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Photo-Oxidation of MiniSOG Related to Cultured Neurons

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We use this protocol and it's working.

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Abstract

This is a general protocol is for photo-oxidizing neuro-vesicle proteins with MiniSOG. We developed a genetically encoded probe to identify glutamatergic synaptic vesicles at the levels of both light and electron microscopy (EM) by fusing the mini singlet oxygen generator (miniSOG) probe to an intralumenal loop of the vesicular glutamate transporter-2.

Guidelines

We use the monomeric MiniSOG and not the td-miniSOG. The intrinsic fluorescence of MiniSOG is weak, and it photo-bleaches VERY quickly. In some cases, we have to rely on an additional fluorescent tag fused to MiniSOG to confirm expression. Therefore, we do not recommend exposures using regular lamps, but only with low power laser right before photo-oxidation. However, the photo-oxidation is not impaired after photo-bleaching.

Materials

List of buffers and solutions used:

- Fixative (2.5% Glut.,2.5 mM CaCl2 in 0.1 M Cacodyl. buffer pH7.4)
- Washing buffer: 0.1 M Cacodylate pH 7.4
- Blocking buffer: 50 mM Glycine, 10 mM KCN, 20mM aminotriazole in cacodylate buffer
- Fix 1% osmium tetra-oxide in 0.1 M Cacodyl. buffer

- **DAB (free base - MW 214.27) (Cat. No. D8001-10G Sigma)**

DAB solution (make 1 mL more than what is needed because some is lost during filtering):

- Dissolve 5.4 mg free base DAB in 1 mL 0.1 M HCl (final concentration 25.24 mM)
- Filter (0.22 um) into 9 mL of 0.1M Cacodylate or Blocking buffer

Before start

SET UP FOR PHOTO-OXIDATION

1.Filters we use for miniSOG
Leica Blue Excitation : 11513878
Ex:450-490
DM 510
Em:LP 515

Semrock Lucifer Yellow Filter : LuciferYellow-B-000 Filter Set
Ex:FF01-438/24
DM:F482-Di01
Em:FF01-538/40

Lamp House
We use 150W Xenon Lamp House. It is very strong and miniSOG can be oxidized with weaker lamp. It is a good idea to try photo-oxidation with your existing lamp house system. Just avoid UV excitation, which will cause DAB to precipitate. 490nm LED source may be good for miniSOG since it can excite miniSOG well. 1000mW 488 nm is very strong and should be enough or need to attenuate depend on how big illumination area is.

Objective Lens
Our microscope has three objectives:
63x Oil 1.30
63x Water 1.15
20x multi immersion 0.7

1 Plate cells in MaTek dishes.

2 Fix in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature for 5 min. Then, for one hour on ice.
IMPORTANT: Keep Everything cold!

3 Wash 5x for 1 min in 0.1M cacodylate buffer.

4 Block for 30 min on ice with blocking buffer.

5 Wash 3x in 0.1M cacodylate buffer for 1 min.

6 Block in 5 – 10 mM mersalyl acid for 30 min.

7 Wash 3x for 1 min with 0.1M cacodylate buffer.

8 Remove solution from plate and replace with DAB solution.

Microscope

9 At the microscope, set the temperature of the stage at 4˚C. Set the MaTek dish on the proper holder. Install oxygen, find target area with 63X objective lens, take initial picture (fluorescence + DIC).

10 Turn oxygen ON to blow onto surface of sample solution. (Regarding oxygenation: periodically replacing with freshly oxygenated solution is fine too when working with cultured cells).

11 Illuminate target area to photo-oxidize until light browning. **Important:** miniSOG is fast (2 to 10 minutes depending on protein). Optimal timing needs to be determined by investigator and will require the EM analysis of the samples at different reaction time.

12 Take final picture after photo-oxidation. Go to next area in the same dish and repeat, usually 3-4 areas per plate, making sure they are equally spaced to facilitate separation and cutting of

individual blocks after polymerization.

13 When done, remove dish from holder, wash immediately 5x for 1 min in 0.1 M cacodylate buffer, on ice.

14 Fix in 1% osmium tetroxide in 0.1M cacodylate buffer. Leave 30 min on ice.

15 Wash 5x for 1 min in ddH_2O.

16 Optional step: Add filtered 2% UA (uranyl acetate) in ddH2O at 4˚C for 1hr on ice. Wash 5x for 1 min in ddH_2O.

17 Dehydrate: 20-50-70-90-100-100% cold Ethanol (1 min each). Then 100% dry ethanol at room temperature 3x for 1 minute.

18 Infiltration and Embedding: Prepare Durcupan: A:B:C:D=11.4:10:0.3:0.1 g

18.1 Add 50% ethanol: 50% Durcupan to dish. Leave for 30 min.

18.2 Pour out and add 100% Durcupan. Leave for 1 hour.

18.3 Add new 100% Durcupan resin, leave for 1 hr. Scrape out with a pair of plain wood applicators (Product No. 23-400-102 Fisherbrand) and without touching the cover slip containing the cells.

18.4 Add new 100% Durcupan resin, leave for 1 hr. Scrape out with a pair of plain wood applicators (Product No. 23-400-102 Fisherbrand) and without touching the cover slip containing the cells.

18.5 Add new 100% Durcupan resin, leave for 1 hr. Scrape out with a pair of plain wood applicators (Product No. 23-400-102 Fisherbrand) and without touching the cover slip containing the cells.

18.6 Add new 100% Durcupan resin, place in vacuum oven to polymerize for 48 hrs (60˚C).