2

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Photo-oxidation Using MiniSOG with EM Preparation of Transfected Culture Cells V.2

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1

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Abstract taken from Plos Biology Journal: *A Genetically Encoded Tag for Correlated Light and Electron Microscopy of Intact Cells, Tissues, and Organisms*

Electron microscopy (EM) achieves the highest spatial resolution in protein localization, but specific protein EM labeling has lacked generally applicable genetically encoded tags for in situ visualization in cells and tissues. Here we introduce “miniSOG” (for mini Singlet Oxygen Generator), a fluorescent flavoprotein engineered from *Arabidopsis* phototropin 2. MiniSOG contains 106 amino acids, less than half the size of Green Fluorescent Protein. Illumination of miniSOG generates sufficient singlet oxygen to locally catalyze the polymerization of diaminobenzidine into an osmiophilic reaction product resolvable by EM. MiniSOG fusions to many well-characterized proteins localize correctly in mammalian cells, intact nematodes, and rodents, enabling correlated fluorescence and EM from large volumes of tissue after strong aldehyde fixation, without the need for exogenous ligands, probes, or destructive permeabilizing detergents. MiniSOG permits high quality ultrastructural preservation and 3-dimensional protein localization via electron tomography or serial section block face scanning electron microscopy. EM shows that miniSOG-tagged SynCAM1 is presynaptic in cultured cortical neurons, whereas miniSOG-tagged SynCAM2 is postsynaptic in culture and in intact mice. Thus SynCAM1 and SynCAM2 could be heterophilic partners. MiniSOG may do for EM what Green Fluorescent Protein did for fluorescence microscopy.

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List of Buffers and Solutions Used:

- *0.1M Sodium Cacodylate Buffer Recipe for 100ml:
  - 65.7 ml of DDH2O + 1ml of 0.204 M CaCl2 + 33.3ml of 0.3M sodium cacodylate pH 7.4

- DAB solution
  - (0.0052grams DAB in 100µl 1.0N HCL and then add 900µL DDH20). Sonicate for 20 minutes. Add 9.0mL 0.1M sodium cacodylate buffer pH 7.4. Place on Ice. Remove solution after contrast development.

- Durcupan Epoxy Resin Components
A. 11.4 grams (Product No. 102384658 Sigma Aldrich)
B. 10 grams (Product No. 102418273 Sigma Aldrich)
C. 0.3 grams (Product No. 102131679 Sigma Aldrich)
D. 0.1 grams (Product No. 101886488 Sigma Aldrich)
Mix well to the point it homogenous and minimize air bubbles. Let sit for 20 minutes before using.

Durcupan resin epoxy in liquid form is a carcinogen, please wear proper PPE.

Sodium Cacodylate contains arsenic, therefore it is toxic. Wear proper PPE.

Uranyl acetate and glutaraldehyde is toxic, wear proper PPE.

Osmium tetroxides are very powerful oxidizers, they must be used in the fume hood.

1 Cells are grown on glass bottom microwell MatTek culture dishes (35 mm Glass Bottom Dishes No. 0 / Poly-d-lysine coated / γ-Irradiated)

(Part no. P35G-0-14-C, MatTek Corp)

2 Transfected Cells are fixed with 37oC 2% glutaraldehyde (Product No: 18426 Ted Pella, Inc.) in *0.1M sodium cacodylate (Product No. 18851 Ted Pella, Inc.) buffer pH 7.4 with 2.0 mM CaCl2 for 5 minutes at room temp and then for 55 minutes on ice.

3 Wash 5X with 0.1M sodium cacodylate buffer pH 7.4 with 2.0 mM CaCl2 for 2 minutes each on ice.

4 Block with 50 mM glycine, 10 mM KCN, 10 mM aminotriazole and 0.4mM H_2O2 in 0.1M sodium cacodylate buffer pH 7.4 with 2.0 mM CaCl2 for 20 minutes on ice. Added H_2O2 just

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before using.

5 Wash 2X with 0.1M sodium cacodylate buffer pH 7.4 with 2.0 mM CaCl2 for 2 minutes on ice.

6 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.

7 Collect initial picture (confocal fluorescent image + DIC). Excitation beam 488nm is used for the confocal fluorescence collection.

8 Add DAB with 0.22um Vented Millex -GS 25mm sterile filter (Product No. SLGSV255F, Millex). Let sit for 5 minutes. Install oxygen. 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).

9 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.

10 Photooxidation using GFP filter (Ex:450-490nm, DM:510nm and Em:515nm LP) with intense light from a 150W xenon lamp with a stream of pure oxygen gently bubbling the DAB solution.

11 Turn off oxygen. Take final picture after photo-oxidation. Go to next area in the same dish and repeat, turning on the oxygen again, usually 3-4 areas per plate, and making sure they are equally spaced to facilitate separation and cutting of individual blocks after polymerization.

12 When done, remove dish from holder and wash 5X with 0.1M sodium cacodylate buffer pH 7.4 with 2.0 mM CaCl2 for 2 minutes on ice.

13 Post-fix cells with reduced 1-2% osmium tetroxides (Product No. 19150 Electron Microscopy Sciences) (0.8% potassium ferrocyanide + 2.0 mM CaCl2) in 0.1M sodium cacodylate buffer pH 7.4 for 30 minutes on ice.

14 Wash 3X with 0.1M sodium cacodylate buffer pH 7.4 with 2.0 mM CaCl2 for 1 minutes on ice.

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15 Wash 5X with cold DDH_2O, dehydrate 20, 30, 70, 90, 100% ethanol on ice one minute each.

16 Dehydrate with 100% dry ethanol 3X 1 minute each at room temperature.

17 Infiltrated with (1:1 ratio) 100% dry ethanol and Durcupan epoxy resin for 30 minutes (see materials for product information). With lid on and place on rocker. Make sure both components are mixed completely where the mixture is homogenous.

18 Pour out the 1:1 ratio solution and add 100% Durcupan epoxy resin for overnight on a rocker.

19 Next day, add 3X Durcupan epoxy resin for one hour each. When changing resin, scrape out with a pair of plain wood applicators (Product No. 23-400-102 Fisherbrand) and without touching the cover slip containing the cells. After the final change, place in vacuum oven at 60Co for 48 hours.

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