# PROTOCOL FOR PHOTOCONVERSION OF DAB BY REASH

After cells are stained with ReAsH they are fixed with 2% glutaraldehyde (EM grade) in 0.1M sodium cacodylate buffer pH 7.4 for 15 minutes. The fix is added at 35 °C and then the dishes are placed on ice.

Cells are washed in buffer for 5x1 minute and background staining is blocked with a solution of 10mM KCn, 10mM aminotriazole, 0.01% hydrogen peroxide, and 50mM glycine in 0.1M cacodylate buffer for 5 minutes on ice.

Cells are rinsed 3x1 minute in buffer and the area of interest is identified by fluorescence microscopy using a standard rhodamine filter cube. An inverted epifluorescence microscope with a confocal attachment is preferred. Note: avoid bleaching the region of interest. A strongly fluorescent signal is required. Use high n.a. optics.

A solution of 1mg/ml DAB (Sigma) in cacodlyate buffer that is on ice and bubbled with O2 is added to the cells and after a few minutes, a Hg or Xe light source is used to illuminate the area at full intensity using a rhodamine cube.

The region is monitored by transmission light and the solution is changed every minute or two. A light brownish reaction product should begin to appear after 5-15 minutes.

The reaction is stopped by halting the illumination. Several areas on each dish can be reacted. However, after a period of 5-10 minutes the DAB solution will temporarily reduce the overall ReAsH brightness. If required the sample can be rinsed with buffer to restore the brightness. When a new area is located, replace the buffer with the DAB solution.

After photoconversion, the cells are rinsed in buffer 5x3 minutes and postfixed in 1% OsO4 in cacodylate for 30 minutes on ice.

Cells are rinsed in ice cold DDW 5x1 minute and are dehydrated as follows: ice cold ETOH: 10, 20, 50, 70, 90% for 2 minutes each followed by 100, 100% ETOH for 2 minutes each at room temp.

Cells are infiltrated with Durcupan ACM resin (EMS Corp.) as follows: 1:1 ETOH-Durcupan for 30 minutes followed by 100%, 100%, 100% Durcupan for 1 hour each.

Resin is polymerized at 60°C for 24 hours.

## ADDITIONAL INFORMATION FOR MAKING SOLUTIONS:

[1]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!

[2]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!

[3]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.

[4]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.

[5]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.

## MORE INFORMATION:

There are a number of small but very important details which need to be addressed in order for the photooxidation to be successful. For instance, one must use an inverted microscope to ensure that high n.a. oil immersion optics can be used and still have the solution the cells or tissues are bathed in freely exchangeable. We generally use a Zeiss Axiovert equipped with a xenon or mercury source and a standard rhodamine filter cube. For imaging, the microscope is attached to a BioRad MRC-1024 confocal system.

Use Sigma DAB (the water soluble tetrahydrochloride (cat# D5637) is what we recommend). We buy it in the 1 or 5 gram size and rapidly dissolve it in DDW at a concentration of 10mg/ml, aliquot 1ml each into eppindorf tubes, cap them and freeze them in LN2 and store them in a -80 freezer. This way we have a uniform batch of reagent that is stable for years. We simply remove a tube from the freezer and dissolve it into ice cold buffer for a final concentration of 0.5 mg/ml. This solution is made a fairly short time before use (it is stable for several hours) and is continually bubbled with O2. Once the area of interest is identified the solution is added and the region is irradiated with the most intense light you can obtain with your set up. The solution is changed every minute or two during illumination and generally speaking, as viewed with transmitted light the reaction product begins to appear in the 4-12 minute range.

One source of background is from the mitochondria and peroxisomes, which can spontaneously photooxidize the DAB after a few minutes. This is greatly reduced with prior fixation with 2% glutaraldehyde followed by KCn and aminotriazole treatment for 5 minutes.

## MORE INFORMATION

If you experience problems I would recommend getting familiar with the photoconversion process by starting out photoconverting the dye bodipy-ceramide which loads into the Golgi apparatus. Here is a protocol:

# MATERIALS AND METHODS:

## BODIPY-CERAMIDE LOADING

Cultured NRK cells (or other types) grown on MatTek dishes are incubated in 50uM Bodipy-ceramide (Molecular Probes Inc.) in normal media for 30 min at 37°C. Cells are washed 5x1 minute in media and placed back in the incubator for 20-30 min. Cells are fixed with 2% glutaraldehyde in 0.1M sodium cacodylate pH 7.4 for 30 minutes at 4°C. Using a standard rhodamine filter set you will see that the Golgi apparatus is brightly stained in all the cells.

## PHOTOCONVERSION

A solution of DAB (Sigma, 1mg/ml in 0.1M sodium cacodylate) is placed on ice and bubbled with pure oxygen. Fluorescently labeled cells are incubated in diaminobenzidine tetrahydrochloride (Sigma) for 5 minutes at 10°C. Using an inverted microscope and high n.a. optics, the fluorescent signal is photoconverted to a reaction product by illumination with full intensity green light from a xenon or Hg source using a standard rhodamine filter set for 4-6 minutes. The sample is monitored periodically using transmitted light during this process and the solution is refreshed every 1-2 minutes. The illumination is halted when a light brownish reaction product begins to appear in place of the fluorescence.

## POST PROCESSING

Following conversion, the cells are rinsed with cacodylate and post fixed in 1% $OsO_4$ in cacodylate for 30 minutes. After several brief washes in ice cold DDW the cells are stained 1-24 hours in 1% aqueous uranyl acetate. The cells are then dehydrated in an ethanol series and embedded in Durcupan ACM resin (Electron Microscopy Sciences) and polymerized at 60°C for 24 hours.

After you become comfortable with this near failure-proof protocol, you can move to using ReAsH labeled cells