Immunoperoxidase on Cultured Cells

Day One:
1) Make fixatives, A and B Epon stocks, and PB.

2) Fix cells on dishes by replacing the media with either Nakane for 4 hours or 0.05% Glut. + 3% Para. for one hour, at RT.

3) After ~5 min replace fixative.

4) Wash for 15 to 20 min in 0.1M PB/0.5% BSA, pH 7.4 --- over 5 changes.

5) Permeabilize with 0.1M PB/0.5% BSA/SAP (generally 0.05% or 0.01%) for 10 to 15 min.

6) Add primary antibody in 0.1M PB/ 0.5%BSA/SAP + 0.02% Azide. Incubate in level humid chamber overnight. Optimally have the first two hours at RT and then put the chamber in the cold room for the evening wrapped around the sides with parafilm. (~0.5 ml per dish is the minimal volume needed to cover and keep cells from drying out.)

Day Two:
NO AZIDE!!!
7) Wash with 0.1M PB/0.5%BSA/SAP for 15 min --- many changes.

8) If necessary add bridge in 0.1M PB/0.5% BSA/SAP and incubate for one hour.

9) Take DAB out of freezer and make buffers.
0.2M Cac stock = 42.8 g/ 1 l.
0.2M Tris stock = 24.23 g/ 1 l (generally make ~250 ml)

10) Wash for 30 min in 0.1M PB/ 0.5% BSA/SAP through 5-6 changes.

11) Add peroxidase conjugate to cells and incubate for one hour.

12) Wash for ~20 min in 0.1M PB/0.5% BSA/ SAP, ~ three changes.

13) Wash for five min in 0.1M PB/0.5% BSA (No Saponin) through numerous changes (~3).

14) OPTIONAL Wash in 0.1M Sodium Cacodylate buffer + 7.5% Sucrose, pH 7.4, for five min through two changes.
(100 ml 0.1M Cac + 7.5% Sucrose = 50 ml Cac stock, 7.5 g sucrose, and fill to 100 ml with ddH20.)

15) Fix with 4% Glutaraldehyde in 0.1M NaCacodylate + 2% Sucrose, pH 7.4, at RT for one hour.
10 ml = 5 ml 0.2M Cac stock + 4 ml 10% glut + 0.75 g sucrose

16) Make DAB.

17) Wash for 30 min in 0.1M Cacodylate + 7.5% Sucrose, pH 7.4, through ~3 changes.

18) Rinse with Tris --- 0.05M Tris + 7.5% Sucrose, pH 7.4, for five min through ~3 changes.
0.05M Tris + 7.5% Sucrose = 50 ml 0.2M Tris stock + 140 ml ddH2O + 15 g sucrose; remember to adjust to pH 7.4

19) Perform DAB rxn. with freshly made and filtered, through a 0.22mm Millipore filter with 10 ml syringe, DAB, placed into a 10 ml disposable tube, wrapped in Al. foil, put on ice.
0.2% DAB in 50 mM Tris, pH 7.2-7.4 = 30 ml 0.05M Tris
+ 7.5% Sucrose, pH 7.4
+ 60 mg DAB (2mg/ml)

Use and label DAB glassware and keep on ice. It is important to adjust the pH and not to overshoot it. This generally requires about 1 drop of 10N NaOH.

Monitor rxn. with Inverted Phase Scope and keep track of times.
Start rxn. by adding H202 to a final concentration of 0.005- 0.01%
(no more than 5 drops, one at a time).
Make H202 by mixing 50 ul of 30% H202 into 3 ml ddH20 = 0.5% sol'n H202.
Keep on ice.
Add 1 lambda aliquots to 1 ml DAB =

0.005% H202.
Shake Dish.
Wait and Observe.
(Check cells on Phase 10x; move obj. bet. 0 and click--just above click = iris, which changes illumination.)

20) Stop rxn. by rinsing cells 2x fast with Tris (0.05M Tris + 7.5% sucrose, pH 7.4)

21) Put cells in NaCac buffer --- dump, and add 400 ul, and then scrape.

22) Pellet cells (alot about one hour's time).
Gently scrape cells with rubber policeman and pipet into 400 ul "needle" tubes. Spin in Microfuge for 10 min. Pry pellet out of tube into a larger test tube (or mincing dish) filled with Cac buffer. To pry out pellet, first cut tip off tube away far from pellet. Pry with a scissor, pen or other dull, pointed instrument. When pellet starts to float, cut off top of tube.

23) Post-fix cells with reduced 0s04 for one hour one ice, light tight, under hood.

Reduced 0s04 = 5 ml 0.2M Cac
+ 2.5 ml 4% 0s04
+ 0.1 g KFeCN (10 mg/ml --- Add just before use)
+ ddH20 to 10 ml

= 1% 0s04 + 1% KFeCN in 0.1M Cac, pH 7.4

24) Rinse with Cac 'til clear. Take resin out of refrigerator.

25) Dehydrate cells with graded series of ethanol (70, 95, 100, 100) quickly EA (~1 min).

26) Place in 50% PO/50% Epon (can be old) for 30 min. on wheel with caps off.

27) Embed in 100% Epon rotatting on the wheel with caps off, 2x30 min. (If destroying pellet, don't change Epon.)

28) Put typed or pencil-written label and sample in capsule with a wooden stick and place in 60o oven overnight.

Day three: