyde for 30 minutes.

v) Quench residual fixative using 10 mM glycine in PBS solution for 15 minutes.

vi) If only transfected FPs are used, proceed to PALM imaging. If immunolabeling native proteins or nucleic acids, prepare sample according to standard immunofluorescence protocols and optimize for each molecule of interest. If staining cellular structures with fluorescent dyes, follow manufacturer protocol. Note: Many fluorescent labeling/staining protocols will have to be optimized for the highly sensitive nature of single molecule localization imaging.

**C) Unroofed cells • TIMING 2 - 3 d**

i) Place clean and dry Au nanoparticle coated coverslips (See Box 1) in a 6-well plate with the gold side up. Coat with whatever surface coating your cells prefer to stick to and grow well on. For HeLa cells we coat coverslips with 0.01% poly-L-lysine solution for 20 minutes.

ii) Adherent cultured cells should be seeded onto the coverslip allowing room for growth to reach a final confluency of 80% for imaging (growth rate will be different for different cell lines).

iii) Transfection should be done using conditions and reagents suitable and optimized for your specific cell line. We use Lipofectamine 2000 or TurboFect according to the manufacturers’ instructions. If doing 3D localization, transfecting with myristoylated-PS-CFP2 to demark the membrane plane is useful for 3D alignment.

iv) Fill two wells of a 6-well plate with stabilization buffer and two wells with 4 ml of stabilization buffer containing 0.5-2% paraformaldehyde.

v) Prepare sonicator with 1/8” tapered microtip that is in good condition and tightly secured to the horn of the sonicator. Place your 6-well plate immediately below the tip of the sonicator and set your sonicator to the appropriate settings. We use a single 400 ms pulse at the lowest output setting of our sonicator but the settings will need to be tested for your specific sonicator. **TROUBLESHOOTING**

vi) Take your cells from the incubator and immediately rinse for a minute in each well containing stabilization buffer.

vii) Place the coverslip in a well containing paraformaldehyde, bring the sonicator tip down about 5 mm above the coverslip (should be right below surface of liquid) and sonicate within 5 secs of the cells being in fixative. **TROUBLESHOOTING**

viii) Move the coverslip to the well containing fresh paraformaldehyde and let sit in fixative for 20 mins.
ix) If labeling your protein of interest with antibodies, use a typical antibody labeling procedure that will work for your specific antibodies. **TROUBLESHOOTING** Permeabilization reagents like Triton-X should not be used, as the target is already accessible.

x) Image immediately or store at 4 °C overnight. **CRITICAL.** Do not store the cells for more than 24 hrs prior to imaging. Longer storage will result in sample degradation.

D) OsO₄-resistant labels, GMA resin embedding • TIMING 5 – 7 d

i) Under chemical hood, add 2 ml pre-warmed CLEM fixative to cell culture in 35 mm Petri dish. **TROUBLESHOOTING**

ii) Place Petri dish on ice, blocking light, for 1 hr.

iii) Wash with ice-cold 0.1 M PB three times for 3 mins on ice. **TROUBLESHOOTING**

iv) Pre-warm water bath to 42 °C.

v) Cut a piece of 2% agarose gel and microwave the gel to a clear liquid, and place in the 42 °C water bath.

vi) Add 1 ml of 1% BSA to the primary-fixed cells and scrape the cells of the dish using a rubber policeman.

vii) Collect all cells in a 1.5 ml microcentrifuge tube and centrifuge at 3000 g for 3 min at room temperature.

viii) Remove most of the supernatant, leaving enough to cover the cell pellet.

ix) Place the microcentrifuge tube with cell pellet in the 42 °C water bath.

x) Add 1 ml clear 2% agarose liquid, stir gently in the water bath.

xi) Centrifuge at 14,000 g for 2 min at room temperature and put on ice for 30 min to let the agarose solidify.

xii) Use a scalpel to cut the agarose-embedded cell pellet into 0.5-1 mm³ pieces and store in 0.1 M PB.

xiii) Post-fix in desired concentration of OsO₄ (e.g. 0.5% or 1%) in a fume hood for 1 hr, rotating, blocking light. **CAUTION** Fumes of osmium tetroxide are toxic; open the reagent only in an appropriate fume hood.

xiv) Rinse with water three times for 15 mins to remove the excess OsO₄, rotating and blocking light during wash.

xv) In fume hood, stain and stabilize in 1% UA for 1 hr. Protect from light.

xvi) Dehydrate in an ethanol series of 30%, 50%, 70%, 95% for 15 min each, rotating, blocking light. **TROUBLESHOOTING**

 xvii) Place sample in 95% ethanol in a freeze-substitution unit set to -20 °C for 1 hr.

 xviii) Infiltrate with 30% GMA for at least 6 hrs at -20 °C.

 xix) Infiltrate with 70% GMA for at least 6 hrs at -20 °C.

 xx) Infiltrate with 100% GMA overnight at -20 °C.

xxi) Infiltrate with fresh 100% GMA two more times for at least 6 hours each time.

xxii) Add 0.15% (vol/vol) N,N-Dimethyl-p-toluidine to GMA. Pre-cool the GMA stock to -20 °C solution and quickly mix with N,N-Dimethyl-p-toluidine otherwise GMA will polymerize quickly and non-uniformly. Let polymerization run at -20 °C for 24 - 48 hrs. Increase the temperature to 0 °C (5 °C/hr). **PAUSE POINT.** Samples can be stored in the dark at -20 °C for 1 month until sectioning, but it is advisable to use fresh samples due to potential fluorescence decay. Blocks should be stabilized at room temperature for 30 min before sectioning. For FP-expressing cells limit exposure to light as much as possible when sectioning. **TROUBLESHOOTING**

E) OsO₄-resistant labels, High-pressure freezing/freeze substitution • TIMING 5 – 7 d

UNCLASSIFIED
i) Remove culture medium and quickly add 2 ml pre-warmed CLEM fixative to cell culture in 35 mm Petri dish. Avoid exposure of cells to air. TROUBLESHOOTING

ii) Place Petri dish on ice, blocking light, for 1 hr.

iii) Wash with ice-cold 0.1 M sodium cacodylate buffer three times for 3 min on ice. TROUBLESHOOTING

iv) Coat the flat side of a Type B specimen carrier with hexadecene.

v) Add 1 ml of 1% BSA to the Petri dish. Scrape the cells off while keeping the dish on ice and collect in a 15 ml conical centrifuge tube.

vi) Pellet cells by centrifuging at 3000 g for 10 min at 4 °C.

vii) Wash the pellets with 20% BSA and resuspend in small volume (15 µl of 20% BSA.

viii) Add 0.8 µl cell suspension to the 0.1 mm-deep well of the Type A specimen carrier. Wet the edges of the well so no air bubbles will be trapped.

ix) Take the hexadecene-coated Type B specimen carrier flat side-down with fine forceps. Touch the flat side against a piece of filter paper to remove excess hexadecene and place it over the 0.1/0.2 mm disc.

x) Load the carrier assembly into the sample holder and freeze in HPF machine per manufacturer’s instructions. Store samples in cryovials (labeled, and punctured in the cap and in the side wall to ensure liquid nitrogen access) in liquid nitrogen. PAUSE POINT Samples can be stored in liquid nitrogen indefinitely.

xi) Under liquid nitrogen, force open the frozen sample carrier assembly with the tips of a pair of pre-cooled forceps. TROUBLESHOOTING

xii) Transfer the Type A specimen carrier containing the sample to a 2 ml Nalgene cryovial containing 1 ml freeze substitution medium. Program the FS unit according to the following program.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Temp. changing rate (°C/hr)</th>
<th>Duration (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-140</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>-140 to -90</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>-90</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>-90 to -45</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>-45</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>-45 to -20</td>
<td>5</td>
<td>5</td>
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<tr>
<td>-20</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>-20 to -45</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

xiii) Wash with 95% ethanol six times over a period of 3 hrs at -45 °C.

xiv) Infiltrate with 30% GMA resin at -45 °C for at least 6 hrs to overnight.

xv) Infiltrate with 70% GMA resin at -45 °C for at least 6 hrs to overnight.

xvi) Infiltrate in 100% GMA at -45 °C three times for 6 hrs each. The last infiltration can be done overnight.

xvii) Pre-cool the GMA stock solution to -45 °C, and add N,N-dimethyl-p-toluidine to a final concentration of 1.5µl/1ml GMA. Immediately return to -45 °C after quickly mixing.
Transfer the specimen carrier to an embedding capsule filled with the catalyzed GMA. Make sure that the sample side (0.1 mm-deep well) is facing up.

Run polymerization at -45 °C for 24 hours, and increase the temperature to 0 °C (5 °C/hr). PAUSE POINT Samples can be stored in the dark at -20 °C until sectioning. Blocks should be stabilized at room temperature for 30 min before sectioning. Limit exposure to light as much as possible when sectioning.

Super-resolution imaging: Imaging will proceed in a similar manner for all samples at this point with two major variables: 1) Whether two-dimensional or three-dimensional (iPALM) will be used and 2) Whether endogenous fluorescent proteins (PALM) or dye-labeled antibodies (STORM/dSTORM) are used. If iPALM will be used, a coverslip sandwich will need to be created for the dual objectives. If STORM/dSTORM is to be performed, then a reducing buffer will need to be made prior to imaging. Many different reducing buffers have been described. See Materials for the STORM/dSTORM blinking buffer we typically use.

Identifying the same areas in both PALM and EM is made easier by either using a coordinate registration system or through the acquisition of low and high-magnification images of the sample using differential interference contrast (DIC) microscopy (Box 2, Figure 1). If you are not using a coordinate registration system, you can use a diamond objective marker to make a circle on the bottom of the coverslip around the region that you imaged. In the case that you will be making unroofed cell platinum replicas, we suggest you make a 4 mm circle with your region in the center (Box 2, Figure 2).

A) iPALM imaging. Since dual, opposing objectives are used for iPALM the sample will need to be sandwiched between two coverslips. We typically use #1.5 Ø=25mm coverslip for the bottom, and #1.5 Ø=18mm coverslip for the top. This way it is easy to identify the top side, seal the sandwich, and pry the coverslip sandwich open after measurements.

i) Place small drops of epoxy near the outer edge of the glass coverslip to be used as top.

ii) Place the top coverslip, with the epoxy drops down, onto the coverslip with the sections. The surface of the section containing coverslip should be covered in approximately 100 µl of buffer (either PBS or dSTORM buffer).

iii) Press down firmly, but gently, on the top coverslip to create the sandwich.

iv) Seal around the edge of the top coverslip using Vaseline. This can be done by ejecting Vaseline through a syringe needle.

v) Clean the top and bottom glass surface of the sample sandwich using Q-tip and microscope objective cleaning fluid.

B) Post PALM imaging: Clean the oil off your coverslip thoroughly with 70% ethanol or microscope objective cleaning fluid. If you are doing iPALM, your iPALM coverslip sandwich can be taken apart by cleaning off the Vaseline, adding PBS around the sandwich to keep it wet, and using a very thin sheet of metal (we use a 0.0015” thickness gauge) to slide between the two coverslips just around the edge and pry the top coverslip off.
3) **EM Imaging**: The samples will need to be processed further for EM imaging. Again, the protocol one follows will depend on the initial sample preparation (Step 1) as well as the EM modality to be used (i.e., TEM, ET, SEM, FIB-SEM)

A) Staining of Tokuyasu cryosections

i) Staining for 2D SEM • TIMING 1 hr

1. Wash three times with PBS for 1 min each at room temperature.
2. Aspirate PBS and add 2% OsO₄/1.6% potassium ferrocyanide in 0.1 M phosphate buffer. Incubate for 15 mins at room temperature.
   !CAUTION Osmium tetroxide is a very strong oxidizing fixative and a heavy metal toxin. It must be handled with extreme caution in a fume hood wearing protective clothing, gloves, and goggles.
3. Wash three times for 2 mins each with deionized water.
4. Aspirate water and add 0.6% UA in 1.6% polyvinyl alcohol. Incubate for 15 mins at room temperature protected from light.
5. Wash three times for 3 secs with 2% polyvinyl alcohol.
6. Aspirate polyvinyl alcohol and add 0.0075% Sato’s triple lead in 2% polyvinyl alcohol for 15 mins at room temperature.
7. Wash three times for 3 secs with 2% polyvinyl alcohol.
8. Place coverslip on a slide spinner and spin at 5000 rpm for 10 secs.
9. Remove coverslip and place in a desiccator until ready to image.

ii) Staining for FIB-SEM TIMING 15 mins

1. Wash three times with PBS for 1 min each at room temperature.
2. Put three drops of 200 µl 0.5% UA in 1.8% methylcellulose on Parafilm that is placed on an aluminum block on ice.
3. Grabbing the coverslip with forceps, invert it so that the section side is down and place it on the first drop for 3 secs, transfer to the second drop for 3 secs and then transfer to the third drop. Incubate for 10 mins.
4. Grab the coverslip with forceps and drag the edge of the coverslip over Whatman 50 hardened paper until the methylcellulose solution is wicked away from the coverslip surface.
5. Once methylcellulose is dried, cover the sections with a small drop of cyanoacrylate.
6. Image using FIB-SEM.

?TROUBLESHOOTING

B) Whole cell mount EM preparation • TIMING 3 – 6 hrs

i) Remove sample from fluorescence microscope and wash with PBS.

ii) Post-fix the specimen with 2% glutaraldehyde in PBS buffer for 1-2 hrs. PAUSE
   POINT Fixation can be performed overnight (12 – 16 hrs) at 4 °C.

iii) Rinse specimen with PBS and then with deionized water.

iv) Apply 1% OsO₄ in deionized water as secondary fixative for 1-2 hrs. Optional:
   Osmium solutions can be reduced with potassium ferrocyanide to increase reactivity and help stabilize membranes further. ?TROUBLESHOOTING

v) Rinse specimen thoroughly with deionized water to remove residual osmium salts.

vi) Dehydrate specimen with an increasing ethanol series by exchanging the following solutions:

   a) 15% EtOH, 5 minutes
   b) 25% EtOH, 5 minutes
   c) 35% EtOH, 5 minutes
   d) 50% EtOH, 5 minutes
(e) 60% EtOH, 5 minutes
(f) 75% EtOH, 5 minutes
(g) 85% EtOH, 5 minutes
(h) 95% EtOH, 5 minutes
(i) 100% EtOH, 2 × 5 minutes
(j) 100% EtOH dried over molecular sieve, 3 × 5 minutes

vii) Desiccate specimen using a CO₂ critical point drying system according to the manufacturer's protocol. **TROUBLESHOOTING CRITICAL** If the user does not have access to a critical point drying system, hexamethyldisilizane (HMDS) can be used to dry the sample. In this case, the following wash solutions should be used after the ethanol series. **CAUTION** HMDS wash solutions should be handled in a chemical fume hood.

(a) 25% HMDS/75% dried EtOH, 5 mins
(b) 50% HMDS/50% dried EtOH, 5 mins
(c) 75% HMDS/25% dried EtOH, 5 mins
(d) 100% HMDS, 3 × 5 mins
(e) Overlay 50-100 µl of fresh HMDS and allow specimen to air dry slowly in the hood.

**PAUSE POINT** Sample can be stored desiccated under vacuum until SEM imaging can be performed.

viii) Coat specimen with 5-10 nm of Au or Pt/Ir using sputter coating system following manufacturer protocol. **CRITICAL** Angular shadowing of the coating will aid in more complete coating of cellular structures and minimize charging effects during SEM imaging.

ix) Image by SEM.

C) Platinum replica of unroofed cells • TIMING 3-5 hrs
i) Post-fix your specimen by placing it in 2% glutaraldehyde in PBS and incubating at 4 °C for 20 mins to 36 hrs.

ii) Transfer coverslip to freshly made 0.1% tannic acid in deionized water for 20 mins at room temperature.

iii) Rinse coverslips four times with deionized water.

iv) Transfer the coverslip to 0.1% UA for 20 mins at room temperature. **CAUTION.** UA is an alpha emitter and heavy metal toxin. UA is a cumulative toxin. Wear protective clothing, gloves and goggles.

v) Rinse coverslips four times with deionized water.

vi) Place your critical point dryer holder in a water filled beaker that has a metal mesh shelf and a magnetic stir bar below the shelf.

vii) Add your sample to the critical point dryer holder making sure that there are spacers that allow liquid to flow above and below your coverslip.

viii) Move the sample into 15% EtOH in your other magnetic stir bar-containing beaker and stir for 5 mins to start the gradual dehydration process. Continue the process with 30%, 50%, 70%, 80%, 90%, 100% EtOH, each for 5 mins. Do the 100% EtOH three times rinsing out the beaker each time with 100% EtOH to remove residual water.

ix) Place the sample holder into the critical point dryer in 100% EtOH and dry the sample according to the manufacturer’s instructions. In our Tousimis Samdri-795, we purge the chamber with liquid CO₂ for 20 minutes. **TROUBLESHOOTING**
x) Cut the sample to fit into the freeze fracturer using a diamond scriber to cut the coverslip around the etched circle region. It should be small enough to fit on your freeze fracturer stage. We use a lab marker to make dark dots on the bottom side of the coverslip making the region more visible. We then use a razor blade to guide our diamond scriber during cutting.

xi) Place your sample on your freeze fracturer stage and use two pieces of ~1 mm² double-stick tape to adhere the sample to a flat stage.

xii) After placing your sample in the freeze fracturer, use the manufacturer’s instructions to rotary shadow 2-4 nm of platinum/carbon onto your sample at a 17° angle (90° is no tilt). Then coat with carbon at 90° to stabilize the Pt/C coat (~5-10 nm).

xiii) Image your coverslip with a 10x phase contrast objective to be sure you can find the region that you previously imaged and you know what the region looks like after drying and coating (Box 2). You will use this image to help you find your cells of interest on your grid after the replica has been transferred.

xiv) Cut the sample again. Before lifting, the sample needs to be ~4 mm in diameter so you should be able to cut exactly around where the original diamond objective marking was. Cut with the diamond scriber again to assure that the sample is this size. It should fit completely within the 5 mm inoculation loop but not be so small that it can move around a lot within the loop. The region that you imaged should be directly in the center. It is possible to cut the sample down to this size the first time that you cut, which would make this step unnecessary. We prefer to cut twice because the larger piece gives room to handle the coverslip on and off of the double stick tape with tweezers without scratching the region of interest.

xv) Glow discharge TEM grids (75-mesh, with formvar and carbon coating) for one minute according to the manufacturer’s instructions.

xvi) If you are doing tomography, you should add 10 nm gold fiducials to the grids; these will enhance your tomography reconstruction. Dip your grids into a 10 nm gold solution or 10 nm gold conjugated antibody for two minutes, rinse with water, and place on filter paper to dry. You may need to optimize the density of gold labeling by changing the concentration of gold. If not doing tomography, you should not add 10 nm gold fiducial markers as it will add unnecessary spots to your image.

xvii) Place the grids on filter paper.

xviii) Place the cut sample, replica side up, on the surface of 5% hydrofluoric acid (HF) (diluted in pure water from 49% stock solution) in a 12-well plate. Wait for the glass coverslip to fall to the bottom of the well while the replica stays at the surface. Use a transfer pipette to replace the HF with water by successive dilutions while keeping the replica at the surface. **CRITICAL.** You must be delicate and avoid the replica touching the pipette or riding up the side of the well. Bringing the solution down to ~1.5 ml and adding ~5 ml of water 10-15 times has been sufficient for us. **CAUTION.** Hydrofluoric acid is extremely toxic and should be used with gloves in a fume hood. Any contact with skin requires immediate medical attention.

**TROUBLESHOOTING**

xix) Lift the replica onto the grid by bringing a 4 mm Pt inoculation loop into the liquid and underneath the replica to lift it up out of the water. Bring the inoculation loop with replica down onto the grid and keep it there for 3 secs until the replica has stuck to the grid and the liquid has been soaked up by the filter paper.

**TROUBLESHOOTING**

xx) Image the grid with a 10x phase contrast objective and find where the cells are on the grid (Box 2). **CRITICAL.** There is a good chance that at least one or maybe two of the regions of interest have been obscured by a grid bar. You will not be able to correlate these cells.
xxi) Image by TEM or perform electron tomography

D) For plastic embedded sections, a same-section or consecutive-section approach can be taken. For the consecutive section approach, cut two consecutive sections, placing one on a TEM grid and the other on a coverslip for PALM. The section on the TEM grid can be stained as below, starting at step viii. For imaging the same section in both PALM and TEM, the section will need to be moved from the pioloform-coated coverslip (Box 1) to a TEM grid. This can be achieved by the following protocol steps.

TROUBLESHOOTING
i) After PALM imaging, using a razor, score the fiducial side of the coverslip with a rectangular area surrounding the sections.
ii) Place 1-2 drops 1.2% HF along one edge of the scored rectangle.
iii) Tilt the coverslip slightly and let the HF seep under the pioloform film.
iv) Fill a glass jar with water. Once the HF solution has reached the other edge of the scored rectangle, dip the coverslip slowly into the water to float the pioloform membrane.
v) Place a non-coated Synaptek slot grid on the floating film to capture the sections in the center of the opening slot.
vi) Lower a piece of Parafilm onto the floating film to pick up the grid.
vii) After drying, carefully punch out the grid from the Parafilm with fine tweezers.
viii) Place a drop of 1% UA on a piece of Parafilm in a Petri dish. Put the TEM grid on top of the 1% UA droplet, section side-down, for 10 min. Protect from light.
ix) Pass the grid over 3 deionized water droplets for washing.
x) Dry the grid with filter paper by touching the edge of the grid.
xi) Place a piece of Parafilm in a Petri dish, with a few NaOH pellets inside the dish.
xii) Cover the Petri dish and wait 5 min for NaOH to absorb excess carbon dioxide from the air in the Petri dish.
xiii) Dispense a drop of Sato’s triple lead (filtered through 0.2 μm filter) on the Parafilm.
xiv) Place the grid on top of the lead droplet, section side-down. Put the lid back on the Petri dish and stain for 3 min.
xv) Rinse quickly with deionized water.
xvi) Dry the grid with filter paper by touching the edge of the grid. The grid is now ready for TEM imaging.

4) Registering and transforming PALM and EM data sets.
A) Registering two-dimensional data sets (Note: this is used only when the same sample/section is imaged in both light and electron microscopy, i.e. not when consecutive sections are imaged. Consecutive sections have sufficiently different structures that they must be manually registered.)

i) After PALM data has been collected and processed, determine the coordinate pairs \( \{X_i^{Palm}, Y_i^{Palm}\} \) of multiple reference fiducial points (Au nanoparticles).

ii) Identify the same reference fiducial points in EM micrographs and determine their coordinate pairs \( \{X_i^{EM}, Y_i^{EM}\} \).
iii) Determine the transformation coefficients $Kx, y_{ij}$ based on the corresponding reference sets $\{X_i^{P\text{ALM}}, Y_i^{P\text{ALM}}\}$ and $\{X_i^{E\text{M}}, Y_i^{E\text{M}}\}$ (Use POLYWARP function in IDL or CP2TFORM function in Matlab).

iv) Use the transformation coefficients $Kx, y_{ij}$, to calculate the new (transformed) PALM coordinates for reference fiducial points $\{X_i^{P\text{ALM mapped to } E\text{M}}, Y_i^{P\text{ALM mapped to } E\text{M}}\}$

v) Calculate the registration errors for each fiducial point as:
$$\varepsilon_i = \sqrt{(X_i^{P\text{ALM mapped to } E\text{M}} - X_i^{E\text{M}})^2 + (Y_i^{P\text{ALM mapped to } E\text{M}} - Y_i^{E\text{M}})^2}$$

vi) Compare the determined registration errors $\varepsilon_i$ for reference fiducial points with acceptable registration tolerance $\varepsilon_{\text{tolerance}}$ ($\varepsilon_{\text{tolerance}} = 20$ nm is reasonable). If $\max(\varepsilon_i) \geq \varepsilon_{\text{tolerance}}$, exclude the point with maximum registration error from the reference sets $\{X_i^{P\text{ALM}}, Y_i^{P\text{ALM}}\}$ and $\{X_i^{E\text{M}}, Y_i^{E\text{M}}\}$ used to determine the transformation coefficients (assuming that the error is due to a fiducial object that is a single dipole source). After this, repeat steps iii through vi. This will result in a new set of transformation coefficients $Kx, y_{ij}$ with a new corresponding set of registration errors $\{\varepsilon_i\}$.

vii) Once the condition $\max(\varepsilon_i) < \varepsilon_{\text{tolerance}}$ is met, use the transformation coefficients $Kx, y_{ij}$ to calculate the new (transformed) PALM coordinates for all fluorescent localizations.

viii) Determine the registration error as the average of the registration errors of all fiducial markers:
$$\sigma_{\text{reg}} = \langle \varepsilon_i \rangle$$

B) 3D (i) PALM and EM image registration

i) Identify the fiducial points.

ii) Assuming that the fiducial points are Au nanoparticles on the surface of the coverslip, transform the entire 3D PALM data set so that all fiducial points lie in the same average plane of $z=0$. There may be small local variations between the vertical locations of fiducial points, so this transformation is not intended to put them exactly on the same plane. This transformation should be simple and global. We use a shear transformation, done with the following steps:

1) Linear regression is used to find a best-fit plane through all fiducial points.

2) Shear transformation is applied, such that $x$- and $y$- coordinates remain unchanged, but $z$ coordinates are changed so that the plane defined in step (1) coincides with the plane defined by condition $z=0$.

iii) Perform 2D image registration of PALM and EM data sets leaving $z$ coordinates unchanged.

iv) Identify the features inside the volume of the sample, which can be detected in both PALM and EM data sets (ideally close to the top surface of the sample). These features may be fluorescantly labeled proteins with locations easily identifiable in EM (such as clathrin or membrane proteins in the case of correlative iPALM-TEM of...
unroofed cells\textsuperscript{22}, or random sparse fluorescence throughout the volume of the section (as in the case of iPalm-FIB-SEM of thick cryo-sections\textsuperscript{18}).

v) Determine the scaling coefficient required to make these features overlap. In the example of iPalm-TEM of unroofed cells this means adjusting the scaling factor so that the membrane proteins overlap vertically with the Pt replica of the membrane. In the example of iPalm-FIB-SEM of thick cryosections the scaling is adjusted so that the random sparse fluorescence falls off at the same vertical location as the boundary of the section observed in the 3D EM data set.

vi) Apply the scaling coefficient to the entire 3D PALM data set.

An example of the ability to identify gold in both fluorescence and EM channels can be seen in Figure 2.

?TROUBLESHOOTING

Anticipated Results (Figure 3)

Tokuyasu cryosectioning (Figure 3a-c)
The user should expect accurate localization of your fluorescently labeled protein or dye within a context-rich electron micrograph. In particular, membranes are well defined and visible in electron microscopy of Tokuyasu cryosections. If performing 3D CLEM, the fluorescent probes should be observable within a 3D volume (Figure 3 b,c). However, due to shrinkage of the sample one would expect significant collapse of the z height. This can be adjusted and appropriately aligned to give excellent 3D CLEM results.

Whole-cell Mount (Figure 3d)
If this protocol is performed properly, the user should expect two-dimensional correlative SEM and super-resolution point localization images detailing the topology of the specimen surface as well as the nanoscale distribution of the labeled molecule of interest \textsuperscript{21}. The SEM/fluorescence images should be registered within <30 nm (often below 10 nm with sufficient fiducial coverage within the field of view) of one another, enabling a detailed topological analysis.

Unroofed cells (Figure 3e)
In general platinum replicas will show the complex architecture of the plasma membrane with its associated cytoskeleton, exocytic, endocytic structures, and membrane proteins. Clathrin coated pits can be clearly identified in TEM by their distinctive geometric basket patterns and caveolae appear as distinctive 80 nm-striped vesicles. Filaments of the actin cortex (appearing 8-12 nm in width) are also generally prominent features of these images.

When this correlative protocol is performed properly, you should be able to observe high quality PALM and platinum replicas. With smaller cells (15-20 \textmu m) you should be able to correlate the entire adhering membrane. With larger cells, you should limit yourself to 20 \textmu m regions for correlation to avoid large-scale imperfections in the film on the grid and EM image stitching. The final correlation should have 20-nm accuracy or better. If you are using antibodies for fluorescence labeling, there should be small (~20 nm) blobs in your EM that co-localize with your fluorescence localizations.

Resin-embedded samples (Figure 3f)
Both standard chemical fixation with aldehyde cross-linking and high-pressure freezing/freeze substitution (HPF-FS) should produce well-preserved cellular samples with good ultrastructure. Addition of OsO₄ for samples with fluorescent labels resistant to this treatment (such as mEos4²⁰) should markedly improve ultrastructure preservation, including that of plasma and nuclear membranes, endoplasmic reticulum, Golgi apparatus and mitochondrial cristae. Chemical fixation is compatible with larger samples than HPF-FS, which typically requires samples smaller than 100 µm in order to achieve uniform freezing without ice crystals.

The protocols described here should maintain fluorescence of the FP probes used, either targeted to fill specific organelles or the cytoplasm, or by fusion to specific proteins of interest. The user should expect good ultrastructure preservation, and the post-embedding use of aldehyde quenchers such as borohydride and cyanoborohydride should decrease autofluorescence without perturbing ultrastructure.
BOX 1. Coverslip Preparation and Coating.

1) Coverslip Preparation

A) Clean the coverslips (#1.5, 18 or 25 mm, from Werner Instruments) in basic Piranha (RCA clean) at 80 °C, stirring, at least 2-3 hours (can be done overnight). Then rinse with deionized water and blow-dry.

B) Put coverslips into cover-slip support blocks in individual small Petri dishes. Write numbers near the edge of the top surface for later identification.

C) Cover the surface with 75 µl fresh-made 0.1% (w/v) poly-L-lysine for 15 minutes. It should cover most of the top surface. After 15 minutes rinse with deionized water and blow-dry.

D) Cover the surface with 75 µl 0.5% (1:200 diluted in deionized water) of stock Au nanoparticles or nanorods for 15 minutes. After 15 minutes rinse with water and blow-dry. Most versatile are bare Au nanorods 25 x 57 nm from Nanopartz. Sometimes larger 80 nm Au nanospheres are useful (they are brighter at 500-600 nm emission wavelength and are sometimes easier to see in SEM). If fluorescent imaging is done at 800 nm (such as with Alexa Fluor 750), then bare Au 25 x 75 nm nanorods from Nanopartz should be used. Depending on application and image area, the target surface density of nanoparticles should be 10,000-100,000 mm⁻². The concentration of Au nanoparticles may need to be adjusted to achieve this.

E) Deposit silicon dioxide (SiO₂) or indium tin oxide (ITO) using a sputtering deposition system. For SEM, deposit ITO (ITO is conductive and will help with avoiding charging during SEM imaging). ITO thickness should be selected so that the resistance of the coverslip surface (measured by connecting two multimeter probes near the opposing edges of the coverslip) is below 5 kOhm. For TEM, deposit 20 nm of SiO₂.

2) Pioloform coating of coverslips

A) Clean coverslip by adding 0.5% HF on each side and hold over sink for 30 seconds. Rinse with deionized water and blow-dry with compressed air.

B) Attach a clean coverslip on a glass slide using double-sided tape in a way that it can be easily removed. Place the slide in the DiffSpin™ 2 Slide Spinner.

C) Put 100 µl of 1% pioloform onto the coverslip and immediately start the spinner at maximum speed. Spin for 1 min before use.

D) Glow discharge the coverslips: 25 mA, 10 sec.

E) Coat the coverslips with 100 µl 0.1% poly-L-lysine for 30 min.

F) Dilute 80 nm gold fiducials with water to 15% and sonicate for 10 min.

G) Rinse off poly-L-lysine with Milli-Q water and blow-dry the coverslips.

H) Put diluted fiducials on coverslips and incubate for 20 min.

I) Rinse with deionized water and blow-dry.
Correlative LM and EM imaging is usually performed sequentially: LM imaging is done first, followed by additional sample staining and treatment, and then EM imaging. One important and potentially challenging step is finding the areas where LM imaging was performed for subsequent EM imaging. “Blind” searching in high-resolution EM for a structure that resembles what was imaged during LM can be very time-consuming and ultimately unsuccessful. A better procedure for finding the previously imaged area must be established. This can be done in two different ways:

1) If both microscopes are equipped with sample coordinate registration software and sample holders (such as in Box 2, Figure 1a, b), and the sample did not need to change substrates (e.g., from coverslip to grid), then the procedure is straightforward:
   A) Coordinates of every site imaged during LM are recorded.
   B) Knowing the coordinates of the imaged site in LM sample reference and the transformation coefficients (which can be established using a simple test sample with a few distinct features), the coordinates in EM sample reference are calculated.

2) If the conditions set in (1) above cannot be met, then creating a set of intermediate-zoom optical image maps can be very helpful. Since most cells and thin sections are optically transparent, differential interference contrast (DIC) becomes very useful. It is highly desirable to have the DIC imaging modality available in the LM setup (it is very useful not only for this step, but in general to check sample quality before imaging). If DIC is not available, a fluorescence image map could also be used if all cells are fluorescently labeled with a membrane or similarly uniform stain. The procedure we use is:
   A) Once each LM data acquisition is complete, take a DIC image of the LM imaged area (preferably DIC image of slightly expanded area if possible).
   B) Once all LM acquisitions have been performed on a sample, take low-zoom DIC image of the sample to create a look-up map of the imaged sites.
   C) Using this look-up map, search for the imaged sites in EM.

An example of these steps is shown in Box 2, Figure 1c-d. Box 2, Figure 1c shows the 10x DIC image taken after the sample has been removed from the LM microscope, prior to post-staining and EM imaging. Box 2, Figure 1d presents the 120x DIC image taken in the LM microscope after LM imaging. Having this map greatly simplifies searching for the areas that have been previously imaged.

3) For the purposes of making a platinum replica for TEM, this process is also aided by a circle that is etched on the bottom of the coverslip with a diamond objective marker after fluorescence imaging (Box 2, Figure 2). The procedure is:
   A) A diamond objective marker is used to etch a 4 mm circle around the region that was imaged in fluorescence and a zoomed out view of the region is imaged in DIC to create a map of the cells imaged (Box 2, Figure 2a).
   B) After coating the sample with platinum and carbon, the region within the etched circle is again mapped, this time with 10x phase contrast (Box 2, Figure 2b).
   C) The etched circular region is cut out and the replica is lifted onto a grid. The grid can then again be imaged with 10x phase contrast to identify the location of your cells on the grid. Colored rectangles indicate cells imaged in fluorescence (Box 2, Figure 2c).
BOX 3
Expanded discussion of registering and transforming (i)PALM and EM data sets

The registration of data sets acquired in different modalities is essential for correlative (i)PALM and EM. EM images may have different nm/pixel scales as compared to (i)PALM data sets; in addition they may be shifted and tilted. The uncertainties due to imperfect image registration must be accounted for when estimating compound localization accuracy; usually these are treated as independent and are added in quadratures:

\[ \sigma_{tot} = \sqrt{\sigma_{loc1}^2 + \sigma_{loc2}^2 + \sigma_{reg}^2}, \]

where \( \sigma_{loc1} \) and \( \sigma_{loc2} \) are the localization accuracies in each data set, and \( \sigma_{reg} \) the accuracy of registration.

Since (i)PALM data sets are vector based, and EM images are typically pixel maps, it is easier to register and transform (i)PALM data to overlay it with EM data. The registration can be performed using the coordinates of objects (fiducials) that are detectable in both modalities. The procedure is fairly straightforward for 2D registration and we discuss this first.

2D PALM and EM image registration

We found that Au nanoparticles are very good fiducial markers since they behave as bright, single-dipole emitters during fluorescent imaging and are also electron-dense, so they can be registered in both fluorescent microscopy and EM images. Once the (i)PALM and EM imaging have been performed, the coordinates of the same Au nanoparticles can be determined with accuracy on the order of 2-5 nm in both data sets. As a result, we have corresponding coordinate pairs \( \{X_i^{\text{PALM}}, Y_i^{\text{PALM}}\}, \) and \( \{X_i^{\text{EM}}, Y_i^{\text{EM}}\}. \) These two sets are used to establish the transformation procedure for the rest of the data. We found that bilinear mapping works very well for PALM-EM registration:

\[
X_i^{\text{EM}} = K_{x00} + K_{x01} \cdot X_i^{\text{PALM}} + K_{x10} \cdot Y_i^{\text{PALM}} + K_{x11} \cdot X_i^{\text{PALM}} \cdot Y_i^{\text{PALM}},
\]

\[
Y_i^{\text{EM}} = K_{y00} + K_{y01} \cdot X_i^{\text{PALM}} + K_{y10} \cdot Y_i^{\text{PALM}} + K_{y11} \cdot X_i^{\text{PALM}} \cdot Y_i^{\text{PALM}},
\]

where \( \{X_i^{\text{PALM}}, Y_i^{\text{PALM}}\}, \) and \( \{X_i^{\text{EM}}, Y_i^{\text{EM}}\} \) are as above, while \( K_{xyij} \) terms are the transformation coefficients. The bilinear terms \( K_{xii} \) and \( K_{yij} \) are usually very small. In choosing imaging sites, we recommend selecting areas that contain as many fiducials as possible. The transformation coefficients \( K_{xj}, y_{ij} \) can be determined using POLYWARP function in IDL or using CP2TFORM function in MATLAB. This step requires a minimum of 4 coordinate pairs to determine the transformation coefficients; more are desirable in order to reduce the registration error. Au nanoparticles occasionally form clusters, which tend to exhibit wavelength-dependent, higher order multi-pole radiation patterns, resulting in erroneous localization results. It is usually relatively easy to identify and exclude these fiducials during processing. But in order to perform this iterative procedure, the initial reference sets must exceed the minimum size. It is recommended to start with at least 20 fiducial points identifiable in PALM and EM data sets. Then, for a ~30 µm field of view, the achievable average registration error is typically below 10 nm.

3D (i)PALM and EM image registration

Registration of 3D (i)PALM and EM images is more complicated. The main problem is sample deformation during transfer from the aqueous environment of PALM to the vacuum of an EM. This sample deformation can occur in any direction and is a serious limitation for CLEM in general. Making the sample as thin as possible helps minimize the lateral shrinkage. This can be achieved by working with relatively thin (<1 µm) sections or unroofed cells. We believe that the most promising approach to minimizing shrinkage of sections is plastic embedding. Other
methods that we tried still suffer from some degree of vertical shrinkage. Fortunately, due to very high sample aspect ratio (tens of microns lateral to ~0.5 µm vertical), this shrinkage may be considered uniform, and the registration can be done with a constant scaling factor applied to the (i)PALM data set in the vertical direction (z-dimension). Ideally one would like to create fiducial points either throughout the volume of the sample or on both top and bottom surfaces of the sample. This would allow for precise quantitative 3D registration of multiple data sets. Unfortunately, we have not been able to create stable fiducial markers on the top surface of cryosections or unroofed cells. Au nanoparticles tend to detach and move during the PALM experiments unless they have been covered by a layer of SiO₂, ITO, or by a section itself (in all of these cases they create good fiducial points in the plane of the bottom surface of the sample).

We have successfully used a feature-selection approach to establish the vertical scaling for registering 3D PALM and EM data sets. Such features need to clearly define an object in the volume of the sample (ideally as close to the top surface of the sample as possible – or even better – the top surface itself) and must be identified in both imaging modalities with high vertical precision.
Table 1: Comparison of the correlative super-resolution fluorescence/electron microscopy protocols.

<table>
<thead>
<tr>
<th>Sample Process</th>
<th>Super-resolution Fluorescence Imaging Method</th>
<th>Electron Microscopy Imaging Method</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tokuyasu Cryosectioning (100 nm section thickness)</td>
<td>PALM</td>
<td>SEM or TEM</td>
<td>- Rapid</td>
<td>- Delicate hydrated sections - 2D</td>
</tr>
<tr>
<td>Tokuyasu Cryosectioning (500-750 nm section thickness)</td>
<td>iPalm</td>
<td>FIB-SEM</td>
<td>- 3D</td>
<td>- Technically demanding</td>
</tr>
<tr>
<td>Whole-cell mount</td>
<td>iPalm and/or dSTORM</td>
<td>FIB-SEM</td>
<td>- Aids iPalm interpretation</td>
<td>- Shrinkage limits registration quality - Limited to surface structures</td>
</tr>
<tr>
<td>Platinum Replica of Unroofed Cells</td>
<td>iPalm and/or dSTORM</td>
<td>ET or SEM</td>
<td>- 3D data - High-contrast visualization of membrane processes</td>
<td>- Limited to surface structures</td>
</tr>
<tr>
<td>Resin embedding (with Os staining)</td>
<td>PALM/iPalm</td>
<td>SEM or TEM</td>
<td>- Excellent ultrastructure - Serial sectioning capability</td>
<td>- Requires OsO₄-tolerant FP - FP properties still somewhat compromised</td>
</tr>
</tbody>
</table>

UNCLASSIFIED
<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.A</td>
<td>Large folds in Tokuyasu cryosections</td>
<td>Not using correct cryosectioning equipment.</td>
<td>Use a sharp diamond knife designed for cryo-ultramicrotomy. Hold the loop perfectly steady when picking up the sections</td>
</tr>
<tr>
<td>1.A</td>
<td>Tokuyasu cryosections have large holes</td>
<td>Section retrieval solution not correct</td>
<td>Use a 1:1 methylcellulose:sucrose solution for section retrieval</td>
</tr>
<tr>
<td>1.A</td>
<td>Little to no fluorescence in Tokuyasu cryosections</td>
<td>Fluorescent proteins are degraded.</td>
<td>Sections should be imaged by fluorescence within 24 hours after Tokuyasu cryosectioning.</td>
</tr>
<tr>
<td>1.B.i</td>
<td>Loss of cardinal axis system on coverslip</td>
<td>The marking step is susceptible to removal by solvents used in the SEM sample preparation protocol.</td>
<td>Use marking method that is not susceptible to removal by solvents such as etch marking with a diamond knife.</td>
</tr>
<tr>
<td>1.C.v</td>
<td>Sonication blows away all of the cells</td>
<td>The cells weren't adhered strongly enough</td>
<td>Try a stronger adhering surface. Sonication was too strong Decrease power on sonicator or length of time sonicating</td>
</tr>
<tr>
<td>1.C.vii</td>
<td>None of the cells were unroofed</td>
<td>Cells were fixed too long prior to sonication</td>
<td>Sonicate more quickly after placing in fixative The sonication was not strong enough. Sonicate stronger and closer, or make sure your sonicating tip is secured tighter to the sonicator horn, and is in good condition.</td>
</tr>
<tr>
<td></td>
<td>The unroofed cells are very broken up like shards of cells</td>
<td>Cells didn’t uniformly adhere to surface.</td>
<td>Treat the cells with poly-L-lysine and a hypo-osmotic shock prior to lifting, which will help the cells stick down more uniformly. Please see[22] Intracellular structure is getting fixed together and tearing the cell apart. Try sonicating in stabilization buffer without fixative and then quickly transferring to fixative.</td>
</tr>
<tr>
<td>1.C.ix</td>
<td>The antibody labeling doesn’t look good</td>
<td>One of the antibodies is bad.</td>
<td>Try a new antibody The concentration has not been optimized Try altering the concentration of antibodies</td>
</tr>
<tr>
<td>1.D.xxii</td>
<td>GMA not polymerizing at -45 °C</td>
<td>Too much uncatalyzed GMA left when adding catalyzed one</td>
<td>Bring the temperature up to -30 °C or -20 °C</td>
</tr>
<tr>
<td>1.E.xix</td>
<td>GMA blocks are too soft for ultramicrotomy</td>
<td>GMA is not prepared correctly</td>
<td>Make sure instructions for preparation of GMA are followed correctly. Be especially careful that the</td>
</tr>
<tr>
<td>1.E.xi</td>
<td>Frozen specimen carrier assembly cannot be split open</td>
<td>Specimen carrier was not coated with hexadecene</td>
<td>Ensure the flat disc is coated with hexadecene.</td>
</tr>
<tr>
<td>1.E</td>
<td>Poor sample morphology with artifacts and/or over-extraction in high-pressure frozen cells</td>
<td>Incorrect practice of high-pressure freezing</td>
<td>Do not expose cells to air at any point. Avoid air bubbles when placing Type B specimen carrier over Type A. After HPF, transfer the specimen carrier immediately to liquid nitrogen. After the sample is frozen with HPF, precool forceps in liquid nitrogen before touching the specimen carrier. Minimize temperature changes when changing media during washing and infiltration.</td>
</tr>
<tr>
<td>3.A</td>
<td>Uneven contrast across Tokuyasu cryosections</td>
<td>Dehydration artifact.</td>
<td>Be sure sections are not exposed to air dehydration prior to final drying step.</td>
</tr>
<tr>
<td>3.A</td>
<td>Precipitate on Tokuyasu cryosections</td>
<td>Heavy metal stain precipitate</td>
<td>Make sure staining solutions are fresh. Filter UA and lead solutions just prior to use. Be sure salts are removed through water washes before UA/lead staining.</td>
</tr>
<tr>
<td>3.B.vi</td>
<td>Membranes not preserved well (cracking or holes in membrane surface) as assessed by SEM imaging.</td>
<td>Sample not dehydrated properly</td>
<td>Extend the length of ethanol dehydration steps to 10 minutes each. Ensure that EtOH dried over molecular sieve is used in the final wash steps to eliminate any residual water remaining in the sample.</td>
</tr>
<tr>
<td>3.B.ii</td>
<td>Fixation proceeded too long causing swelling and rupture.</td>
<td></td>
<td>Reduce fixation times with 2% glutaraldehyde and 1% OsO₄ to 1 hour each.</td>
</tr>
<tr>
<td>3.B.iv</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.B.vii</td>
<td>Critical point drying step did not remove all EtOH from sample upon liquid CO₂ exchange and prior to CO₂ evaporation.</td>
<td>Proceed with additional exchanges and extend the mixing times between EtOH and liquid CO₂. Subsequently extend</td>
<td></td>
</tr>
<tr>
<td>3.B.vii</td>
<td>Cell samples too brittle for critical point drying.</td>
<td>Test the HMDS drying/desiccation procedure.</td>
<td></td>
</tr>
<tr>
<td>3.B.iv</td>
<td>Not enough OsO₄ to properly stabilize membranes.</td>
<td>Reduce OsO₄ with Potassium Ferrocyanide prior to secondary fixation of specimen.</td>
<td></td>
</tr>
<tr>
<td>3.C.ix</td>
<td>Sample looks melted in EM</td>
<td>There was trace water present during critical point drying</td>
<td></td>
</tr>
<tr>
<td></td>
<td>There was trace water present during critical point drying</td>
<td>Make sure you are using 100% dry ethanol during dehybridation. Open a new bottle before each dehydration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The critical point dryer is not functioning properly.</td>
<td>Check to make sure critical-point dryer is operating at the appropriate temperatures and pressures and has enough CO₂.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The sample dried out prior to critical point drying or became wet after critical point drying.</td>
<td>Be sure your sample never dries out prior to critical point drying, and gets rotary shadowed immediately after CPD to avoid rehydration from moisture in the air.</td>
<td></td>
</tr>
<tr>
<td>3.C.xviii</td>
<td>There are black spots around the grid bars in EM.</td>
<td>Hydrofluoric acid reacted with the grid.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hydrofluoric acid reacted with the grid.</td>
<td>Do more dilutions of the hydrofluoric acid prior to moving your replica onto the grid</td>
<td></td>
</tr>
<tr>
<td>3.C.xix</td>
<td>The replica falls to the bottom of the well in the hydrofluoric acid.</td>
<td>The sample was placed upside down in the HF.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The sample was placed upside down in the HF.</td>
<td>Check the orientation of the coverslip prior to placing on the HF. The cell surface generally appears rough and should be facing up.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The HF is contaminated from the plastic.</td>
<td>Check what types of plastics are coming into contact with the HF. Some polystyrenes will dissolve contaminants into your HF, making the surface tension lower.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The replica breaks into pieces when lifted out of solution or placed on the grid</td>
<td>The carbon layer is too thin.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The carbon layer is too thin.</td>
<td>Coat with more carbon to make the replica more stable.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The replica sticks to the wall of the 12-well plate and breaks.</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>As soon as you see the replica approach the wall of the well, you should add more water to help it move away from the wall.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>As soon as you see the replica approach the wall of the well, you should add more water to help it move away from the wall.</td>
<td>The replicas should be constantly observed during this</td>
<td></td>
</tr>
</tbody>
</table>

UNCLASSIFIED
<table>
<thead>
<tr>
<th>3.D</th>
<th>Section has too many wrinkles on the grid</th>
<th>Grids are hydrophobic</th>
<th>Use glow discharge to make grids hydrophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.D.iii</td>
<td>Hydrofluoric acid solution flows over sections</td>
<td>Excessive hydrofluoric acid used.</td>
<td>Use less hydrofluoric acid solution. Tilt the coverslip at a slight angle and let the hydrofluoric acid slowly seep under the pioloform membrane.</td>
</tr>
<tr>
<td>3.D.iv</td>
<td>Some of the pioloform still adheres to the coverslip</td>
<td>Coverslip not scored cleanly.</td>
<td>When scoring the coverslip, use a ruler to help score a clean line.</td>
</tr>
<tr>
<td>3.D.v</td>
<td>Pioloform film moves too much on water surface</td>
<td>Too much airflow in the room.</td>
<td>Minimize airflow in the room by working away from vents and limiting the movement of people and equipment.</td>
</tr>
<tr>
<td>3.D.xiv</td>
<td>Dirty precipitate on grid</td>
<td>Precipitation of lead.</td>
<td>Lead reacts with carbon dioxide. Avoid airflow as much as possible during the lead staining procedure. Ensure grid is dry before touching the lead solution.</td>
</tr>
<tr>
<td>4</td>
<td>Cellular specimen does not register to the corresponding fluorescent image with high fidelity, but Au fiducials are registered well and with a very small uncertainty.</td>
<td>The SEM sample preparation and dehydration procedure caused movement of the cellular specimen with respect to the coverslip (Au fiducial) surface.</td>
<td>Thick portions of the specimen often experience more movement on the nanometer scale upon desiccation. Choose regions of the cell to image by fluorescence that are thinner and less prone to movement/distortion upon desiccation.</td>
</tr>
</tbody>
</table>
| 4 | Poor image registration or high residual uncertainty in SEM/fluorescence image alignment (based on fiducial alignment). | Au nanoparticle density is too low or non-reproducible | Choose region of interest during fluorescence image acquisition that is surrounded by many (>20) surface-exposed Au fiducials. Plating cells at lower density can help find unobstructed fields of view with detectable fiducials. Use new stock of Au nanoparticles. Au nanoparticles have a shelf life of a few months (80 nm nanospheres) to a few weeks (25 x 75 nm and 25 x 57 nm nanorods). Put a higher concentration of
gold on your coverslips. Make sure the coverslips are cleaned properly. Do not recycle coverslips.

Make fresh 0.1% poly-L-Lysine solution (shelf life ~ 1 week) so that Au nanoparticles adhere to coverslip.

<table>
<thead>
<tr>
<th>All fiducials are confined to one region of the image, biases the registration towards that region.</th>
<th>Choose cells of interest by fluorescence that have even (non-clustered) spatial distributions of fiducials.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4</strong> Registration of FIB-SEM and iPALM data of Tokuyasu cryosections is not accurate</td>
<td>Shrinkage of sample Tokuyasu cryosections are subject to significant shrinkage in the z-dimension during dehydration. Try using CPD or HMDS drying or place fiducials on top of the section.</td>
</tr>
</tbody>
</table>
Figure 1. Flowchart of the protocols featured in this paper. See text and Table 1 for the details, advantages and limitations, and anticipated results for each procedure.

Figure 2. Example of using gold nanoparticles to align fluorescence and electron microscopy datasets. (a) Scanning electron micrograph where gold nanoparticles can be observed as white dots (arrows) due to their high electron densities. (b) Fluorescence signal from gold nanoparticles (arrows) with a DIC image showing cell outlines. Note this image shows diffraction-limited fluorescence for illustration purposes as a super-resolution fluorescence rendering would make the gold nanoparticles difficult to see. Scale bars = 5 μm. Figure adapted from18.

Figure 3. Examples of anticipated results. (a-c) Examples of CLEM using Tokuyasu cryosectioning adapted from18,19. These images show the localization of the mitochondrial DNA binding protein (mitochondrial transcription factor A, TFAM) genetically fused to the paFP mEos2 rendered in red or magenta. (b-c) Three-dimensional CLEM showing an x-y plane in (b) and a y-z plane in (c) sectioned through the volume as indicated by the hash marks in (b). (d) Example results for whole-cell mount method showing correlated iPALM volume of HIV Gag protein fused to mEos2 and SEM. (e) Example result for unroofed cells/platinum replica using iPALM image of fluorescently labeled clathrin correlated with an electron tomogram. (f) Example of resin-embedded sample showing OsO4-resistant mEos4a with a mitochondrial matrix localization signal correlated to TEM. Scale bars = 500 nm.

Box 2, Figure 1. Sample holders for fluorescent microscopy (a) and for SEM (b), which allow for precise referencing of the imaged areas. The coverslip has a notch ground using a 1/16” diamond bit near its top (indicated by a red arrow in the inset). Both sample holders have two fixed 1/16” posts (indicated by green arrows), which assure unambiguous sample placement, allowing for precise coordinate referencing. (c) 10x DIC image of a Tokuyasu cryosection of NIH 3T3 mouse fibroblast cells used for correlative PALM and EM imaging. This can be used as a look-up map to find the area imaged in PALM when performing EM. (d) 120x DIC image of the area imaged during the PALM step (area inside the box in (c)).

Box 2, Figure 2. Identifying imaged areas when using platinum replicas. See text for details of each step. Adapted from22.
References

39. Zhang, M. et al. Rational design of true monomeric and bright photoactivatable


