A protocol for Epon-embedding-based correlative super-resolution light and electron microscopy

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Abstract

This protocol describes a detailed step-by-step sample preparation procedure for Epon based post-embedding correlative super-resolution light and electron microscopy (SR-CLEM). The newly developed fluorescent protein, mEosEM, is the core in this protocol. The advantage of this method is to simultaneously obtain high-quality LM and EM images from the same ultrathin section of Epon-embedded sample after conventional chemical fixation.

Introduction

Combining the nanoscale localization information from super-resolution light microscopy with ultrastructure context from EM, SR-CLEM is a versatile imaging tool. Fluorescent probe and labeling strategy that can balance the LM fluorescence, EM contrast and structural preservation are in great demand. Genetically encoded fluorescent proteins (FPs) are widely used in SR-CLEM for its specificity and biocompatibility. However, the strong oxidizer osmic acid (OsO_4) and the hydrophobic embedding resin Epon will destroy the fluorescence of existing FPs. With mEosEM, a newly developed PCFP that has OsO_4 and Epon embedding resistance, the same-section SR-CLEM under conventional chemical fixation condition with FP is enabled. The procedure described in this Protocol Exchange chapter focuses on detailing the SR-CLEM process of same-section Epon-embedding sample using mEosEM, and is related to the Nature Methods manuscript NMETH-BC38466B titled: "An Epon embedding- and osmium acid-resistant fluorescent protein for superresolution CLEM".

Reagents

1. 8% (wt/vol) PFA, aqueous (Electron Microscopy Sciences, cat. no. 15707)
2. 8% (wt/vol) Glutaraldehyde (GA), aqueous (Electron Microscopy Sciences, cat. no. 16020)
3. Osmium tetroxide, crystalline (Electron Microscopy Sciences, cat. no. 19110)
4. SPI-Pon 812 Kit, (SPI, cat. no. 02663-AB)
5. Uranyl acetate (Electron Microscopy Sciences, cat. no. 22400)
6. Pioloform (Ted Pella, cat. no., 19244)
7. Acetone (Sinopharm Chemical Reagent, cat. no. 10000418)
8. Ethanol, (Sinopharm Chemical Reagent, cat. no. 10009259)
9. Lead nitrate (Electron Microscopy Sciences, cat. no. 17900)

10. Sodium citrate (Electron Microscopy Sciences, cat. no. 21140)
11. Poly-L-Lysine (BEIJING LABLEAD BIOTECH, cat. no. P1399-25mg)
12. Hydrofluoric acid (Sinopharm Chemical Reagent, cat. no. 10011108)
13. 80-nm gold nanoparticles (Corpuscular, cat. no. 790120-010)
14. 1,2-Dichloroethane (Sinopharm Chemical Reagent, cat. no. 10008618)
15. Coverslips (Fisher Scientific, cat. no. 12-545-102)
16. Ammonium hydroxide (J&K Scientific, cat. no. 335213)
17. Anhydrous ethyl alcohol (J&K Scientific, cat. no. 441863)
18. Mowiol 4-88 (Sigma-Aldrich, cat. no. 324590)
19. DABCO (1,4-diazabicyclo-[2,2,2]-octane) (Sigma-Aldrich, cat. no. D2522)

Equipment

1. Ultra-microtome (Leica, EM UC6)
2. Uncoated slot Grids (Electron Microscopy China, cat. no. AGS2075)
3. 120 kV transmission electron microscope (FEI, model no. Spirit)
4. Super-resolution fluorescence microscope. Homemade based on Olympus X71 body
5. Chemical fume hood

Procedure

1. Transfect cells with an appropriate plasmid expressing mEosEM using Lipofectamine® 2000.

2. Cells were trypsinised 36h after transfection using Trypsin (using PBS wash 2 times) and collected in a 1.5-ml microcentrifuge tube and centrifuged at 3,000g for 3 min at room temperature.

3. In a chemical fume hood, remove the supernatant and add 1 ml of prewarmed fixative (4% paraformaldehyde and 2.5% glutaraldehyde in 100 mM PBS, pH 7.2) to the 1.5-ml microcentrifuge tube. Use a toothpick to make the cell pellet into 0.5–1 mm^3 pieces and incubate at 4 °C for 12 h, blocking light.

4. Wash the cell pellets with ice-cold H_2O three times for 5 min on ice, blocking light.

5. Postfix the cell pellet in 1% OsO_4 in a fume hood for 1 h on ice, blocking light.

6. Wash the cell pellet with ice-cold H_2O three times for 5 min on ice, blocking light.

7. Cells were stained *en bloc* with 2% UA for 1 h on ice, blocking light.

8. Wash the cells with ice-cold H_2O three times for 5 min on ice, blocking light.

9. Dehydrate cells in increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 100%) for 6 min each on ice and further dehydrate cells in 100% acetone one time for 6 min at room temperature, blocking light.

10. Prepare Epon resin (50% Epon812, 30.5% NMA, 18% DDSA, 1.5% DMP-30).

11. Infiltrate the cell pellet with 25% Epon in acetone at room temperature for 1 h on shaker, blocking light.

12. Infiltrate the cell pellet with 50% Epon in acetone at room temperature for 2 h on shaker, blocking light.

13. Infiltrate the cell pellet with 75% Epon in acetone at room temperature for 3 h on shaker, blocking light.

14. Infiltrate the cell pellet with 100% Epon at room temperature two times for 12 h on shaker, blocking light.

15. Embed the cell pellet in 100% Epon at 60 °C for 12 h, blocking light.

16. Coverslip cleaning. Coverslips were washed in a mixture of 250 ml of H_2O, 50 ml of ammonium hydroxide, and 50 ml of hydrogen peroxide in a 500 ml beaker for 2 h at 142 °C. After being rinsed 6 times in H_2O, the coverslips were carefully transferred to a beaker containing anhydrous ethyl alcohol overnight and dried on a clean bench.

17. Pioloform coating of coverslips (Figure 1). To coat a coverslip with Pioloform, we placed a glass slide on the desktop centrifuge using double-sided adhesive tape and then mounted a coverslip on the glass slide. Then, 75 µl of 1% Pioloform in chloroform was applied to the coverslip before spinning for 3 min. This treatment produced a thin layer of Pioloform on top of the coverslip. The coverslip was then coated with 250 µl of 0.1% fresh poly-L-lysine for 1 h. Then, 80-nm gold nanoparticles were diluted with H_2O to 25% and sonicated for 15 min. The poly-L-lysine was rinsed off with H_2O, and the coverslip was blow-dried. Finally, the diluted gold nanoparticles were placed on the coverslip for 1 h,

and the coverslip was rinsed with H_2O and blow-dried[1].

18. Cut sections to 100 nm using Ultra-microtome (Leica, EM UC6). Place a section on the center of the pioloform coating of coverslip.

19. Mowiol imaging buffer. Add Mowiol 4-88 (2.4 g) and H_2O (6 ml) to glycerol (6 g), stir to mix and leave for 12 h at room temperature. Add 0.2 M Tris-Cl (12 ml, pH 8.5) and heat at 50°C for 20 min. After the Mowiol dissolves, add DABCO to 2.5% to reduce fading.

20. To find the section, draw a circle using marking pen around the section on the other side of the pioloform coated coverslip. Add 10 µl Mowiol imaging buffer on the section and coat another coverslip on the section. Place the coverslips in the chamber and make sure that the drawn circle is on the top side.

21. Add one drop of oil on the objective and place the chamber with the coverslips and section on the imaging stage. Incubate for 30 min to recover the fluorescence. Find the drawn circle and the section using 10 × DIC. Take the SMLM imaging using 100 ×,1.49 NA oil objective (Olympus PLAN APO) plus a 1.6 × intermediate magnification with an image pixel size of 100 nm. 2000–5000 frames were recorded by an electron-multiplying charge-coupled device (EMCCD) camera (Andor iXon DV-897 BV) in frame transfer mode with exposure time of 50-ms/frame. Excitation light was generated from a Sapphire laser (Coherent) of 488 nm, at 0.6-2 kW/cm^2. Activation light was generated from an OBIS laser (Coherent) of 405 nm, at 5 W/cm^2, with 1 s/pulse and 10-ms exposure time. In order to acquire the signal of gold nanoparticles, increased excitation light of 10-20 kW/cm^2 were applied at the end of the SMLM data acquisition.

22. After the SMLM imaging, take different magnification DIC images (160 ×, 100 ×, 40 ×, 16 ×, 10 ×).

23. Clean the oil using 100% ethanol.

24. Separate two coverslips in H_2O carefully and rinse the pioloform coated coverslip to remove Mowiol imaging buffer.

25. Score a rectangular area surrounding the image section in the coverslip.

26. Add 10 µl of 12% HF on the scored rectangle.

27. Tilt the coverslip slightly and let the HF seep into the Pioloform film.

28. Fill a glass jar with water. Put the coverslip slowly into the water to float the Pioloform membrane carefully.

29. Put an uncoated slot grid on the floating film to capture the section in the center of the opening grid.

30. Coat the glass slide with Parafilm.

31. Use Parafilm coated glass slide to pick up the floating film with the grid.

32. After drying, carefully remove the grid from the Parafilm.

33. Add a drop of 2% UA on a piece of Parafilm on the desk. Put the slot grid on the top of the 2% UA droplet, section side down, for 15 min, blocking light.

34. Pass the slot grid over nine deionized water droplets for washing.

35. Dry the slot grid with filter paper.

36. Place a piece of Parafilm in a Petri dish. To absorb excess carbon dioxide from the air in the Petri dish, add a few NaOH pellets inside the dish and cover the Petri dish for 5 min. Add a drop of Sato's triple lead (filtered through a 0.2-µm filter) onto the Parafilm. Put the slot grid on the top of the lead droplet, section side down. Put the cover back on the Petri dish and stain for 5 min.

37. Pass the slot grid over nine deionized water droplets for washing.

38. Dry the slot grid with filter paper. The slot grid is now ready for TEM imaging.

39. TEM imaging. Find the same cell using different magnification DIC images (160 X, 100 X, 40 X 16 X, 10 X). TEM images were taken using MORADA G3 camera on FEI Spirit TEM operated at 100 kV with 1 s exposure time.

40. SMLM analysis was performed using custom routines in MATLAB as described previously[2], with a modification to remove the background and the noise. The reconstruction parameters were shown in Figure 2.

41. eC-CLEM was used to register the LM image with EM image using gold nanoparticles as fiducial

makers using non rigid (2D or 3D) mode[3]. The whole predicted error map given by eC-CLEM could be used to evaluate the registration accuracy.

Time Taken

It takes four days to finish the whole process.

References

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

Figure 1. Schematic diagram of pioloform coating of coverslip.png
Figure 2. The reconstruction parameters.png