Chapter 22

Nanometer-Resolution Fluorescence Electron Microscopy (Nano-EM) in Cultured Cells

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Abstract

Nano-resolution fluorescence electron microscopy (nano-fEM) pinpoints the location of individual proteins in electron micrographs. Plastic sections are first imaged using a super-resolution fluorescence microscope and then imaged on an electron microscope. The two images are superimposed to correlate the position of labeled proteins relative to subcellular structures. Here, we describe the method in detail and present five technical advancements: the use of uranyl acetate during the freeze-substitution to enhance the contrast of tissues and reduce the loss of fluorescence, the use of ground-state depletion instead of photoactivation for temporal control of fluorescence, the use of organic fluorophores instead of fluorescent proteins to obtain brighter fluorescence signals, the use of tissue culture cells to broaden the utility of the method, and the use of a transmission electron microscope to achieve sharper images of ultrastructure.

Key words Nano-fEM, Protein localization, Correlative light and electron microscopy, Fluorescence electron microscopy, Super-resolution fluorescence microscopy, Fluorescence nanoscopy, Photoactivated localization microscopy (PALM), Ground-state depletion and single-molecule return (GSDIM), Stochastic optical reconstruction microscopy (STORM), Direct stochastic optical reconstruction microscopy (dSTORM)

1 Introduction

Proteins play essential roles in cellular functions; to fully understand their roles, we must know where they are localized relative to subcellular structures. Previously, there have been two methods for localizing proteins to subcellular structures: immuno-electron microscopy and fluorescence microscopy. Immuno-electron microscopy localizes proteins at an ultrastructural level using gold-labeled antibodies [1–5]. However, it has many drawbacks, including a reliance on antibodies that can work on plastic sections, poor survival of epitopes, poor preservation of morphology, high nonspecific background,

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and a spatial displacement of up to 38 nm (the molecular length of two antibodies) [1, 2, 6]. Moreover, only a minority of the proteins are labeled due to the inaccessibility of antigens in the plastic.

Fluorescence microscopy, on the other hand, has become the method of choice for protein localization. In particular, genetic tagging of proteins with green fluorescent protein (GFP) [7–9] is very useful because every protein is labeled and can be imaged in living cells. However, two drawbacks limit its ability to locate proteins in a cell: resolution and cellular context. The light from a fluorescent protein can be only focused to a 450 nm spot on a camera. If there were only two proteins in an image, they could theoretically be distinguished if they were separated by greater than 250 nm, but typically many proteins are labeled and the image is really no more than an incoherent micron-sized ghost at high magnification. Thus, the diffraction limit means that proteins, typically on the order of 4 nm in diameter (the diameter of GFP), cannot be associated with cellular organelles, which are on the order of 40 nm in diameter (such as synaptic vesicles or Golgi cisternae). In the last few years, several super-resolution techniques have been developed that can resolve fluorescence at resolutions below the diffraction limit [10–16]. Single-molecule-based techniques include photoactivated localization microscopy (PALM) [10], fluorescence photoactivation localization microscopy (fPALM) [11], stochastic optical reconstruction microscopy (STORM) [13], direct stochastic optical reconstruction microscopy (dSTORM) [14], and ground-state depletion microscopy followed by individual molecule return (GSDIM) [12]. Despite the variations in acronyms, these techniques overcome the diffraction barrier by imaging single molecules one at a time [17]. The location of individual molecules is then calculated and mapped to reconstruct an image. In this chapter, we will use the terms PALM, for imaging individual fluorescent proteins, and dSTORM, for imaging organic fluorophores using ground-state depletion (*see* **Note 1**). These imaging techniques break the diffraction limit of conventional fluorescence microscopy and can localize a protein with a precision of 10 nm standard error of the mean (SEM) [18, 19] (*see* **Note 2**).

The second drawback with fluorescence microscopy is that it lacks cellular context. A precisely localized pinpoint of fluorescence in a black background is not useful. Combining these new subdiffraction fluorescence methods with electron microscopy merges the protein localization advantages of fluorescence microscopy with the structural definition of electron microscopy [20].

To localize GFP in ultrathin sections, one must maintain GFP fluorescence during the harsh osmium fixation steps, the dehydration steps, and the incubation and polymerization in acidic resins. Recently, we developed a method to preserve fluorescence by reducing fixative concentrations, maintaining hydration, and compromising on ideal polymerization conditions [20]. In short, transgenic worms

expressing proteins tagged with fluorescent proteins are subject to high-pressure freezing and freeze-substitution. These animals are embedded into glycol methacrylate resin at –20 °C. Ultrathin sections are cut using a microtome and collected onto pre-cleaned cover glasses. These sections are first imaged on a super-resolution light microscope and then post-stained with uranyl acetate and imaged using backscattered electrons on a scanning electron microscope (SEM). Using this approach, we successfully localized histones, mitochondrial membrane proteins, and liprin-alpha to their proper organelles in a cell [20]. However, several improvements can be implemented to increase the sensitivity and the utility of our methods.

There are five problems associated with the original protocol we published. First, ~40 % of fluorescent proteins were lost during the fixation. Second, photoactivatable fluorescent proteins suitable for PALM imaging are not ideal. EosFP and its variants are the most reliable fluorophores for PALM. However, they emit green fluorescence before photo-conversion and red fluorescence after photo-conversion, covering two large spectra of visible light. Thus, multicolor imaging is extremely difficult. Third, the number of photons collected from each EosFP molecule before bleaching is very low (~300), reducing the calculated precision of the protein location by the computer. Fourth, the protocol was originally developed using the nematode *Caenorhabditis elegans*, optimal conditions for other biological samples such as cultured cells were not known. Fifth, despite the well-preserved morphology, the resolution of images generated by collecting backscattered electrons in a scanning electron microscope is limited by the size of the electron beam scanning the sample and leads to blurry images at high magnification (*see* Fig. 1a, c).

Therefore, since the original publication, we have improved our methods to solve these five problems. First, we fine-tuned our protocol to preserve up to 90 % of the fluorescence without compromising the fixation quality [21]. Second, to overcome the paucity of suitable fluorescent proteins with PALM imaging and demonstrate the potential for the method to localize multiple proteins at a time, we imaged the fluorescent protein, citrine, that is not photoactivatable or photoconvertible using a ground-state depletion scheme [21]. Third, we labeled with an organic fluorophore, Alexa-647, capable of generating many more photons and hence higher precision, and imaged using a dSTORM scheme although this approach is not quantitative (*see* Fig. 2a, b and **Note 3**). Fourth, we demonstrated that this method could be readily applied to specimens other than *C. elegans* using the protocol (*see* Fig. 2). Fifth, we developed a method to image using a transmission electron microscopy to provide higher-resolution details of ultrastructure (*see* Figs. 1 and 2). Here, we describe the improved protocol in detail.

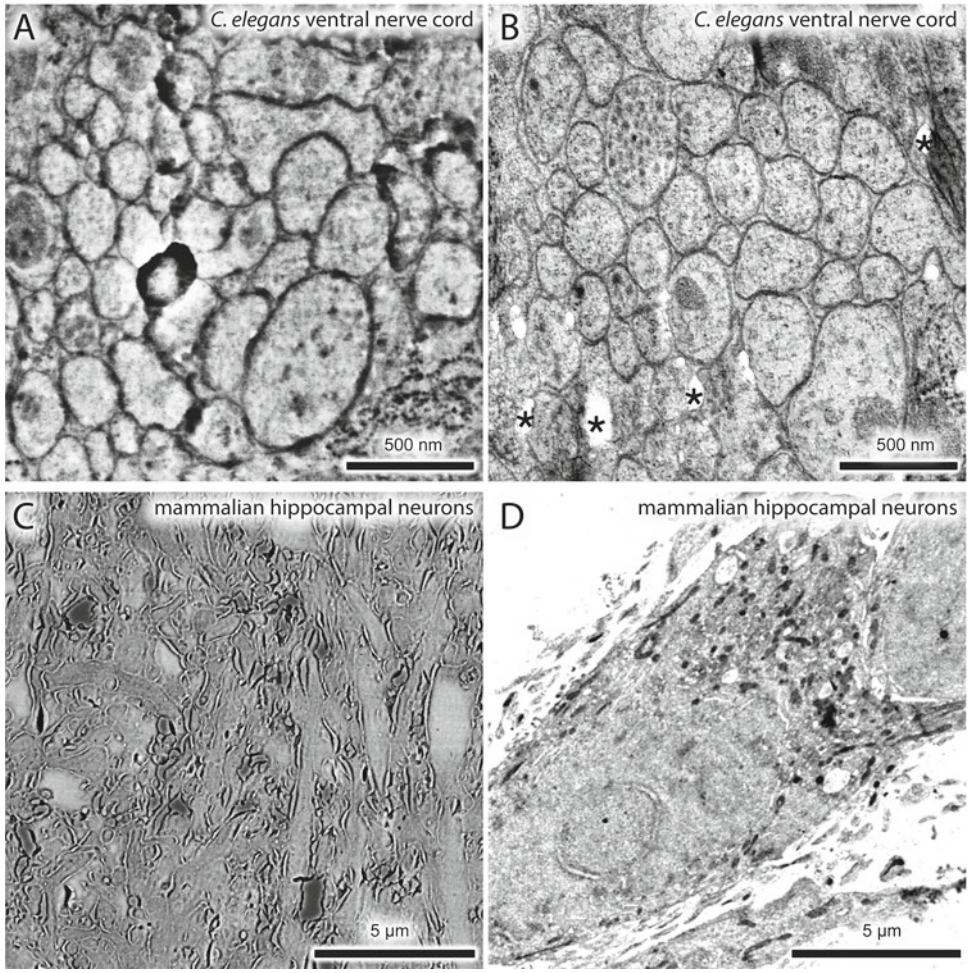

Fig. 1 Comparisons between SEM and TEM images. A nematode expressing a fluorescently labeled transgene was processed for nano-fEM, and the adjacent sections were imaged using SEM (**a**) and TEM (**b**). Mouse hippocampal neurons were labeled with Alexa-647 transferrin. Labeled transferrin binds transferrin receptors and is endocytosed. Cells were processed for nano-fEM and imaged using SEM (**c**) and TEM (**d**). The subcellular details are clearly visible in the TEM images. *Asterisk* indicates holes in a section created during sectioning due to incomplete infiltration or polymerization of the GMA resin. For the complete list of artifacts observed with GMA plastic, please refer to Watanabe and Jorgensen [21]

2 Materials

2.1 High-Pressure Freezing for C. elegans

1. High-pressure freezer (Baltec HPM010, ABRA/Boeckeler Instruments Inc., Tucson, AZ; *see* **Note 4**).
2. Specimen carriers type A (Technotrade International, Manchester, NH).
3. Specimen carriers type B (Technotrade International, Manchester, NH).

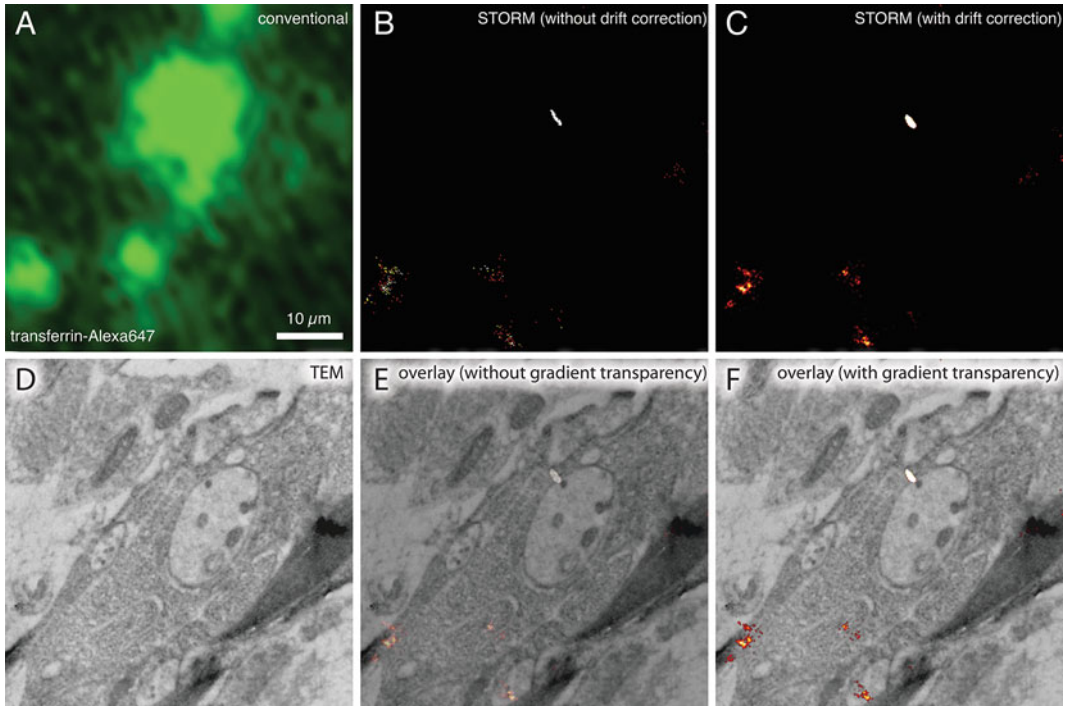

Fig. 2 dSTORM images of plastic sections of cultured mouse primary hippocampal cells with fluorescently labeled transferrin. Wide-field fluorescence image (**a**), dSTORM image without drift correction (**b**), dSTORM image with drift correction (**c**), and TEM image (**d**) from the same GMA thin section (50 nm) of cell cultures incubated with Alexa647-labeled transferrin. The sample drift during imaging was corrected based on the positions of TetraSpeck beads in the first image of the acquired series. (**e**, **f**) Correlative dSTORM—TEM image showing transferrin localization. The transparency was applied to the whole STORM image in (**e**), whereas the transparency was applied only to the background black pixels in the STORM image in (**f**). Note that the STORM signal intensity is not compromised using a gradient transparency in (**f**). The section was incubated in imaging buffer containing oxygen scavengers and imaged using a fluorescence microscope equipped with an EMCCD camera and appropriate laser lines and filter sets. For the dSTORM image, a total of 10,000 frames (30 ms/frame) were collected and processed using ImageJ with the QuickPALM plug-in [27]

4. Hexadecene (Sigma-Aldrich, St. Louis, MO).
5. Forceps.
6. Paintbrush #00.
7. Whatman filter paper.
8. Liquid nitrogen (160 l).

2.2 High-Pressure Freezing for Cell Cultures

1. High-pressure freezer (HPM100, Leica Microsystems, Wetzlar, Germany; *see* **Note 5**).
2. Sapphire disks (6 mm diameter, Wohlwend GmbH, Sennwald, Switzerland).
3. Specimen carriers type A (6 mm diameter, Leica Microsystems, Wetzlar, Germany).

4. Culture media: DMEM medium (90 % DMEM, 10 % FCS, 10 μg/ml penicillin, and 1 μg/ml streptomycin), FUDR stock solution (19.94 mg 5-fluoro-2'-deoxyuridine, 49.82 mg uridine, and 10 ml DMEM), and NBA medium (97 % NBA, 2 % B27, 1 % Glutamax, 10 μg/ml penicillin, and 1 μg/ml streptomycin).
5. Recording media: BASE+(140 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$, 4 mM $MgCl_2$; pH adjusted to 7.5–7.6; osmolarity adjusted to ~300 mOsm).
6. Transferrin from human serum, Alexa Fluor 647 Conjugate (Invitrogen, Grand Island, NY).
7. Sapphire disk holder middle plate (6 mm diameter, Leica Microsystems, Wetzlar, Germany).
8. Sample holder half cylinder (6 mm diameter, Leica Microsystems, Wetzlar, Germany).
9. Forceps (insulated).
10. Ethanol (absolute).
11. Liquid nitrogen.
12. Whatman filter paper.

2.3 Freeze-Substitution

1. Automated freeze-substitution unit (AFS 2, Leica Microsystems, Wetzlar, Germany).
2. Cryovials.
3. 50 ml screw cap conical tubes.
4. 15 ml screw cap conical tubes.
5. Acetone (glass distilled, Electron Microscopy Sciences, Hatfield, PA).
6. Ethanol (absolute).
7. Osmium tetroxide (crystals, 1/10 g, Electron Microscopy Sciences, Hatfield, PA).
8. Potassium permanganate (Electron Microscopy Sciences, Hatfield, PA).
9. Uranyl acetate (Polysciences, Warrington, PA; *see* **Note 6**).
10. Disposable transfer pipette (7.5 ml).
11. Disposable transfer pipette (1.5 ml).
12. Disposable Pasteur pipette (borosilicate glass).
13. Pipetman and tips (P1000, P200, P20).

2.4 Plastic Embedding

1. Glycol methacrylate kit (GMA, 2-hydroxyethyl methacrylate, low acid and TEM grade, SPI, West Chester, PA).
2. *N,N*-Dimethyl-*p*-toluidine (EMD Chemicals, Philadelphia, PA).
3. Scintillation vials with screw caps (20 ml, Electron Microscopy Sciences, Hatfield, PA).

4. ACLAR film (Electron Microscopy Sciences, Hatfield, PA).
5. BEEM capsule (polypropylene, EBSciences, East Granby, CT; *see* **Note 7**).
6. 3/8" disk punches (Ted Pella Inc., Redding, CA).
7. 25 ml serological pipette.
8. 10 ml serological pipette.
9. Petri dish (35 mm × 10 mm).

2.5 Cover Glass Cleaning

1. Cover glass (#1.5, round, Warner Instruments, Hamden, CT).
2. Sulfuric acid.
3. Hydrogen peroxide.
4. Cover glass rack (Teflon; Invitrogen, Grand Island, NY).
5. Sonicator.
6. Glass beaker (500 ml).

2.6 Cover Glass Coating

1. Microarray high-speed centrifuge (Arrayit, by Microarray Technology, Sunnyvale; *see* **Note 8**).
2. Pioloform (Ted Pella Inc., Redding, CA).
3. Chloroform.
4. Wax.

2.7 Sectioning

1. Ultramicrotome (UC6, Leica Microsystems, Wetzlar, Germany).
2. Diamond knife (ultra jumbo, 45°, 4.0 mm; DiATOME, Hatfield, PA).
3. Glass strips (Ted Pella Inc., Redding, CA).
4. Glass knife boats (Ted Pella Inc., Redding, CA).
5. Nail polish (clear).
6. Perfect loop (Electron Microscopy Sciences, Hatfield, PA).
7. Hair tool for manipulation of plastic sections.
8. Razor blade (double edge; Electron Microscopy Sciences, Hatfield, PA).
9. High-profile microtome blades (Leica Microsystems, Wetzlar, Germany).

2.8 PALM Imaging

1. Zeiss PALM microscope (ELYRA P.1, Zeiss, Jena, Germany; *see* **Note 9**).
2. Gold nanoparticles (call for 2× concentrated solution; Microspheres-Nanospheres Inc., Cold Spring, NY).
3. Canned air.
4. Attofluor cell chamber for microscopy (Invitrogen, Grand Island, NY).

2.9 Cover Glass Mounting for dSTORM Imaging

1. Twinsil (two-component silicone glue, Picodent, Wipperfürth, Germany).
2. Concave blank microscope slides (Celestron, Torrance, CA).
3. Imaging buffer (50 mM Tris (pH 8.0), 10 mM NaCl, 0.5 mg/ml glucose oxidase, 40 μg/ml catalase, 10 % (w/v) glucose, and 10 mM MEA).

2.10 dSTORM Imaging

1. Zeiss PALM microscope (ELYRA P.1, Zeiss, Jena, Germany; *see* **Note 9**).
2. TetraSpeck fluorescent microspheres (0.1-μm diameter, Invitrogen, Grand Island, NY).

2.11 Staining

1. Uranyl acetate (Polysciences, Warrington, PA).
2. Syringe (10 ml).
3. Syringe filter (0.22 μm; Millipore, Billerica, MA).

2.12 Retrieval of Sections from Cover Glass

1. Hydrofluoric acid (Sigma-Aldrich, St. Louis, MO).
2. Parafilm.

2.13 SEM Imaging

1. FEI Nova Nano (FEI, Hillsboro, OR; *see* **Note 10**).
2. Backscattered electron detector (vCD, FEI, Hillsboro, OR).
3. Carbon fiber cord (Electron Microscopy Sciences, Hatfield, PA).
4. Carbon sputter device (SCD005, Baltec).
5. SEM pin stub (Ted Pella Inc., Redding, CA).
6. Carbon conductive tape (double coated; Ted Pella Inc., Redding, CA).

2.14 Image Alignment

1. Photoshop (CS6, Adobe, San Jose, CA).

3 Methods

3.1 Preparing Sapphire Disks for Culturing Cells

1. Wash the disks in ethanol (absolute) and let them dry in air.
2. Apply a thin layer (10–30 nm) of carbon coat using a carbon sputter [22, 23]. The sapphire disk should be fairly dark. Note that cells will be cultured on the carbon-coated surface.
3. To keep a track of the carbon-coated surface during the subsequent manipulation, mark the carbon-coated surface by scratching out an asymmetrical letter such as “F” or “G” [24]. If finding particular cells (e.g., fluorescence-positive cells) is required, a finder grid (Leica Microsystems) can be placed on top of the sapphire disk to generate a specific pattern on the surface when carbon coating (*see* Fig. 3a, b).

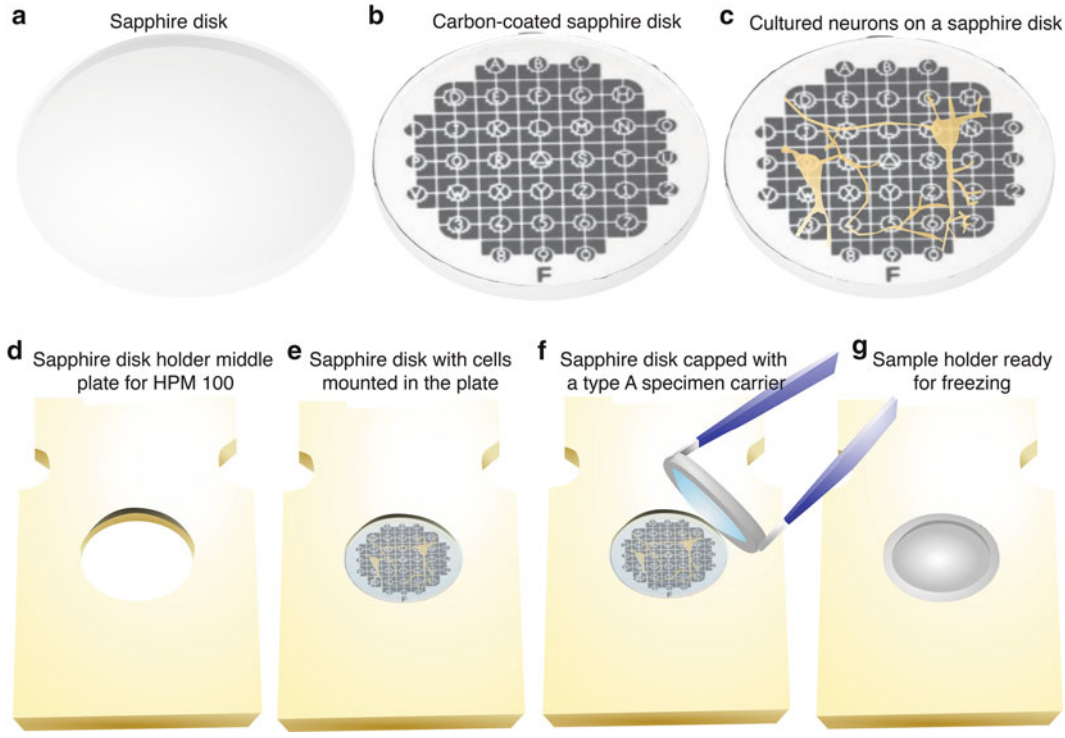

Fig. 3 Preparing cell cultures for high-pressure freezing. (**a**) 6 mm-diameter sapphire disks are rinsed in ethanol before use. (**b**) A carbon-coated sapphire disk with a finder grid pattern. A finder grid was placed on top of a sapphire disk before carbon coating. (**c**) Mouse hippocampal neurons cultured on a coated sapphire disk after the treatment with poly-L-lysine. Cells can grow as well on sapphire disks as on a cover glass. (**d**) A middle plate for the HPM100 that can hold a sapphire disk. (**e**) A sapphire disk mounted directly in the plate. Note that the countersink holds the sapphire disk in place. (**f**) A type A specimen carrier, overfilled with culture media, placed on top of the sapphire disk. Make sure there are no air bubbles trapped in the chamber of the specimen carrier. (**g**) A sample holder ready for freezing

4. Bake the disks in an oven (120 °C) overnight.
5. Place sapphire disks into a 12-well culture plate (we typically place two in each well), apply poly-L-lysine for 5 min, and let it dry for 1 h.
6. Apply ultraviolet light for 20 min.
7. The sapphire disks are now ready for culturing. For culturing neuronal cells, we followed a protocol described in Pyott and Rosenmund [25]. Follow the appropriate protocol for your particular cell type (*see* Fig. 3c).

3.2 Transferrin-647 Uptake

1. To prepare transferrin conjugates, we followed a protocol provided by Invitrogen (http://tools.invitrogen.com/content/sfs/manuals/mp02871.pdf). In short, we mixed 5 mg transferrin-647 with 1 ml deionized water.
2. Dilute the transferrin-647 solution to 5 ng/ml by adding 1 µl of the solution into 1 ml of recording media (BASE +).

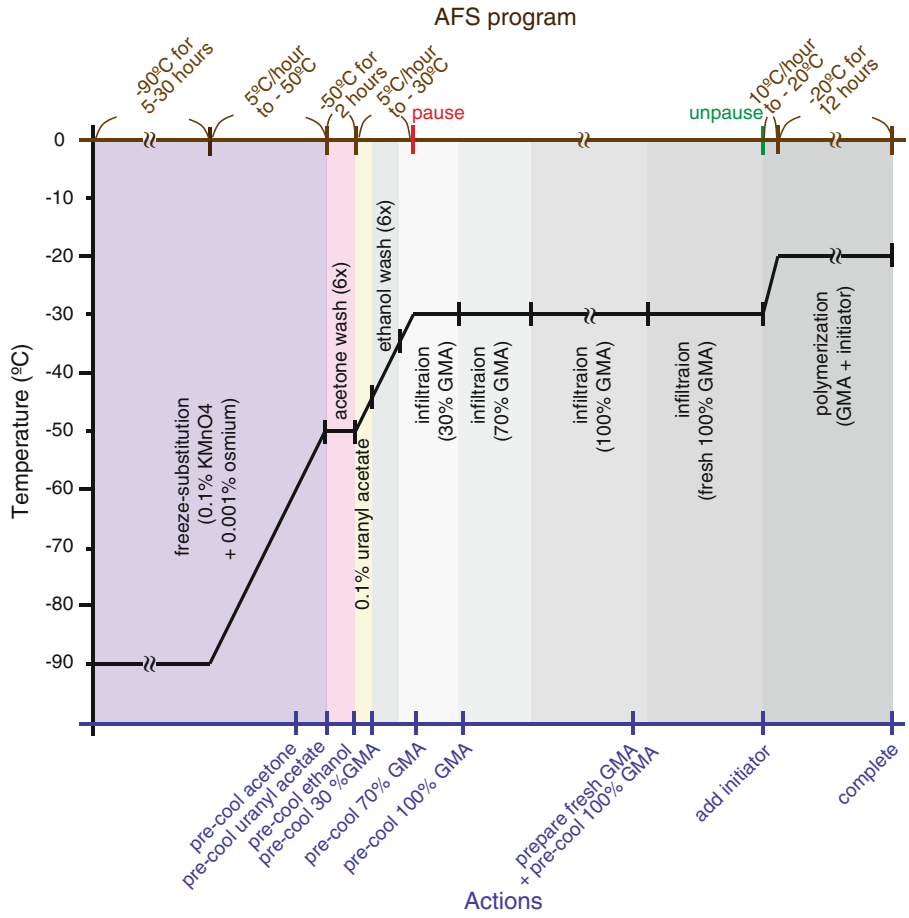

Fig. 4 An overview of freeze-substitution and plastic embedding incubations for the AFS program. The *y-axis* of the graph shows temperature. The *black line* in the graph indicates the temperature change through the freeze-substitution and plastic embedding procedures. The graph is shaded with colors to indicate different solutions used in the particular steps: *purple*, 0.1 % KMnO4 + 0.001 % osmium tetroxide + 5 % water + anhydrous acetone; *pink*, 95 % acetone; *yellow*, 0.1 % uranyl acetate in 95 % acetone; *orange*, 95 % ethanol; and *gray*, GMA solutions. The *brown-colored x-axis* at the top shows how to program the AFS (–90 °C for 5–30 h, 5 °C/h to –50 °C, –50 °C for 2 h, 5 °C/h to –30 °C, pause/unpause at –30 °C, 10 °C/h to –20 °C, and –20 °C for 12 h). The *blue-colored x-axis* at the bottom indicates the actions one should take at a particular time point in the program

3. Apply the diluted solution to cell cultures for 2 h.
4. Wash cells with recording media several times.

3.3 High-Pressure Freezing

1. Prepare the automated freeze-substitution device (Leica AFS2) by filling the tank with liquid nitrogen, programming the freeze-substitution protocol as shown in Fig. 4, and initiating the program. Note that the program must be paused while the chamber is cooled down to –90 °C and unpaused when all the samples are loaded into the chamber.
2. Prepare the fixatives (20 ml) by adding 1 ml milliQ water, 20 mg potassium permanganate, and 20 ml osmium tetroxide

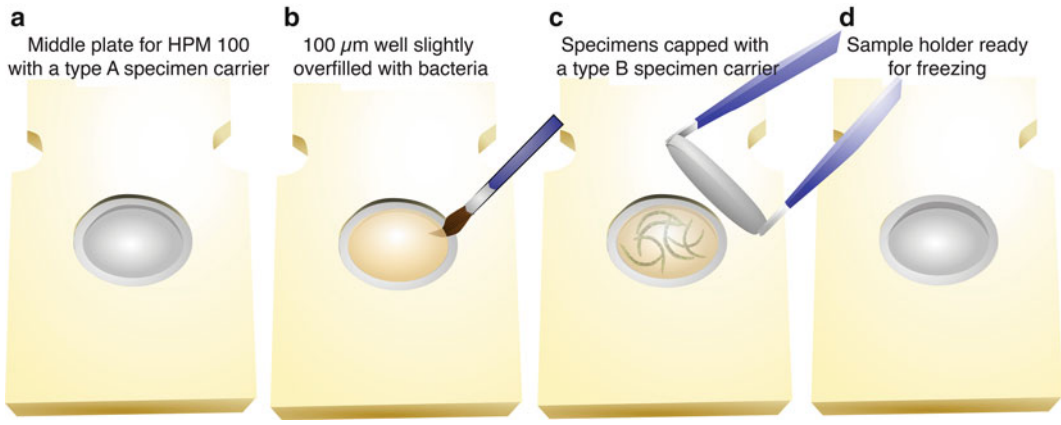

Fig. 5 Preparing *C. elegans* for high-pressure freezing. (**a**) A type A specimen carrier (3 mm diameter) mounted in the middle plate for the HPM100 freezer with its 100 μm well facing up. (**b**) A 100 μm well of a specimen carrier filled with bacteria using a paint brush. Note that the well is slightly overfilled. (**c**) The specimen carrier capped with a flat side of a type B specimen carrier after placing the worms in the well. (**d**) A sample holder ready for freezing

(1 % in anhydrous acetone; *see* **Note 11**) into a 50 ml conical tube and filled with anhydrous acetone (*see* **Note 12**) up to the 20 ml mark. Aliquot 1 ml of this solution into each cryovial, and submerge the cryovials into liquid nitrogen immediately. Store the vials under liquid nitrogen until use.

3. Mount the specimens for high-pressure freezing (HPM 100; for other high-pressure freezers; *see* **Note 13**). For *C. elegans*, fill a 100 μm well of a type A specimen carrier (3 mm diameter; *see* Fig. 5a) with bacteria using a paint brush (*see* Fig. 5b), and place transgenic animals on top of the bacteria (*see* Fig. 5c). Make sure the well is slightly overfilled. Cap the well with the flat side of a type B specimen carrier (3 mm diameter; *see* Fig. 5c, d). For cell cultures, mount a sapphire disk (6 mm diameter) directly onto a sapphire disk holder middle plate (*see* Fig. 3d, e). Make sure the surface that cells are cultured on faces up. Dip a 100 μm well side of a type A specimen carrier into the recording solution so that the well is filled (overfilled) with the solution (*see* Fig. 3f). Place the specimen carrier gently onto the sapphire disk (*see* Fig. 3f, g). Now, specimens are ready for freezing (*see* Figs. 3g and 5d).
4. Freeze specimen according to the instructions provided by the instrument's manufacturer.

3.4 Freeze-Substitution and Plastic Embedding

1. Once frozen, transfer specimens into a cryovial containing fixatives under liquid nitrogen.
2. Place the cryovials in the chamber of AFS.
3. Unpause the program.

4. When the temperature reaches −60 °C, place a scintillation vial containing 95 % acetone (made up of 5 % milliQ water and anhydrous acetone) into the chamber.
5. When the temperature reaches −50 °C, remove the solutions from each vial and add precooled 95 % acetone using a precooled glass pipette. Repeat the process five more times with each spaced by 15 min. Place a scintillation vial containing 0.1 % uranyl acetate in 95 % acetone to precool the solution.
6. At the end of **step 5**, replace acetone in the vials with 1 ml of precooled 0.1 % uranyl acetate solution. Place a scintillation vial containing 95 % ethanol (made up of 5 % milliQ water and anhydrous acetone).
7. After 1 h incubation with uranyl acetate, replace the solution and wash specimens with 95 % ethanol (*see* **Note 12**) for six times over 2 h. Note that **steps 8–10** should be carried out concurrently with this step.
8. Prepare GMA resin for infiltration by mixing 22.3 ml glycol methacrylate, 10 ml butyl methacrylate, 1 ml milliQ water, and 0.2 g benzoyl peroxide (*see* **Note 14**). Do not prepolymerize the GMA resin as instructed by the manufacturer. These components are simply mixed, and no further processing is required before use.
9. Prepare 30 and 70 % GMA resin by mixing GMA resin with 95 % ethanol.
10. Place a scintillation vial containing 30 % GMA resin in the chamber of AFS at least 1 h before use.
11. At the last step of ethanol washing (**step 7**), replace ethanol with precooled 30 % GMA resin. Incubate the specimens in 30 % resin for 3–5 h (*see* **Note 15**). Place a scintillation vial containing 70 % GMA resin in the chamber of AFS.
12. Replace 30 % resin with 70 % resin, and incubate specimens for 4–6 h (*see* **Note 15**). Place a scintillation vial containing 100 % GMA resin in the chamber of AFS.
13. Replace 70 % resin with 100 % resin and incubate specimens overnight.
14. On the next day, transfer specimens into caps of BEEM capsules (*see* **Note 16**). Fill the embedding cap with 100 % resin immediately after the transfer.
15. Replace the resin two additional times with each change spaced at 2 h.
16. To polymerize GMA resin, add *N,N*-dimethyl-*p*-toluidine into the GMA resin at 1.5 μl/1 ml GMA resin (*see* **Note 17**). Replace the GMA resin in the embedding cap with the GMA resin with *N,N*-dimethyl-*p*-toluidine.

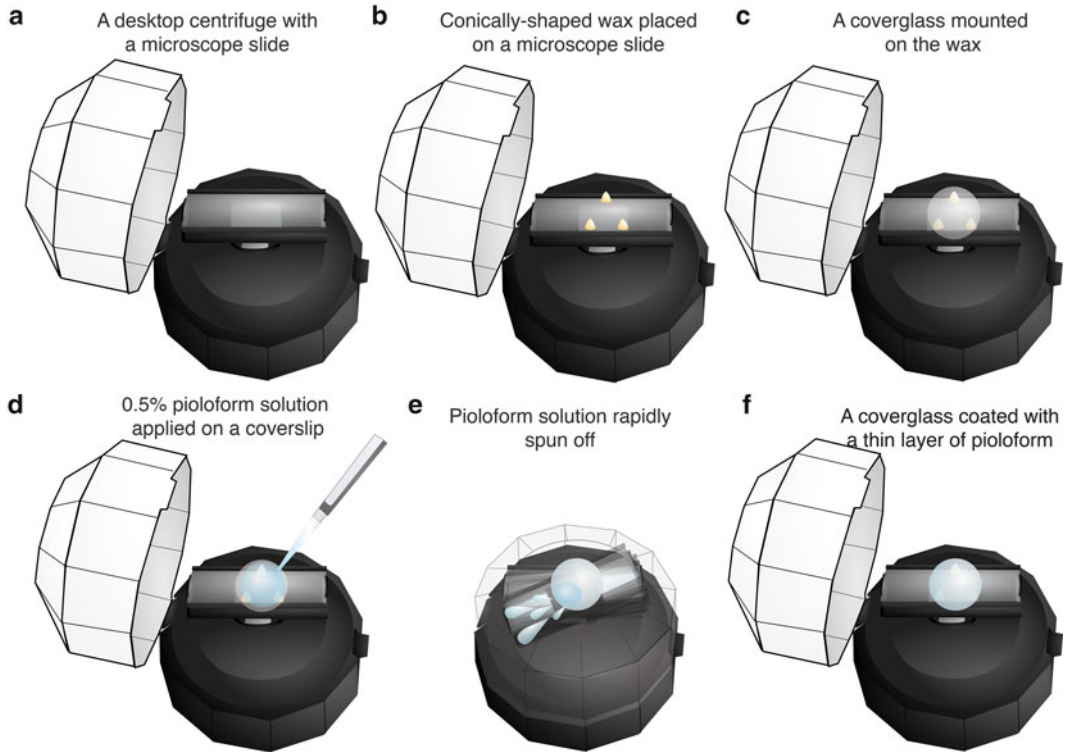

Fig. 6 Coating a cover glass with TEM-compatible film (Pioloform). (**a**) A microscope slide inserted into a desktop centrifuge. (**b**) Three wax cones placed on a cover glass to serve as a mount for the cover glass. Note that the wax cones are placed so that the center of the cover glass does not touch the wax. (**c**) A round cover glass placed on top of the wax cones. (**d**) 0.5 % Pioloform solution (250 ml) applied on a coverslip. (**e**) Pioloform solution spun off by closing the lid immediately after the application of Pioloform solution. (**f**) A cover glass coated with a thin layer of Pioloform

17. Allow the plastic to polymerize overnight, even though reaction is complete within 30 min (*see* **Note 18**).
18. If not processed immediately, samples should be wrapped in foil and stored in a vacuum bag at –20 °C.

3.5 Cover Glass Cleaning and Coating

1. Immerse cover glasses in piranha solution (three parts sulfuric acid and one part hydrogen peroxide) for 1 h (*see* **Note 19**).
2. Rinse cover glasses with milliQ water for six times.
3. Sonicate cover glasses for 30 min.
4. Rinse cover glasses with milliQ water for six times.
5. Dry cover glasses in air.
6. To coat a cover glass with a film compatible with TEM imaging, mount a cover glass in a desktop centrifuge as shown in Fig. 6a–c.

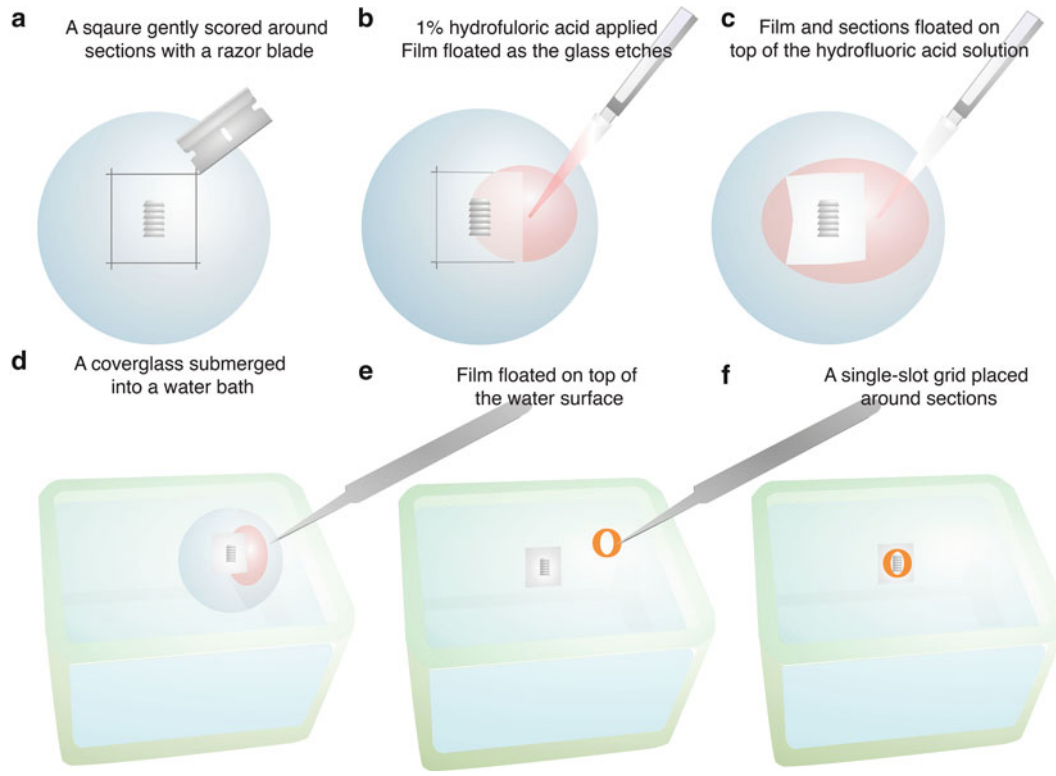

Fig. 7 Retrieving sections from a cover glass for TEM imaging. (**a**) A Pioloform film scored using a razor blade. The cuts should be made far from where the sections are located. (**b**) 1 % hydrofluoric acid applied to the cover glass. Note that the solution is applied gently from the side of the cover glass. The glass will be etched slightly as soon as the solution is applied, causing the film to float onto the solution. (**c**) A film containing sections fully detached from the cover glass and floated on top of the solution. (**d**) A cover glass submerged into a water bath. (**e**) The film floating on top of the water surface. (**f**) A single-slot grid placed on top of the film so that the sections appear inside the window of the grid

7. Apply 250 μl of 0.7 % Pioloform solution (in chloroform) to a cover glass (*see* Fig. 6d), and spin it off immediately (*see* Fig. 6e). This treatment leaves a thin layer of Pioloform on top of the cover glass (*see* Fig. 6f). The film along with sections can be retrieved after the super-resolution imaging *(see* Fig. 7).

3.6 Sectioning

1. Sectioning is carried out at room temperature. Extra cautions should be paid not to expose the specimens to sunlight or bright room light. Locate a region of interest (*see* **Note 20**), and trim the block so that the sectioning surface is in a trapezoid shape with a dimension of less than 100 μm high and 1 mm long.
2. Set up the microtome as instructed by the manufacturer. Mount the specimens on the microtome.
3. After aligning the specimen with a diamond knife, start cutting 50–70 nm-thick sections with the cutting speed set at 1.6 mm/s.

4. After cutting a sufficient number of sections, collect sections onto a cover glass with or without the Pioloform film by immersing the cover glass into a water bath, touching the edges of sections to the center of the cover glass, and sliding the cover glass gently out of the water bath.
5. Place the cover glass in a dark place, and allow it to air dry. Once dried, store the cover glasses at −20 °C in a vacuum bag until further processing.

3.7 PALM Imaging of tdEos

1. Place a small drop (~50 μl) of 100 nm gold nanospheres solution (1.2×10^{10} particles/ml; *see* **Note 21**) directly on top of the sections. After 30–60 s, blow the drop to the edge of the cover glass and blot with a Kimwipe. Gold nanospheres serve as fiducial markers for aligning fluorescence and electron micrographs and can also be used to correct sample drift during image acquisition.
2. Hydrate the sections for 2 min by placing a small drop of milliQ water directly on top of the sections, then blow to the edge of the cover glass and blot.
3. Start PALM microscope and associated software. 561, 488 and 405 nm lasers should be turned to standby mode.
4. Load cover glass into a cover glass holder (cell chamber), sections facing up, and place on microscope stage over the low-power objective. If no cover glass holder is available, the cover glass can be fixed to a microscope slide by a few spots of nail polish. The sections should be sandwiched between the cover glass and the slide, and the assembly placed into the microscope so the cover glass is towards the objective. Apply immersion oil on the cover glass.
5. Using wide-field illumination and a low-power objective, locate the sections.
6. Switch to the high-power TIRF objective. Low-power 488 nm laser light can be used to locate a region of interest, but this should be performed only briefly and only if necessary, as it will reduce the available fluorescence signal during image acquisition.
7. Using maximum intensity 561 nm light, illuminate the region of interest for ~2 min to bleach the autofluorescence.
8. For image acquisition, use minimum intensity 405 nm illumination to activate fluorophores and maximum intensity 561 nm illumination for readout. The frame rate should be set to 20 Hz or greater. We typically collect 6,000 frames for each region, but this number can be adjusted to suit the specifics of the experiment. For example, if recording of every molecule in the region is required, more frames may be necessary.
9. If the activated molecules are sparse, increase the intensity of 405 nm laser (*see* **Note 22**).

10. Collect images from each section on the cover glass. During long imaging sessions, the sections may become dehydrated resulting in weakened signal; rehydrate as in **Step 2** every ~30 min.
11. After acquisition, images should be processed by PALM software. Both sum TIRF (a collection of all the fluorescence signals acquired) and PALM images should be created. The sample shift during image acquisition is corrected based on the locations of gold particles in the first image. To eliminate background fluorescence, which is typically long-lived, we filter out molecules that are active for >500 ms; tdEos usually bleaches before 500 ms under these illumination conditions. The final image only shows molecules with high localization precision (below 35 nm) to filter out background fluorescence because background fluorescence is typically dim and localization precision is directly related to the quantity of photons received.

3.8 dSTORM Imaging

1. Place a small drop of 100 nm TetraSpeck fluorescent microspheres (9.0×10^9 particles/ml; *see* **Note 21**) directly on top of the sections. Blow the solution off to the edge of the cover glass and absorb it with a Kimwipe. TetraSpeck fluorescence microspheres also serve as fiducials for image alignment and sample drift (*see* Fig. 2b, c).
2. Prepare dSTORM imaging buffer (*see* **Note 23**) [26].
3. Mount the cover glass onto a microscope slide with a concave surface. First, fill the concave surface with the imaging buffer, and then apply a few drops of the imaging buffer onto the cover glass containing sections. Carefully flip the cover glass and mount it onto the microscope slide so that there are no air bubbles trapped in the chamber. Wipe out the excess buffer using a Kimwipe. To fix the cover glass onto the microscope slide and seal solutions from the air, apply Twinsil to the edges of cover glass and let it dry for ~5 min after mixing the two components of Twinsil solution (*see* **Note 24**).
4. For dSTORM imaging, turn on the microscope and associated software. Turn on 405 and 641 nm lasers to standby mode.
5. Locate the sections and regions of interest using a low-power objective lens and a bright-field illumination.
6. Switch to a high-power objective lens. Turn on the 641 nm laser at its lowest power and locate the region of interest.
7. Collect ~10,000 frames at 30 ms/frame with 641 nm laser set at its maximum power. Turn on the 405 nm laser as necessary (*see* **Note 25**).
8. Process the images with the PALM/STORM software (QuickPALM [27] was used to process dSTORM images in this chapter). Because fluorescence from Alexa-647 dyes is

much brighter than tdEos fluorescence, signals are highly distinctive from the background fluorescence, and thus subtraction of background as described in Subheading 3.7, **step 11** is not necessary. For sample drift correction using QuickPALM, *see* Fig. 2b and c and also **Note 26**.

3.9 SEM Imaging

1. Prior to SEM imaging, the sections must first be post-stained with uranyl acetate to increase contrast. With 2.5 % uranyl acetate in milliQ water passed through a 0.2 μm filter, place a drop directly on top of the sections. After 4 min, thoroughly but gently rinse the sections with filtered milliQ water. Allow to air dry.
2. To prevent charging by the electron beam, the sections should be carbon coated. Use a carbon sputter to deposit a thin layer of carbon, until the surface of the cover glass becomes dark brown. Use a piece of carbon tape to ground the surface of the cover glass by affixing one end to the carbon-coated surface and contacting the base of the metal specimen stub with the other end.
3. Electron micrographs of the sections can now be obtained from backscattered electrons. We typically image with specimen current set at 0.11 nA, the accelerating voltage at 5 keV, the landing energy at 3 keV, and the immersion mode "on" (*see* **Note 27**).
4. Collect both low-magnification and high-magnification images for each section. The low-magnification images will be used as a guide for alignment with the fluorescence micrographs and the high magnification to define the fine ultrastructure. Invert the grayscale to achieve a look similar to a TEM micrograph.

3.10 TEM Imaging

1. Following fluorescence imaging, sections should be post-stained with uranyl acetate as described in Subheading 3.9, **step 1**.
2. The sections then must be retrieved from the cover glass for a TEM imaging. To retrieve sections, first cut a square around the sections using a razor blade (*see* Fig. 7a). Second, apply 1 % hydrofluoric acid to the cover glass (*see* Fig. 7b, c). Once the glass is etched a little bit, the Pioloform film and sections will float on top of the hydrofluoric solution (*see* Fig. 7b, c). Immerse the cover glass into a water bath so that the film containing sections are floated on the surface of water (*see* Fig. 7d, e). Place a single-slot TEM grid on top of the film so that the sections appear in the middle of the slot window (*see* Fig. 7f). Finally, retrieve the grid by gently laying a strip of Parafilm on top of the grid and Pioloform, followed by carefully withdrawing the Parafilm strip from the water surface in a rolling motion. The grid and surrounding excess Pioloform will come with the Parafilm. Once dry, separate the grid from the excess Pioloform by carefully poking the film at the edge of

the grid with the tips of a pair of forceps. The grid can then be cleanly removed from the excess Pioloform film.

3. After the grid is dried, regular TEM imaging can be performed on these plastic sections. Both low-magnification and high-magnification images should be collected.

3.11 Image Alignment

1. In Adobe Photoshop, open a new 5000 × 5000 pixel window with 300 pixel/in.
2. Paste the low-magnification electron micrograph into the window and transform to fill ~80 % of the window. Be certain to maintain the aspect ratio during transformation (hold down the "shift" key in Windows or Mac).
3. Paste the high-magnification electron micrograph into a new layer and transform to align with the low-magnification micrograph.
4. Paste the sum TIRF micrograph into a new layer and scale, rotate, and transform to align the fiducials. Gold nanospheres should appear as fluorescence in the sum TIRF and as electron density in the electron micrograph. Nonlinear transformations may be necessary (*see* **Note 28**).
5. Paste the PALM image into a new layer. Align perfectly with the sum TIRF by applying identical transformation values.
6. For presentation, it is desirable to apply a transparency to the black background of the sum TIRF and PALM images. In a duplicate file (to avoid manipulation of the original), select "Color range" from the "Select" menu. Select a black background pixel and invert selection. Adjust the fuzziness so that the black pixels surrounding the signal are not selected.
7. Cut and paste the selection into a new layer. This new layer thus contains only the signal, and the previous layer now contains only black background, with blank spots where the signal has been cut out.
8. Apply a 10 % transparency to the background layer.

4 Notes

1. Because of the fluorophores used in the original publications, the acronym PALM is usually associated with imaging fluorescent proteins, whereas STORM is associated with organic fluorophores usually attached to antibodies. All these methods are based on localizing individual molecules. Imaging single molecules requires the spatiotemporal separation of individual molecules in a densely labeled sample. There are two schemes for separating molecules. PALM, fPALM, and STORM rely on

the stochastic activation of fluorophores from a nonresponsive state to a responsive state. On the other hand, dSTORM and GSDIM drive fluorescent molecules into a dark state ("ground-state depletion") and image single molecules that stochastically return to a ground state.

2. There are two important concepts in super-resolution microscopy: resolution and precision. "Resolution" in fluorescence microscopy reflects the ability to determine that the light collected in a field arises from two separate objects. For example, astronomers must resolve two stars in the sky. Biologists must determine that the fluorescence in a cell arises from two adjacent proteins, instead of one. Our ability to distinguish the presence of two labeled proteins is restricted by the diffraction limit: light from a point source can only be focused on a camera to a spot with a diameter of about 450 nm. Two spots cannot be separated once they are closer than about half the wavelength of light—around 200 nm. There are various ways to estimate this distance depending on the wavelength of light and the optics of the microscope; two commonly cited methods are the Abbé limit and the Rayleigh limit. However, PALM and STORM microscopy bypass the diffraction limit by separating the detection of the fluorescent sources in time. Determining that light coming from a spot is composed of two sources is obvious, since they appear at different times. Counting proteins, unlike stars, can be made to be quite easy.

   Resolution is however important when trying to determine the shape and size of an organelle, for example, to distinguish the stacks in a Golgi. For these applications, one must "paint" the organelle with dense enough labeling to know that they comprise a single structure and have a fine enough resolution to know that these molecules do not belong to the adjacent structure. Resolution of current super-resolution microscopes has been reported to be ~20 nm [18, 19].

   "Precision" reflects one's confidence that a protein is actually located at the calculated position. Precision is important when one is trying to determine if two different proteins are located at the same position, by imaging in two colors. Precision is also very important in nano-fEM, since the location of a protein will be correlated with a structure observed in an electron micrograph. Precision relies on fitting a Gaussian distribution to the photons collected on the camera face from a particular tagged protein. The more photons collected, the more accurate the calculation. Localization precision of proteins in PALM images using fluorescent proteins is about ~12–15 nm [20].

3. There are two factors that make STORM and dSTORM imaging not quantitative: antibody labeling density and organic fluorophores. First, antibodies are large and binding occludes

access to neighboring antigens [28, 29], and typically not all proteins are bound to an antibody. Second, unlike fluorescent proteins organic fluorophores blink several times before quenching [31, 32] (*however, see* refs. 28, 29), and thus the same molecules are sampled multiple times [13, 14, 32].

4. Baltec is now a part of Leica Microsystems. The HPM010, however, can only be purchased from ABRA.
5. We used HPM100 for freezing cell cultures, but other high-pressure freezers or freezing methods such as self-pressurized freezing [33] can be used. The HPM100 holds samples as large as 6 mm in diameter, allowing more cells to be examined.
6. The pH of uranyl acetate solution is usually highly acidic (pH 2–5), and fluorescence proteins will be quenched in such conditions. If the pH of the uranyl acetate is low, shorten the incubation duration with uranyl acetate solution in the **step 6** of Subheading 3.4. Uranyl acetate from Polysciences seems to have higher pH (around 6).
7. Unfortunately, polypropylene BEEM capsules have been discontinued. We have not been able to find a replacement source. Although the use of polyethylene is not recommended for glycol methacrylate, we have been successfully polymerized GMA in polyethylene BEEM capsules by placing a layer of ACLAR film (*see* **Note 16**) [20, 21] or a sapphire disk at the bottom of the polyethylene BEEM capsule cap.
8. Any desktop centrifuge that is equipped with a microscope slide holder is likely adequate for removing the excess Pioloform solution off of the cover glass, but this particular desktop centrifuge is capable of reaching its maximum speed rapidly, resulting in the very uniform thin layer of film coated on the cover glass.
9. As discussed in the Introduction, single-molecule-based super-resolution imaging can be performed on any microscope equipped with an EMCCD camera and appropriate laser lines and filter sets. For dSTORM imaging in this chapter, we used a custom-built microscope in the laboratory of Dr. Schmoranzer at Leibniz-Institut für Molekulare Pharmakologie im Forschungsverbund in Berlin, Germany (for details of the microscope setup, *see* ref. 34). Free software for analysis is available from NIH (http://code.google.com/p/quickpalm/ and http://code.google.com/p/palm3d/) [27, 35]. Currently, three commercial PALM/STORM microscopes are available for purchase: Zeiss ELYRA, Nikon N-STORM, and Vutara SR-200.
10. Although most microscopes are capable of imaging backscattered electrons, a scanning electron microscope with a field emission gun and a sensitive backscattered electron detector should be used for the best resolution.

11. We prepare 1 % osmium tetroxide stock solution by mixing 0.1 g osmium crystals with 10 ml anhydrous acetone. We then aliquot it into cryovials and store them in a liquid nitrogen storage tank until use.
12. We found that the use of acetone is required to preserve membrane morphology [20, 21] presumably due to its ability to interact with lipids [36]. However, acetone acts as free radical scavenger and blocks proper polymerization of plastic resins [37]. Therefore, we substitute specimens with ethanol prior to plastic infiltration.
13. Here, we are describing the protocol for Leica HPM 100. For other high-pressure freezers, please refer to Watanabe and Jorgensen [21] and McDonald [24, 38].
14. To dissolve benzoyl peroxide, use a Nutator in a cold room (4 °C) or place the tube on ice while mixing. Otherwise, the solution becomes cloudy. It requires ~5–10 min.
15. The incubation duration can be altered depending on the size of specimens or your schedule.
16. For cell cultures, make sure that the side with cells faces up after transferring. For *C. elegans*, place a disk of ACLAR film at the bottom of the cap so that specimens do not contact the BEEM capsule directly.
17. Once the catalyst, *N,N*-dimethyl-*p*-toluidine, is added to GMA resin, polymerization reaction initiates immediately and is completed within 30 min. Work as quickly as possible while replacing solutions.
18. If the resin is polymerized outside the AFS chamber, the embedding cap should be sealed with ACLAR film. Otherwise, oxygen will block complete polymerization of the plastic. The AFS chamber is filled with nitrogen gas, and thus the resin can polymerize without the ACLAR film.
19. Piranha solution makes the glass surface hydrophilic, making it difficult to retrieve sections in Subheading 3.6, **step 4**. To create a hydrophobic surface, cover glasses should be rinsed in methanol or should be incubated with hexamethyldisilazane (HMDS) gas in a sealed glass beaker.
20. A region of interest in *C. elegans* is located based on the morphology visualized using a stereomicroscope. For example, if we are interested in imaging a nerve ring, we locate the region by the morphology of pharynx in the animal. If there are no distinctive morphological features, we locate the region of interest by fluorescence. If animals are expressing tdEos, we briefly expose animals to blue light (less than 1 s) and leave a knife mark close to where fluorescence is expressed. For cell cultures, locating a cell of interest would be most reliably

performed by marking a sapphire disk with a finder grid pattern (*see* Subheading 3.2, **step 3**). Before performing a high-pressure freezing, locate the cells of interest using a light microscope and remember the position of the cells in the sapphire disks. The carbon layer is transferred into a plastic block along with the cells, and thus the specific pattern generated by the finder grid helps locate the cells of interest in a plastic block after embedding.

21. Gold nanospheres emit fluorescence due to surface plasmon resonance [39]. The 100 nm gold particles absorb green light and emit red light. Its emission spectra overlaps with that of tdEos after photo-conversion and thus can be used to correct the specimen drift during fluorescence imaging. Because gold scatters electrons, the gold particles also mark the electron micrographs and can be used as fiducial marks for aligning the fluorescence and electron micrographs. TetraSpeck beads can be excited at four different wavelengths: 365 nm (UV), 505 nm (green), 560 nm (orange), and 660 nm (red). Because the emission spectra of Alexa-647 overlaps with that of TetraSpeck beads, we used TetraSpeck beads in our dSTORM imaging as fiducial markers. Because these beads are made of non-electron conductive polystyrene, electrons accumulate on the beads, causing incoming electrons to be repelled when imaged under an electron microscope. Thus, these beads also mark electron micrographs. Note that polystyrene beads do not stick to plastic sections nor the glass surface in the absence of buffer, so we dilute the original solution with PBS to 1:200. We obtained 3–5 beads in each imaging frame at this concentration when applied to the specimens for 1 min.
22. During acquisition, the intensity of the activation (405 nm) laser can be adjusted to deliver an appropriate level of fluorophore activation. If the activated signal is meager, increase the power. Note, however, that if activation is too dense and fluorescence spots overlap, the computer will incorrectly calculate the centroid and produce erroneous data.
23. MEA should be stored as a solid at 4 °C and prepared freshly each day as a 1 M stock solution in water. The pH of the solution should be adjusted to ~8 with 1 M HCl.
24. Twinsil is the same silicon material used to create impression molds or dental molds. Its use for mounting coverslips was introduced by Leica Microsystems as an alternative to a nail polish since nail polish contains organic solvents that interfere with dSTORM imaging (http://www.leica-microsystems.com/science-lab/sample-preparation-for-gsdim-protocols-and-tips/).
25. The 405 nm laser increases the rate of blinking but also increases background fluorescence level. Thus, its intensity should be determined experimentally.

26. To account for sample drift using QuickPALM, a square box with a size larger than the displacement of a particle during the image acquisition is drawn around three individual particles. These squares are saved as a region of interest (ROI) in ImageJ. The drift is calculated based on the displacement of the particles. The corrected locations of the particles are saved as a tif file, reloaded into ImageJ, and reconstructed to obtain the final image. For more details, refer to the QuickPALM wiki page (http://code.google.com/p/quickpalm/wiki/Welcome).
27. There are two features in the FEI NovaNano that make it sensitive to backscattered electrons. First, it is capable of introducing a negative bias to the stage, which accelerates backscattered electrons towards the detector and thus improves signal. Second, an electromagnetic field (immersion mode "on") can be applied between the pole piece and the specimen so that electrons scattered by the specimens do not leave the field and thus are collected by the detectors.
28. Aligning the images can be accomplished in Fiji by using TrakEM2 and selecting the fiducials in each image as alignment targets.

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