PROTOCOL FOR CYTOCHROME OXIDASE HISTOCHEMISTRY

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Adapted from Silverman and Tootell 1987*

This protocol has been optimised for use with frozen primate brain tissue (marmoset and macaque).

Tissue preparation steps

1. Transcardial perfusion with heparinised 0.1M phosphate buffered saline followed by 4% buffered paraformaldehyde.
2. Overnight post-fixation in 4% buffered paraformaldehyde at 4^{o}C.
3. Remove brain from skull and block for sectioning.
4. Cryoprotect block with 10%, 20%, 30% sucrose in buffered paraformaldehyde at 4^{o}C.
5. Tissue block frozen onto chuck and sections cut at 40 or 50µm at ¯22- ¯24^{o}C.
6. Tissue sections stored overnight in 0.1M sodium phosphate buffer at 4^{o}C or may be stored in cryoprotectant at -20^{o}C for longer periods of time (at least 6 months, actual limit is still to de determined)

REACTION SUBSTRATE

	Full quantity	Half quantity	Quarter quantity
DISTILLED WATER	125ml	62.5ml	31.25ml
PHOSPHATE BUFFER (0.2M)	125ml	62.5ml	31.25ml
DIAMINO BENZIDINE (DAB)	125mg	62.5mg (0.063g)	31.25mg
CYTOCHROME C	40mg	20mg (0.02g)	10mg
CATALASE	30mg	15mg (0.15g)	7.5mg

PREPARATION OF THE SUBSTRATE

It is advisable to use new disposable plasticware for preparation of the substrate solution and reaction of the tissue. This helps to prevent development of precipitates and obviates the need to decontaminate the equipment.

Dissolve DAB in distilled water and make up to full quantity with 0.2M PB (gives 0.1M solution). Add the cytochrome C and catalase and dissolve. Use 1ml of substrate for small sections (e.g. marmoset) and 2.5 ml for large (e.g. macaque) sections.

METHOD

1. Cut frozen sections into 0.1M Phosphate Buffer, if sections will be stained the day after cutting. Store at 4°C.
   Sections may be stored in cryoprotectant at -20°C if the reaction cannot be completed the following day, however best results are obtained when stained straight after cryosectioning.

2. Rinse in phosphate buffer (0.1M) (10 minutes)

3. Pre-treat in 0.1% cobalt chloride (0.5g in 500ml dH_20) for 15 minutes.

4. Rinse twice in phosphate buffer (10 minutes each)

5. Incubate in DAB substrate (see above) for 2 hours, checking sections after 1 hour. **This step should be done under gentle agitation on an orbital shaker platform placed in a 37°C incubator.** Sections may require longer than 2 hours but time in substrate should not exceed 4 hours or the solution may develop a dark precipitate and need to be exchanged.

6. Once cytochrome oxidase-rich features can be visualised by naked eye, remove sections from substrate and wash in phosphate buffer (10 minutes).

   (Note: monitoring sections including primary visual area [V1] is usually found informative for primate tissue. The reaction should reveal cytochrome oxidase "blobs" in high contrast. Perform this step once the blobs can be visualised as dark spots against a brownish background).

7. Mount sections onto superfrost plus slides in 0.005M PB (or gel-subbed 3' x 2' slides for larger sections)

   (Note: If sections cannot be mounted on the same day, perform an additional wash in PB and store at 4°C).

8. Dry sections overnight

9. Dehydrate and coverslip.

   a. 100% ETOH x 1 minute
   b. 100% ETOH x 1 minute
   c. Xylene with ETOH (5:1) x 1 minute – for large slides/sections only
   d. Xylene x 2 (1 minute each)

TROUBLESHOOTING:

Patchy staining may indicate that the sections have folded during the reaction. For best results, make sure that the sections are spread out in the bottom of the well of a culture plate during treatment in the cobalt chloride (step 3) and reaction in the substrate solution (step 5). Use enough substrate solution to allow the tissue section to be easily flattened out in the bottom of the well with a brush.

The tissue should be kept as cold as possible during post-fixing and cryoprotection, and sent to the fridge as soon as possible after cryosectioning, to minimise enzyme degradation.

Sections stored for long periods of time in the freezer in cryoprotectant should stain, but should be rinsed thoroughly before reaction in the cobalt chloride. They may also require shorter reaction times in the cobalt chloride (5 mins or so) and can be left as long as necessary in the substrate solution. For best results, a fresh substrate solution, pre-warmed to 37^{o}C should be substituted for the old after about 4 hours or when a dark precipitate begins to form in the bottom of the well.

Sections will continue to darken once mounted if the mounting solution is too "salty"; hence we prefer to use diluted (0.005M) PB solution.

Sections that have overstained may be brought back to a differentiated step (to some extent…) by washing in undiluted (37%) formaldehyde solution. Monitor this step visually. Care should be taken to rinse well (multiple changes of PB) or they will continue to lighten after removal from the formaldehyde.

Solutions and Reagents:

0.2M Stock Phosphate Buffer (PB): (dilute 1 in 2 in dH_2O to give 0.1M solution)
NaH_2PO_4. Monohydrate 6.348g/litre – dissolve first then add
Na_2HPO_4 anhydrous 21.851g/litre
Stir until dissolved
Should give a pH of 7.2

Cobalt Chloride Hexahydrate (ICN Biomedicals 195107)
Dissolve 0.5g in 500ml dH_2O

3' 3'Diaminobenzidine tetrahydrochloride (DAB) (Sigma D-5637)
Cytochrome C, Horse heart (Sigma C-2506)
Catalase, Bovine liver (Sigma C-40)

Cryoprotectant solution
1L of 0.05M PB (250ml of 0.2M stock diluted with 750ml dH_2O)
600ml Ethylene Glycol
400ml Glycerol

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