PROTOCOL FOR PHOTOOXIDATION OF DAB BY FM1-43

1) Cells are plated on MatTek dishes that have been treated with 0.1% polyethylenimine[1].

2) Stain cells with FM1-43 as desired.

3) Wash off excess FM1-43.

4) Fix cell with 2% glutaraldehyde in cacodylate buffer[2] for 15 minutes on ice.

5) All solutions are on ice from here on until otherwise noted.

6) Rinse in 0.1M cacodylate buffer 5 x 1 minute.

7) Treat in blocking solution[3] for 5 minutes.

8) Rinse in 0.1M cacodylate buffer 3 x 1 minute.

9) Place in DAB solution[4] and observe with confocal microscope.

10) Record an image (or z-series) of the desired area and then illuminate with ~488 nm light from a Hg or Xe source at maximum intensity. Change the solution every 2-3 minutes. Observe the fluorescence signal in the desired cells and illuminate until a light brown reaction product appears in place of the fluorescence (about 5-10 minutes).

11) Halt the illumination and move to another region of the plate and repeat until several areas are reacted.

12) Rinse the cells with cacodylate buffer 6 x 2 minutes minimum.

13)Place reacted dish on ice until all plates are reacted.

14) Post fix the cells with 1% osmium tetroxide[5] for 30 minutes.

15) Rinse in DDW 3 x 1 minutes.

16) Optional: counterstain in 4% uranyl acetate (Electron Microscopy Sciences #22400) in DDW for 30 minutes

17) Rinse in DDW 3 x 1 minutes.

18) Dehydrate in ethanol as follows: 20, 50, 70, 90% ETOH in DDW for 1 minute each on ice.

19) Remove from ice and dehydrate in 100% ETOH (200 proof) 2 x 1 minute at room temperature.

20) Place in 1:1 ETOH-Durcupan ACM resin[6] for 15 minutes at room temp.

21) Remove resin and replace with 100% Durcupan resin for 30 minutes.

22) Repeat step 21 two times. The final volume of resin in the plate should be about 1/3rd full and place in a 60°C oven for 24-48 hours.

[1]Polyethylenimine (Sigma # P3143) coating of plates: Make a stock solution of 0.1% polyethylenimine in DDW. Add a few drops to the center of the dish and let sit for 1-2 minutes. Rinse in DDW and allow to air dry before using.

[2]Make a 0.3M stock solution of sodium cacodylate buffer (Ted Pella) pH 7.4 and store this in the fridge. Dilute fresh EM grade glutaraldehyde (Electron Microscopy Sciences, cat# 16220) in cacodylate buffer for a final concentration of 2% glutaraldehyde in 0.1M cacodylate. You will need about 1-2 ml per dish. Caution: very toxic!

[3]Blocking solution is 50 mM glycine, 5 mM KCn, 5 mM aminotriazole (Sigma #A8056) in 0.1M cacodylate. This solution can be stored long term in the fridge. Caution: very toxic!

[4]DAB solution is made as follows: Make a stock of DAB (Sigma #D5637 1gm/$16.80) by dissolving 1gm of DAB in 100 ml of DDW(10mg/ml) and rapidly aliquot 1 ml into polyethylene tubes and freeze in LN2 and store in a –80°C freezer until needed. To make the DAB solution for photooxidation, thaw 1 tube of DAB and add to 9 ml of 0.1M cacodylate buffer on ice and bubble with O2.

[5]Make a stock solution of osmium tetroxide (Electron Microscopy Sciences # 19100) by dissolving 1 gm of OsO4 in 24ml of DDW in a very clean glass container. Snap open the vial and drop it into the DDW. Close container and place in another glass container and wrap in aluminum foil and store in a fridge (note: takes 24 hours to dissolve). To make the final solution take 1 ml of stock and add to 3 ml of 0.1M cacodylate buffer. Note: This solution is dangerous and should be used only in a hood.

[6]Durcupan ACM resin (Electron Microscopy Sciences #14040 kit) is made as follows: Into a clean plastic tri pour beaker weigh out 10 gm component A, 10 gm component B, 0.3

gm component C and 0.1gm component D. Do not contaminate stock components by adding back any excess. Mix the resin with a plastic pipette very thoroughly (2-3 minutes minimum). Take a small amount of the resin and mix with 100% ETOH in approximate equal volumes and mix well.

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