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Correlative fluorescence super-resolution localization microscopy and platinum replica EM on unroofed cells

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Summary

Platinum replicas of unroofed mammalian cells can be imaged with a transmission electron microscope (TEM) to produce high contrast, high resolution images of the structure of the cytoplasmic side of a plasma membrane. A complementary approach, super-resolution fluorescence localization microscopy, can be used to localize labeled molecules with better than 20 nm precision in cells. Here, we describe a correlative method that couples these two techniques and produces images where localization microscopy data can be used to highlight specific proteins of interest within the structural context of the platinum replica TEM image. This combined method is uniquely suited to investigate the nanometer-scale structural organization of the plasma membrane and its associated organelles and proteins.

Keywords

Electron microscopy; CLEM; Super-resolution; localization microscopy; PREM; platinum replica; unroofed cells; fluorescence; correlative microscopy; nanoscopy

1) Introduction

In platinum replica electron microscopy (PREM), a biological sample is shadowed with platinum and coated with carbon to create a cast of the surface. The replica is then removed from the sample, and imaged in a transmission electron microscope. This metal replica provides high contrast images of the structure of macromolecular complexes at the surface of the sample. The resolution of PREM, however, can be limited by the platinum granule size and structures must be accessible to the platinum during vacuum-vapor deposition of the metal. PREM has been used to image freeze-fractured cells [1], whole cells [2], single proteins or protein complexes [3], isolated cytoskeletons [4], or unroofed cells [5]. Unroofed cells, like those used in the method described here, are cells that are attached to a substrate and the top of the cell is removed by various mechanical means leaving behind only the adhered membrane and its associated proteins.

Identifying proteins in a PREM image has been challenging because immunogold labeling, the classical EM protein localization technique, relies on the same contrast mechanism as the rest of the metal replica. An alternative protein localization method combines PREM

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with fluorescence microscopy [4]. With this combination, one can highlight regions on the electron micrograph that are enriched in a specific protein. However, correlative fluorescence and PREM was previously limited to diffraction limited fluorescence microscopy which cannot exceed a resolution of 200 nm. Thus, signals from the fluorescence image cannot be assigned with certainty to specific EM structures at the nanometer scale. Super-resolution localization fluorescence microscopy offers a method to exceed the diffraction limit of fluorescence and localize fluorophores with better than 20 nm precision. Correlation of super-resolution localization microscopy data with PREM uniquely allows one to highlight proteins with high precision without obstructing the high contrast structural background of the electron micrograph [6].

Here, we present methods to extend correlative diffraction limited fluorescence PREM with localization microscopy and reproducibly obtain correlation with better than 20 nm accuracy across a $20 \times 20 \mu\text{m}$ image. One key step in obtaining highly accurate and reproducible correlation is the use of stable spatial fiducial markers that do not move with respect to the sample through all of the imaging and processing. The method presented here uses glass coverslips embedded with gold nanorods that are visible during fluorescence imaging [7]. These gold nanorods adhere to the metal replica when it is removed from the sample so that they are also visible in the EM image. A spatial image transformation can then be used to map the fluorescence image to the EM image using the gold nanorods as an independent reference.

In this protocol we describe two-dimensional image correlation. The method can, however, be extended for three-dimensional correlation with three-dimensional fluorescence localization and EM tomography[6]. When doing 3D imaging, we use a fluorescent membrane marker (myristoylated-psCFP2) to align the fluorescent membrane with the membrane visible in the EM tomogram in the axial dimension. Our method uses critical point drying to dry the sample in preparation for PREM. This works well for unroofed cells because the sample is thin and tightly adhered to the coated-glass substrate. However, delicate objects or objects that project into the cell move slightly during the drying process and have increased correlation error. Therefore, this method is likely not suitable for correlating tall or structurally unstable objects. One could also use freeze-etching to dry the samples but this may result in increased processing error and a higher failure frequency.

We have used this technique to study specific proteins associated with clathrin-mediated endocytosis. After unroofing, the distinct honeycomb structure of clathrin on the plasma membrane is exposed. These 100–500 nm cellular structures vary greatly in shape, size, and depth which are visible in the EM image as well as irregularities in the clathrin meshwork, neighboring objects, or membrane deformations. Most proteins associated with clathrin are underneath the meshwork and not obviously visible in the micrograph but their position can be highlighted using localization microscopy.

2) Materials

2.1) Cell Culture and Fluorescence Preparation.

- 1) Gold nanorod embedded coverslips (hestzig.com, part #600–200AuF) (*see Note 1*).
- 2) Cleaning Solution: 200 mL water, 40 mL 30% Hydrogen Peroxide, 40 mL 30 % ammonia.
- 3) Ethanol: 200 proof
- 4) Growth medium without phenol red (*see Note 2*).
- 5) Stabilization Buffer: 70 mM KCl, 30 mM HEPES brought to pH 7.4 with 5 M KOH, 5 mM MgCl₂, 3 mM EGTA. Sterile Filtered. This can be stored at room temperature.
- 6) Sonication Media: 0.5% PFA (fresh from 16% stock amber ampule, Electron Microscopy Sciences) in unroofing stabilization buffer (*see Note 3*).
- 7) Primary Fixative: 2% PFA for in unroofing stabilization buffer
- 8) Sonicator: Branson Sonifier 450 with a 1/8" tapered microtip
- 9) Alexa Fluor 647 labeled antibody or label of choice (*see Note 4*).

2.2) Fluorescence Localization Imaging

- 1) Blinking Buffer: 10% w/v glucose in PBS, 0.8 mg/mL Glucose oxidase (from 80 mg/mL frozen aliquot in PBS), 0.04 mg/mL Catalase (from 10 mg/mL frozen aliquot in PBS), 100mM 2-mercaptoethanol. This must be made fresh within minutes of using. After applying to sample, it needs to be sealed from air (*see Note 5*).
- 2) 25 mm round coverslips
- 3) Attotfluor cell chamber (Life Technologies) (*see Note 6*).
- 4) Inverted fluorescence microscope preferably with localization microscopy software installed and an automated stage. We use a 100× TIRF objective with 1.49NA on a Nikon Eclipse Ti microscope running the Nikon Elements NSTORM module.

¹)These coverslips can also be made by coating a clean coverslip with poly-L-Lysine, adding gold nanorod solution and allowing gold to stick to surface, drying, and sputter coating with SiO₂. [10,6]

²)Purchase phenol red free DM EM with no glutamine and supplement with glutamax for best results

³)Make all fixative fresh from an ampule.

⁴)Any other fluorescence label that works for fluorescence localization microscopy can be used for this method. Alexa fluor 647 on a nanobody gives excellent precision localization.

⁵)This blinking buffer is specific for dSTORM imaging of Alexa Fluor 647. In the case that you are using a photoactivatable protein, blinking buffer would not be necessary. In the case that you are using a different dye, the blinking buffer may need to be different for that dye. [11] If your fluorophore is sensitive to pH, you should use a tris buffer as the base instead of PBS.

⁶)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.

- 5) Diamond objective marker (Leica). Our diamond objective marker is too short for our microscope so we have machined a 14 mm spacer tapped with m25 to receive the marker and m25 threading to fit into our microscope turret.
- 6) 2% glutaraldehyde (fresh from 8% stock in an amber ampule, Electron Microscopy Sciences) in PBS.

2.3) EM preparation and imaging

- 1) 0.1 % w/v tannic acid in water. This needs to be made fresh.
- 2) 0.1% w/v uranyl acetate (Electron Microscopy Sciences). Diluted in water from a 1% stock solution stored in an amber vial in the refrigerator.
- 3) 15%, 30%, 50%, 70%, 80%, 90%, 100% ethanol solutions (Warner-Graham) (see Note 7).
- 4) Critical point drying sample holder. We machined a coverslip holder like Tousimis #8767 but with an extended diameter to accommodate the 25 mm coverslip. Wavy washers (Tousimis 8767-01) can be used as coverslip spacers.
- 5) Dehydration beaker assembly: two 100 mL beakers with a stir bar on the bottom and metal mesh bent to create a shelf in the beakers.
- 6) Critical point dryer (Tousimis Samdri 795) equipped with liquid carbon dioxide.
- 7) Freeze fracturer (JEOL JFD-V or JEOL/RMC 9010 FF machine)
- 8) Light microscope with 10× phase contrast objective
- 9) Glow discharge (Pelco EasiGlow)
- 10) Electron microscope. We use a JEOL 1400 equipped with a high tilt specimen retainer and Serial EM Freeware.[8]
- 11) 5% Hydrofluoric Acid (Fisher Scientific) in water.
- 12) Platinum 4–5 mm loop (Electron Microscopy Sciences)
- 13) Diamond scribe (Electron Microscopy Sciences)
- 14) Formvar/carbon coated 75 mesh copper TEM grids (Ted Pella) (*see* Note 8)
- 15) Filter paper
- 16) 12-well plate (Corning 3512)
- 17) Plastic bulb transfer pipettes (Fisher 13–711-7M) (*see* Note 9).
- 18) IMOD freeware [9]

⁷⁾This needs to be dry ethanol. The 100% ethanol solution should be opened fresh.

⁸⁾If you coat your own grids, 50 mesh with thin bars will be even better than 75-mesh.

⁹⁾We have found that some plastic pipettes cause problems during the replica lifting process. If your replicas start sinking to the bottom of the well instead of floating, try using other plastic. Poly-propylene works better than poly-styrene and non-graduated pipettes are better than graduated pipettes.

3) Methods

3.1) Cell Culture and Fluorescence Preparation

- 1) Boil the gold-embedded coverslips in the cleaning solution for 10 minutes to clean, rinse with clean water, and store in 100% ethanol. (*see Note 10*)
- 2) Coat the gold-embedded coverslips with a surface to which your cells adhere and grow. We use poly-L-lysine for PC12 and HeLa cells.
- 3) Let cells grow on this surface overnight. You may also want to transfect your cells with a plasmid for your gene of interest and a fluorescent protein. When we are doing 3D imaging, we transfect with myristoylated PS-CFP2 for membrane alignment (*see Note 11*).

3.2) Cell unroofing and immunolabeling

- 4) Prepare sonicator with tip that is in good condition and tightly secured to the horn of the sonicator. We set ours to give a 400 ms pulse of the lowest output power setting. This will need to be optimized for your specific sonicator.
- 5) Rinse samples with stabilization buffer.
- 6) Sonicate in sonication media with tip sonicator. We sonicate within 10 seconds of immersion in the media and position the tip 5 mm above the coverslip. This will need to be optimized for your specific sonicator (*see Note 12*).
- 7) Move coverslip to a fresh well containing primary fixative for 20 minutes.
- 8) Antibody label. Any fluorescence labeling scheme that works for localization microscopy will generally work for this technique. This includes antibody labeling, snap-tag labeling, or photo-activatable protein labeling. We use a nanobody bound with Alexa Fluor 647. Post-fix with PFA after labeling (*see Notes 13–14*).
- 9) Rinse cells with PBS. Store overnight in PBS at 4 °C or image immediately. Membranes will degrade with longer storage.

3.3) Fluorescence Localization Imaging

- 10) Place your coverslip in an AttoFluor imaging chamber with freshly made blinking buffer and seal immediately with another 25 mm coverslip on the top lip of the chamber (*see Note 15*).

¹⁰⁾The coverslips will float during boiling if they are not anchored in a coverslip holder. Always keep track of which side contains gold.

¹¹⁾Before unroofing, you want your cells to be very dense. This will allow you to collect as much data as possible from a single coverslip.

¹²⁾If having trouble optimizing your sonication with fixative present, try using no fixative during sonication to start. Also, a brief poly-L-lysine treatment and brief hypo-osmotic shock will cause the membranes to stick to the coverslip better during sonication. [5]

¹³⁾We use Alexa Fluor 647 (AF647) on a nanobody because it gives very good localization precision (<20 nm).

¹⁴⁾Do not use detergent (like Triton-X) when labeling.

¹⁵⁾We seal the chamber by simply sucking the remaining solution from above the coverslip and allowing the two coverslips to make a vacuum seal.

- 11) Place the diamond marking objective in the turret of your microscope prior to imaging (Fig. 1a). It should be set to make a 4 mm diameter circle around your area of interest.
- 12) Create a map ($\sim 1 \text{ mm}^2$) of the area to be imaged. This can be done with a 10 $\times$ DIC objective. (*see* Note 16)
- 13) Create a localization image of 5–9 different regions within your 1 mm² mapped area. Mark on your map each region that has been imaged (*see* Note 17)
- 14) For Alexa Fluor 647, our localization images are generally made with 30,000 frames, 10 ms each, with 10 kW/cm², 647 nm laser intensity. You may require shorter or longer imaging depending on the density of your labeling (*see* Note 18).
- 15) After localization imaging, move your stage to the center of your 1 mm² area and swing the turret to the diamond objective marker. Hold the sample with one hand as you slowly bring the diamond objective marker up to mark the coverslip. When you start to see the spring-loaded tip recess, rotate the objective collar to make a 4 mm diameter etched circle around the area of interest (Fig. 1b).
- 16) Clean the oil off of the sample well with ethanol. Rinse away imaging buffer in PBS. Store your coverslip in 2% glutaraldehyde (in PBS) at 4 °C overnight (minimum 20 minutes) prior to EM preparation.

3.4) Electron Microscopy Preparation and Imaging

- 17) From the 2% glutaraldehyde, transfer your sample into 0.1% w/v freshly made tannic acid in water for 20 minutes.
- 18) Rinse your sample in water four times.
- 19) Stain with 0.1% w/v uranyl acetate (diluted from 1% stock stored in amber vial at 4 °C) for 20 minutes.
- 20) Rinse with water twice.
- 21) Prepare your sample in a critical point drying (CPD) chamber with wavy washers above and below each sample. We generally process 2–9 samples at one time.
- 22) Dehydrate your samples by placing the CPD chamber through increasing concentrations of ethanol: 15%, 30%, 50%, 70%, 80%, 90%, 100%. Use the dehydration beaker assembly to agitate each ethanol bath for 4 minutes. Use the second dehydration beaker assembly to move the CPD chamber quickly to the new concentration without allowing the samples to dry. Bathe the samples in 100% ethanol three times to ensure complete removal of water.

¹⁶⁾A map could alternatively be made using a 100 $\times$ Fluorescence objective to make a 15 $\times$ 15 fluorescence montage.

¹⁷⁾Not all of your regions will make it all of the way through the correlative process. Some regions will land on the grid bar of the TEM grid. Always image at least twice as many cells as you expect to have in your final data.

¹⁸⁾You should see the gold nanorods during imaging (they do not blink like the Alexa Fluor 647).

- 23) Move the chamber to the critical point dryer that is prefilled with 100% ethanol. Perform the critical point drying using manufacturer's instructions. We use a 20 minute purge to ensure all ethanol is replaced with liquid carbon dioxide prior to heating to critical point.
- 24) Remove sample from the critical point dryer and place in a petri dish on filter paper.
- 25) Use a fine-tipped marker to mark dots on the bottom of the coverslip around the etched circle. This will just help you see the etching easily during the next few steps.
- 26) Cut out the area of interest with a diamond pen. We use a razor blade to guide our pen marks and cut a ~ 8–10 mm square.
- 27) Use two small (~1 mm) pieces of double sided tape to place the sample on the freeze fracturer stage.
- 28) Place the sample in the freeze-fracturer and coat with Platinum/Carbon at 17° with rotation. There is no thickness monitor on our freeze fracturer but our images generally have less than 5 nm of Pt/C coating objects of well-known size. Stabilize the Pt/C layer with carbon at 90°C.
- 29) Remove the sample from the freeze fracturer and carefully remove from the double-sided tape.
- 30) Take an image of your area of interest with a 10× phase contrast objective. It should be easy to find in the center of your etched circle. This image will help you find all of your regions after you have lifted your replica onto the grid.
- 31) Cut your sample again before lifting (Fig. 2a–c). During lifting the sample should be a 4–5 mm diameter circle (*see* Note 19).
- 32) Prepare the formvar/carbon coated grids by glow discharging (*see* Note 20).
- 33) Lift your replica from your coverslip by gently setting the coverslip (replica side up) on the top surface of 5% hydrofluoric acid (HF) in a 12-well plate (*see* Note 21) (Fig. 2d). Warning: HF is highly corrosive.
- 34) When you see the coverslip fall to the bottom of the well, the thin replica will remain floating at the top surface (Fig. 2e).
- 35) Replace the HF with water by sucking out all but ~1 mL from the well with a plastic pipette and replacing the volume with water (Fig. 2f). Do this 20 times (*see* Note 22).

¹⁹⁾We cut the sample twice because we have found that the edges of the coverslip get coated with platinum and carbon and may cause breaking during the lifting process. This also allows us to have a larger sample while coating so our tweezers avoid scraping the area of interest while handling.

²⁰⁾Glow discharging the grids will keep the replica from wrinkling as it settles on the grids.

²¹⁾If your replica sinks with the glass, you probably have a contaminant in your HF solution. This can happen if it has come into contact with certain plastics as discussed in Note 9.

²²⁾Doing these successive dilutions with your replica at the surface is a fragile process and you need to avoid touching and breaking the replica. The replica also needs to stay away from the wall of the well.

- 36) Place your EM grid (glow discharged within the last 12 hours) on filter paper.
- 37) Use a loop to lift your replica from the water and set down on your EM grid (Fig. 2g–i). If the coverslip was cut to the size of your loop, your area of interest should land near the center of your grid.
- 38) Image the grid with a 10× phase contrast objective and compare it to your pre-lifted image. You should easily be able to find your regions of interest.
- 39) Image each region of interest in the electron microscope. We use SerialEM on a JEOL 1400 transmission electron microscope at 15,000× magnification to make 8 × 8 montages. This creates a 22 × 22 μm square area (see Note 23).

3.5) Image Processing

- 40) SerialEM montages should be stitched together using IMOD blending.
- 41) A super-resolution image can be created with any localization software package. We use Nikon Elements to create an image with 5 nm pixel resolution.
- 42) Find 3–10 gold nanorods that are visible in both your EM image and your fluorescence image. The nanorods should be well spaced across your EM image and not in a line. You may have to overlay the two images in Adobe Photoshop to find corresponding spots.
- 43) Find the centroid position of each of the three spots in your fluorescence image.
- 44) Find the center of the gold nanoparticle for each that you found in your EM image.
- 45) Use these coordinates to do an affine image transformation on your fluorescence image to map it onto the EM image (see Note 24).
- 46) Overlay the two images and check how well the other gold nanoparticles (not the ones you used for your transformation) aligned. This will allow you to check the quality of your correlation. If the distance between the fluorescence and EM of the other gold nanoparticles is greater than 30 nm, try the transformation again using different gold fiducials. If correlation is still unsuitable, check the EM montaging. (see Note 25–26)

5) References

1. Moor H, Muhlethaler K (1963) Fine structure in frozen-etched yeast cells. *The Journal of cell biology* 17 (3):609–628 [PubMed: 19866628]

²³⁾We do not make correlative images larger than this because we have found that there is increased registration error with larger images. This could be from montaging errors or large scale bending in the grid film.

²⁴⁾We use MATLAB function `imtransform.m` to do this transformation but many other software packages will do image transformations as well.

²⁵⁾Alternatively, if all of the gold in the image was used to do the transformation, the residuals from the fit are informative about the quality of correlation.

²⁶⁾The procedure needs to be tested using a protein of known position. Clathrin is a perfect control because it is easily visible in PREM (Fig. 3). There are some places where there will be reproducibly lower correlation like high up vesicles that are only loosely tethered to the membrane or regions at the very edge of the membrane where there may have been some rolling of the membrane during the drying process. Using a control, one can identify what data are trustworthy for analysis.

2. Lung B (1968) Whole-mount electron microscopy of chromatin and membranes in bull and human sperm heads. *Journal of ultrastructure research* 22 (5):485–493 [PubMed: 5658649]
3. Heuser J, Keen J (1988) Deep-etch visualization of proteins involved in clathrin assembly. *The Journal of cell biology* 107 (3):877–886 [PubMed: 3417785]
4. Svitkina T (2015) Imaging Cytoskeleton Components by Electron Microscopy. *Methods in molecular biology* (Clifton, NJ) 1365:99–118
5. Heuser J (2000) The production of ‘cell cortices’ for light and electron microscopy. *Traffic* 1 (7): 545–552 [PubMed: 11208142]
6. Sochacki KA, Shtengel G, van Engelenburg SB, Hess HF, Taraska JW (2014) Correlative super-resolution fluorescence and metal-replica transmission electron microscopy. *Nature methods* 11 (3): 305–308 [PubMed: 24464288]
7. Shtengel G, Galbraith JA, Galbraith CG, Lippincott-Schwartz J, Gillette JM, Manley S, Sougrat R, Waterman CM, Kanchanawong P, Davidson MW (2009) Interferometric fluorescent super-resolution microscopy resolves 3D cellular ultrastructure. *Proceedings of the National Academy of Sciences* 106 (9):3125–3130
8. Mastronarde DN (2005) Automated electron microscope tomography using robust prediction of specimen movements. *Journal of structural biology* 152 (1):36–51 [PubMed: 16182563]
9. Kremer JR, Mastronarde DN, McIntosh JR (1996) Computer visualization of three-dimensional image data using IMOD. *Journal of structural biology* 116 (1):71–76 [PubMed: 8742726]
10. Shtengel G, Wang Y, Zhang Z, Goh WI, Hess HF, Kanchanawong P (2013) Imaging cellular ultrastructure by PALM, iPALM, and correlative iPALM-EM. *Methods in cell biology* 123:273–294
11. Dempsey GT, Vaughan JC, Chen KH, Bates M, Zhuang X (2011) Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging. *Nature methods* 8 (12):1027–1036 [PubMed: 22056676]

Figure 1). The imaged region is marked with a diamond objective marker. **(a)** The diamond objective marker is shown in the microscope turret with our custom-made spacer attached at the bottom. **(b)** A 4 mm etched circle is shown on the bottom of the coverslip.

Figure 2).

Lifting the replica onto a grid. **(a)** The coverslip fragment is shown after platinum shadowing and carbon coating. **(b-c)** The coverslip is cut to approximately the size of the loop that will be used to lift the replica. **(d)** The coverslip is placed on the top surface of 5% hydrofluoric acid. **(e)** The glass coverslip falls away leaving the platinum/carbon replica floating on top of the surface. **(f)** The hydrofluoric acid is replaced with water by serial dilutions with a plastic pipette. **(g-h)** The replica is lifted from below and **(i)** placed over the top of the TEM grid.

Figure 3).

Correlative data from GFP-Clathrin light chain expressing cells labeled with GFP-nanobody conjugated to Alexa Fluor 647. **(a)** Large zoomed out view of unroofed HeLa cell after correlation. Fluorescence signal is shown in magenta. Scale bar is 1 μm . **(b)** Gold nanoparticle from (a). **(c-e)** Examples of clathrin correlation from (a). **(f)** example of vesicle that moved during critical point drying due to a weak attachment to the membrane. Scale bar in b-f is 200 nm.