Video Article

Nano-fEM: Protein Localization Using Photo-activated Localization Microscopy and Electron Microscopy

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Abstract

Mapping the distribution of proteins is essential for understanding the function of proteins in a cell. Fluorescence microscopy is extensively used for protein localization, but subcellular context is often absent in fluorescence images. Immuno-electron microscopy, on the other hand, can localize proteins, but the technique is limited by a lack of compatible antibodies, poor preservation of morphology and because most antigens are not exposed to the specimen surface. Correlative approaches can acquire the fluorescence image from a whole cell first, either from immuno-fluorescence or genetically tagged proteins. The sample is then fixed and embedded for electron microscopy, and the images are correlated [1-3]. However, the low-resolution fluorescence image and the lack of fiducial markers preclude the precise localization of proteins.

Alternatively, fluorescence imaging can be done after preserving the specimen in plastic. In this approach, the block is sectioned, and fluorescence images and electron micrographs of the same section are correlated [4-7]. However, the diffraction limit of light in the correlated image obscures the locations of individual molecules, and the fluorescence often extends beyond the boundary of the cell.

Nano-resolution fluorescence electron microscopy (nano-fEM) is designed to localize proteins at nano-scale by imaging the same sections using photo-activated localization microscopy (PALM) and electron microscopy. PALM overcomes the diffraction limit by imaging individual fluorescent proteins and subsequently mapping the centroid of each fluorescent spot [8-10].

We outline the nano-fEM technique in five steps. First, the sample is fixed and embedded using conditions that preserve the fluorescence of tagged proteins. Second, the resin blocks are sectioned into ultrathin segments (70-80 nm) that are mounted on a cover glass. Third, fluorescence is imaged in these sections using the Zeiss PALM microscope. Fourth, electron dense structures are imaged in these same sections using a scanning electron microscope. Fifth, the fluorescence and electron micrographs are aligned using gold particles as fiducial markers. In summary, the subcellular localization of fluorescently tagged proteins can be determined at nanometer resolution in approximately one week.

Video Link

The video component of this article can be found at http://www.jove.com/video/3995/

Protocol

1. High-pressure Freezing

1. Prepare the cryo-protectant by adding 0.2 g of BSA into 1 ml of an appropriate medium for your specimen (for example, culture medium for cell cultures, M9 for *C. elegans*). Incubate the tube in a 37 °C water bath until all BSA is dissolved.
2. Prior to freezing, fill the automated freeze-substitution device (Leica AFS) with liquid nitrogen
3. Set the AFS program: -90 °C for 5-30 hr*, 5 °C/hr to -60 °C, -60 °C for 2 hr (or select "pause" option if you are using Leica AFS 2), 5 °C/hr to -30 °C and -30 °C for 72 hr. If the AFS machine is capable of having more than five steps, the -30 °C step should be replaced with a "pause" option set to 0 hr, and two further steps should be added: 10 °C/hr to -20 °C and 24 hr at -20 °C. If the machine is limited to five steps, set up another program in a different memory slot as follows: 10 °C/hr to -20 °C and 24 hr at -20 °C.

   *The time here can be adjusted so that the -60 °C step starts in the early morning.
4. Prepare 1% osmium tetroxide stock solution by mixing 0.1 g osmium tetroxide crystals (EMS, RT19134) with 10 ml of anhydrous acetone (EMS, # RT10016). Prepare the fixatives in a 50 ml conical tube as follows. First, add 1 ml of milliQ water, and then dissolve in it 0.02 g of potassium permanganate (EMS, RT20200). Add 19 ml of acetone and mix it well. Finally, add 20 µl of 1% osmium stock solution into the tube. Acetone is a free-radical scavenger[11] and thus prevents polymerization of plastic, but the use of acetone as a freeze-substitution

medium is necessary for preserving morphology because of its interaction with lipids. Acetone will be substituted with ethanol prior to plastic infiltration. Aliquot 1 ml to each cryovial and keep the fixatives frozen by storing the tubes in liquid nitrogen.
5. Fill a specimen carrier with 20% BSA or bacteria. Both 20% BSA and bacteria serve as cryo-protectants. The choice for the type of the specimen carrier depends on the specimen. For *C. elegans*, use a 100 µm well.
6. Place the specimen in the carrier and freeze it using a high-pressure freezer.
7. After freezing and under liquid nitrogen, transfer the specimen into the cryotube containing fixatives. Make sure the specimen stays under the liquid at all times.
8. Repeat 1.5) - 1.7) until all the specimens are frozen.
9. Transfer all the cryotubes into the AFS unit, and resume the program.

2. Freeze-substitution

1. When the temperature reaches -60 °C, place vials containing 20 ml of 95% acetone into the AFS.
2. When the temperature reaches -50 °C, replace the fixatives with 95% acetone six times over the period of 2 hr.
3. Place a vial containing 20 ml of 0.1% uranyl acetate (Polysciences, #21447-25) in 95% acetone into the chamber and pre-cool it to -50 °C.
4. At the end of the washing step, add ~1 ml of uranyl acetate solution to each vial. Unpause the program if necessary.
5. When the temperature reaches -30 °C, replace the uranyl acetate with 95% ethanol six times over the period of 2 hr.
6. In the meantime, make up 97% glycol methacrylate resin (GMA, SPI Supplies/Structure Probe, Inc., #02630-AA) by mixing 22.3 ml GMA, 10ml n-Butyl Methacrylate, 1 ml milliQ water, and 0.2 g benzoyl peroxide (catalyst). Store it in glass vials (EMS, #72632) and prepare 30% GMA by mixing it with 95% ethanol. **Do not store GMA media in plastic tubes** because the quality of polymerization degrades with the long-term storage in polyethylene-based plastics. Pre-cool the GMA solution to -30 °C.

   Traditional epoxy resins such as Araldite and Epon cannot be used in the protocol since they are anhydrous and acidic which quenches fluorescent proteins. Acrylic resins, such as LR White, can tolerate a small amount of water and can preserve the fluorescent protein, but its acidic pH tends to quench the fluorophore [12]. GMA tolerates, in fact requires, a small amount of water and is alkaline (pH8)[12]. GMA is unfortunately somewhat brittle. Moreover, GMA does not cross-link with tissues like traditional epoxy resins do. These characteristics of GMA cause inconsistency in sectioning quality especially when sectioned thinner than 80 nm.

   Although the shelf life of GMA is about a year, GMA should be used within 3 months of the purchase to ensure its quality.

3. Infiltration and Polymerization

Steps 3.1-3.3 are carried out in the same cryovials used for freeze-substitution. Infiltration and polymerization are carried out at -30 °C in the AFS to preserve the fluorescent protein.

1. Prepare infiltration media by mixing the GMA stock solution with 95% ethanol. Incubate specimens in 30% GMA for 3-5 hr.
2. Incubate specimens in 70% GMA for 4-6 hr.
3. Incubate specimens in 97% GMA overnight.
4. On the next day, make up fresh 97% GMA.
5. Transfer the samples to an embedding mold (EBSciences, #TC). Prepare a disk of ACLAR film (EMS, #50425-10) by using a 3/8" DISC Punch (Ted Pella Inc.) and place the disk in the bottom of the BEEM capsule.
6. Exchange the 97% GMA three times over 6 hr at -30 °C.
7. After the third exchange, add the initiator N,N-dimethyl-p-toluidine (Sigma-Aldrich, #D9912) to GMA at a concentration of 1.5 µl/1 ml GMA and apply this solution to each specimen mold. Immediately position the specimen in the embedding mold in the AFS.

   Note that benzoyl peroxide is the catalyst and is present during all infiltration steps so stirring is not necessary. Benzoyl peroxide does not polymerize the plastic until it is exposed to the chemical initiator N,N,-dimethyl-p-toluidine. The initiator activates polymerization immediately and will polymerize the resin even in tissues within 1 hr. Nevertheless, tissue dropouts may result in deep tissues due to the incomplete polymerization. Moreover, GMA does not crosslink the specimen to the block well, so separate the specimen from the cryoprotectant by tapping on the matrix of bacteria.

   If polymerization is performed outside of an AFS, the embedding mold must be covered with an aclar disk to block exposure to oxygen. GMA does not polymerize completely when exposed to oxygen.
8. Allow the plastic to cure overnight even though it polymerizes within 1 hr.
9. Store the specimen in a nitrogen gas-filled vacuum bag (Ziploc) in the freezer (-20 °C) until further processing so that fluorescent proteins are not exposed to oxygen.

4. Sectioning

1. Sectioning of GMA-embedded specimens can be carried out in a manner similar to that for epon-embedded specimens. Extra caution should be taken not to wet the sectioning surface because GMA is very hydrophilic and the ribbons will be driven into the water bath if they are wetted on both sides.
2. Collect ribbons of sections (50-80 nm) on a glass coverslip if a TIRF microscope is being used for localization. Otherwise, use a grid made for transmission electron microscopy. The cutting speed should be set at1.6 mm/s or higher. Otherwise, a ribbon may not form.
3. Store sections at -20 °C if not imaged immediately. Protect the fluorophores from UV light by covering section holders with aluminum foil.

5. PALM Imaging

1. Set up the PALM microscope according to manufacturer recommendations. The temperature of an EMCCD camera should be set to -70 °C or below.
2. Apply gold particles (#790122-010 - 2x concentrated, microspheres-nanospheres.com) in solution (approximately 50 µl) to the sample to be imaged. Allow the solution to sit for 30 seconds on the coverslip while under a black cover case.
3. Remove the gold solution by blowing it to the edge of the coverslip and absorbing it with a kimwipe.
4. Insert the coverslip into a circular coverslip holder. If needed, apply vacuum grease to the rim to keep the coverslip in place.
5. Apply immersion oil to the underside of the coverslip, directly below the samples.
6. Insert the sample holder into the slot on the stage of the microscope. Take special care not to touch the objectives.
7. Adjust the holder so it is tight and centers the sections above the objective.
8. Locate the sections using a 10x objective lens.
9. Change to the 100x objective lens.
10. Focus on the specimen.
11. Locate a region of interest by observing the strength of the fluorescence signals. Use the 488 nm laser and increase the intensity in the Channels menu to 10%. Focus on the areas of brightest fluorescence. Note that this step is not necessary if the region of interest can be identified in the bright field by eye.
12. Switch the laser to 561 nm and increase the intensity to 100% to bleach out the background autofluorescence. Bleach the sample for ~2 min.
13. If the focus changes during bleaching, allow 5 min to elapse before adjusting the focus and capturing images. This pause allows the temperature to stabilize. If the PALM scope is equipped with an incubation chamber, set the temperature to 20 °C and wait for ~2 hr to stabilize the temperatures in the chamber.
14. When bleaching is complete, activate the 405 nm laser and set it to the lowest intensity.
15. Start collecting the images at 20 frames per second. We typically collect 5,000-6,000 frames per experiment, but the number of frames should be adjusted depending on the goal of the experiment. For example, if all proteins in a region of interest must be imaged, the frame number should be increased.
16. If the signals are sparse or faint, slowly increase the 405 nm laser intensity.
17. During the acquisition process, be sure to keep the samples in focus by carefully adjusting the knob as necessary.
18. When images are collected, the PALM analysis should be performed. For tdEos, we filter out any signals that fluoresce longer than 500 msec because those signals are likely due to autofluorescence.

6. SEM Imaging

1. Prior to SEM imaging, stain the sections using 2.5% uranyl acetate (in water) for 4 min. Wash off the uranyl acetate thoroughly with filtered milliQ water.
2. After the sections are dried, carbon-coat the coverslip using a carbon sputter until the coverslip becomes fairly dark. Apply one end of carbon conductive tape at the edge of the coverslip and the other end on the metal stub so that electrons that accumulate on the surface of the coverslip are grounded.
3. Mount the specimen into the SEM chamber (FEI Nova Nano).
4. Insert the vCD detector.
5. Close the chamber and pump it using the high vacuum setting.
6. Once evacuated, open the column valve so that electron beam is applied to a specimen.
7. Perform a routine alignment of electron beam.
8. Locate the specimen.
9. Once the focus is adjusted, link the specimen stage and bring up the stage to 5 mm below the pole piece.
10. Take a low magnification image of the specimen (~5,000x).
11. Zoom into the region of the interest and obtain high magnification (50,000x) images.
12. Move to the next section, and repeat step 6.10) and 6.11) until all the sections are imaged.

7. Aligning PALM and EM Images

1. Open Photoshop and the acquired SEM images.
2. Create a new window with dimensions of 5,000 x 5,000 pixels and 300 pixels/inch resolution.
3. Copy the low magnification SEM image into the new window (**Figure 1A**).
4. Scale the image so that it fills up the entire window by using the transform manipulation (Command + T for a Mac).
5. Copy the higher magnification SEM images and scale them as necessary.
6. Flip the sum TIRF image horizontally by selecting image rotation from the image drop-down menu bar.
7. Copy and paste the sum TIRF image (from PALM) into a new layer.
8. Scale the image using the transform manipulation and then rotate as necessary to match the fluorescence of the gold particles (white arrows in **Figure 1A**) with the corresponding structures visualized in SEM (**Figure 1B**).
9. Copy the PALM image into a new layer and apply the same transformation (**Figure 1C**). A higher magnification image can be extracted from this image (**Figure 2**).
10. For presentation, copy the transformed PALM image into a new layer. Select the PALM signals but not background by using "color range" in the "select" drop-down menu. Make sure to select a background pixel as a reference pixel and turn on the "invert" option.
11. Cut the desired PALM signals and paste them to a new layer.
12. Apply transparency to the background layer, and set it to 10%. This allows for visualization of the PALM signals by making the background transparent without compromising their intensity (**Figure 2D**).

8. Representative Results

Histone tagged with tdEos can be stably expressed in the nematode *C. elegans*, and the transgenic animals were processed using the protocol described above. The PALM and electron micrographs were acquired from the same section (**Figure 1**). To align the images, the sum TIRF image, which sums the fluorescence over the entire time course, is overlaid on the electron micrograph. The gold nanoparticles appear in both the fluorescence and electron micrographs and can be used to align the two images using the 'transform' function in Photoshop (**Figure 1A and B**). Then, the same 'transform' value was applied to the PALM image (**Figure 1C**). At this magnification, structural detail cannot be distinguished, so we zoomed into a region near the top end of the micrograph (**Figure 2**). In the high magnification image, subcellular details such as a nucleus, a nucleolus, nuclear pores, and endoplasmic reticulum could be observed. Moreover, the tagged Histone molecules are exclusively localized to the nucleus but not to the nucleolus, as expected. The correlative PALM and electron microscopy thus allows for protein localization at the highest resolution.

Five problems can compromise the quality of the images. First, ice crystal damage can distort ultrastructure (**Figure 3A and B**). Placing specimens in a cryo-protectant such as bacteria, which reduces the propagation of ice crystals, can reduce this damage. Nevertheless, one must still screen specimens by electron microscopy and discard those with freezing artifacts. Second, GMA does not cross-link to tissues like epoxy resins, and thus specimens often break loose from the surrounding plastic and stretch, shrink or even fall out of the section (**Figure 3C and D**). Dissection of the sample away from the bacteria or other cryo-protectant before embedding provides for greater adhesion of the plastic to the specimen (**Figure 3C**). Similarly, structures such as lipid droplets in the gut often dissociate from the tissues due to the absence of cross-linking (**Figure 3E**). Third, the incomplete polymerization of plastic causes stretching or folding of tissues (**Figure 3F**); the presence of oxygen in the sample also impedes the polymerization of GMA. Fourth, the poor sectioning quality of GMA often results in an inconsistent morphology (**Figure 3G and H**). GMA sections should be cut at 70 nm or thicker and at a speed of around 1.6 mm/s to minimize sectioning artifacts. Fifth, background autofluorescence from dust on the coverslip or section is inevitable. Autofluorescence from dust can be minimized by using pre-cleaned coverslips and by avoiding dust contamination from kimwipes and filter paper as described in the protocol. The PALM analysis program can edit out signals from the specimen or plastic that fluoresce longer than typical signals from fluorescent proteins (**Figure 2 A and B**). The final image will therefore be free of such artifacts.

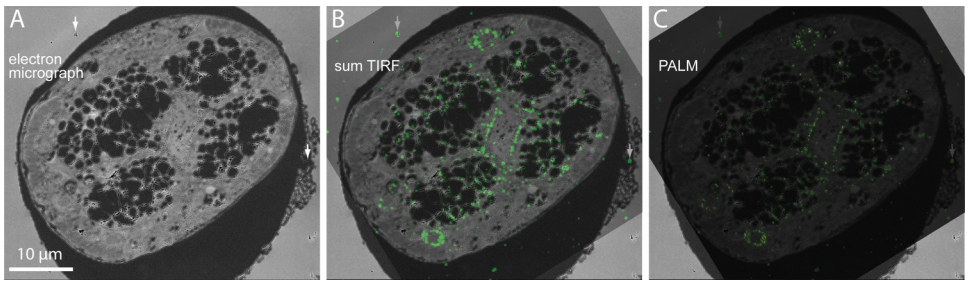

Figure 1. Aligning fluorescence and electron micrographs using gold nanoparticles. (A) A low magnification electron micrograph from a cross section of *C. elegans* expressing the tagged histone tdEos::HIS-11. White arrows indicate 100 nm electron-dense gold nanoparticles applied prior to PALM imaging that serve as fiducial marks. (B) The gold beads fluoresce upon exposure to ~580 nm light and create fiducial marks in the fluorescence image. The sum TIRF image is aligned onto an electron micrograph based on the location of the fiducial marks. The sum TIRF image represents all the photons detected by the camera during the experimental time course. Note that the bright spots on the upper left (white arrow) arise from the clusters of gold particles. (C) A PALM image is then added to the electron micrograph and rotated and translated based on the values determined from the alignment of the sum TIRF image in (B). Click here to view larger figure.

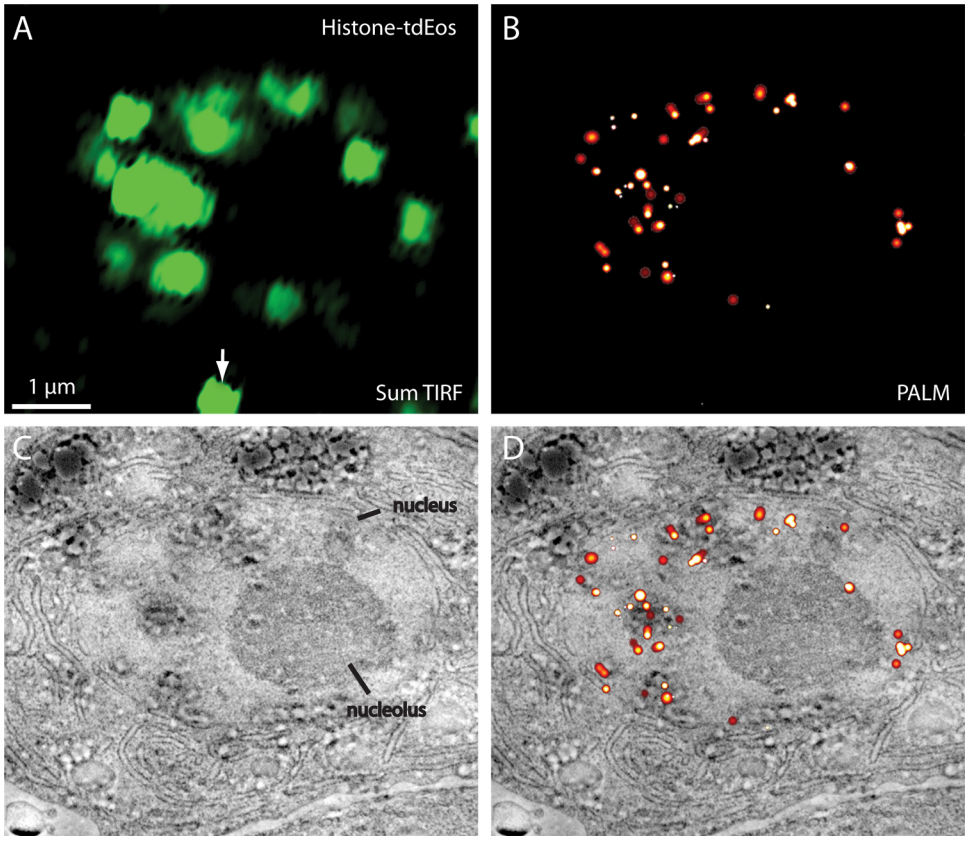

Figure 2. Correlative nano-fEM using Histone fusion proteins. (A) Sum TIRF image of tdEos::HIS-11 acquired from a thin section (70nm). (B) Corresponding PALM image of tdEos::HIS-11. Autofluorescence (white arrow) lasting longer than 500 msec was filtered out by the PALM program. (C) Electron micrograph of a nucleus acquired from the same section. (D) Correlative PALM image and electron micrograph of tdEos::HIS-11. Fluorescence is tightly localized to the chromatin in the nucleus.

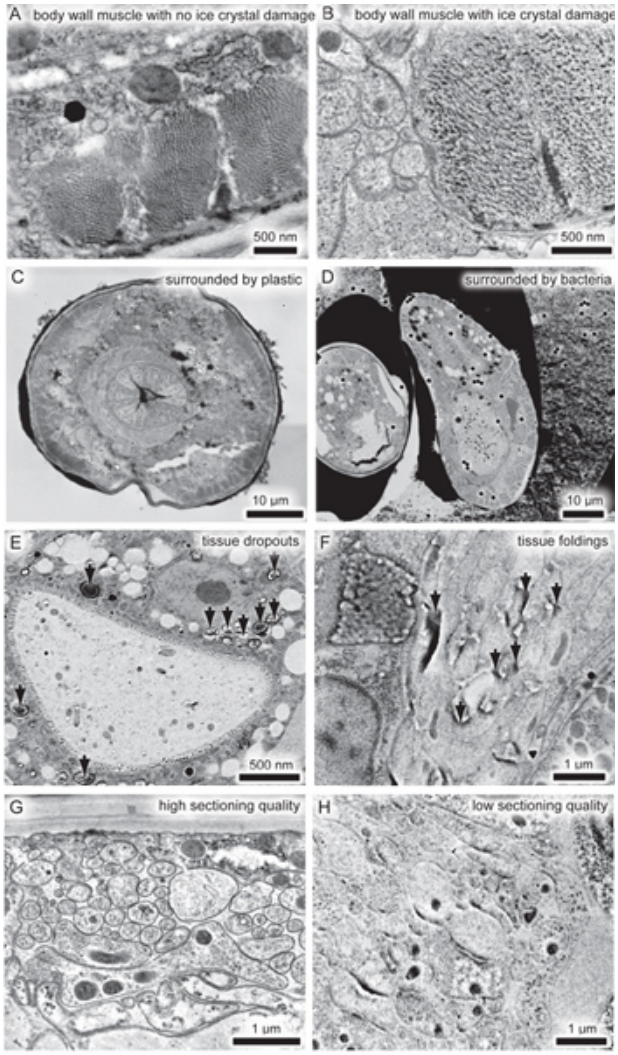

Figure 3. Problems associated with nano fEM. (A) Electron micrograph of a *C. elegans* body muscle without ice crystal damage. (B) Electron micrograph of a body muscle with ice crystal damage. Instead of discrete cross-sections, actin and myosin filaments are collapsed into aggregates due to the formation of ice crystals. (C, D) Low magnification electron micrographs, showing the dissociation of worms from the surrounding media. The section is more distorted in a specimen that is surrounded by the cryo-protecting bacteria (D) than when the specimen is surrounded by plastic (C). The bacterial cryo-protectant in the gallette should be dissected away from the fixed sample before plastic embedding. Note that the animal on the right in (D) was sectioned obliquely, and thus the shape is not due to the distortion of tissues. (E) Electron micrograph of intestine, showing dropouts of tissues (black arrows). (F) Electron micrograph of nerve ring, showing folding of sections due to the incomplete polymerization of plastic (black arrows). (G, H) Electron micrographs of neurons from the same specimen, sectioned on different dates. The preservation of tissues is superb on one day (G), but such morphology is obscured by the inconsistent sectioning quality (H). Click here to view larger figure.

Discussion

Here we describe how to preserve fluorescent proteins in plastic, localize the fluorescent proteins in sections, and image the ultrastructure using electron microscopy. Proteins were localized below the diffraction limit using PALM microscopy to nanometer resolution. To adapt this protocol to particular specimens, the following parameters should be considered: fluorophore, quantification, and alignment.

The choice of fluorescent protein or organic fluorophore depends on the application and the model system. We have tested a variety of fluorescent proteins, including EGFP, YFP, Citrine, mEosFP, mEos2, tdEos, mOrange, PA-mCherry, and Dendra[12]. The preservation of fluorescence from each fluorophore was similar, suggesting that all fluorescent proteins can be preserved using the described method. We chose tdEos because it expressed well in *C. elegans*, proteins remained functional when fused to tdEos, and because its photo-activation characteristics were optimal for PALM microscopy. However, aggregation or failed expression of tdEos has been occasionally observed[12].

Depending on the application, a different fluorophore may be better suited. In many cases, it is not necessary to use a photo-activated fluorescent protein. Simple correlative fluorescence electron microscopy does not require photo-activated fluorescent protein. GFP or organic dyes can be used to image fluorescence from tagged proteins in sections above the diffraction limit. For example, one can image an axon in a

neuropil using fluorescence microscopy and correlate the fluorescence signal with a particular axon in an electron micrograph by imaging the fluorescence on a fluorescence microscope. Other super-resolution techniques, such as stimulated emission depletion microscopy (STED)[12], ground state depletion microscopy followed by individual molecule return (GSDIM)[13], and structured illumination microscopy (SIM)[14], do not require photo-activated fluorescent proteins. Moreover, super-resolution imaging techniques that use organic dyes[9,15,16] or the intrinsic property of fluorescent probes[17] are readily applicable.

In PALM, the number of molecules can be quantified because fluorescence of each molecule is separated spatially and temporally. However, quantification may be misleading for four reasons: oxidation, undercounting, overcounting, and overexpression. First, a fraction of the fluorescent proteins can be denatured or oxidized during sample processing[5,12]. Although ~90% of the fluorescence signal was preserved through fixation and embedding in our protocol, oxidation of the fluorescent protein may occur after the specimen has been sectioned and the surface exposed to oxygen. Second, the activation of photo-activatable proteins is stochastic, and thus multiple molecules can be activated in a given diffraction limited spot[8]. Fluorescence from the multiple molecules will appear as one spot, and thus the total number of proteins will be undercounted. Third, a similar problem can lead to overcounting. In PALM, each fluorescent protein is localized and then "erased" by bleaching. However, fluorescent proteins can return from the dark state without being permanently bleached[18]. Such molecules will then be counted multiple times. Fourth, tagged proteins are expressed as transgenes and are often present in multiple copies, which can lead to overexpression. Therefore, quantification from PALM can be used to estimate but not precisely determine the number of molecules in a given location.

The alignment of a PALM image with an electron micrograph can also be challenging because of the resolution difference in light and electron microscopy and distortion caused by the electron beam. Gold particles serve as tightly localized fiducial markers in electron micrographs. However, fluoresecence from gold particles is not photo-activated, and appears as a large diffraction-limited spot. Thus, the placement of a fluorescence image over an electron micrograph is an estimate. Distortions can also arise from interactions of electrons with the plastic section. Acrylic resins such as GMA are less stable under the electron beam, and the dimensions of the plastic can be altered. Under these circumstances, aligning the fluorescence with ultrastructure may require non-linear transformation of the fiducial markers.

Disclosures

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