

## PhosphoCREB Immunostaining Protocol (Neurons)

1. Plate cells on 35 mm dishes.
2. Treat cells (10 minutes with 10  $\mu$ M Forskolin gives a good response).
3. Remove media.
4. Rinse with PBS.
5. Add 4% paraformaldehyde in PBS (prewarmed to 37°C).
6. Fix cells for 15-30' (no longer than 30') at room temp.
7. Remove fixative, rinse once with 1-2 ml of 10 mM PBS/glycine (prepared fresh) to neutralize PFA.
8. Wash 2 X 5 minutes with PBS/glycine.
9. Remove, add PBS/0.5% NP-40 to permeabilize cells, for 5-10 minutes.
10. Rinse once with PBS.
11. Add 3% BSA in PBS to block non-specific background.
12. Incubate for 45-60 minutes.
13. Remove blocking solution, add anti-phosphoCREB (antibody 2520, IgG fraction) at 1:1000 dilution (0.7  $\mu$ g/ml stock) with 3% BSA. The Ab concentration should probably be titrated for optimal signal in different cells.
14. Put coverslip on cells and incubate overnight at 4°C. Keep plates in a humidified container (wet paper towel), esp when using small of Ab.
15. Rinse once with PBS, then remove the coverslip.
16. Wash once with PBS for 10 minutes.
17. Add biotinylated Secondary antibody (anti-rabbit, Vectastain) (1:200) in 3% BSA/PBS for 1h at room temp. *After 40', make ABC reagent.*
18. Rinse once with PBS.
19. Wash 10 minutes with PBS.
20. Incubate for 30 minutes with 0.5–1 ml of ABC reagent (VectaStain kit, Vector Laboratories) at room temp.
  - 2 drops solution A into 5 ml PBS, mix, then add 2 drops solution B, mix (prepare 30 minutes before).
21. Rinse with PBS.
22. Wash with PBS for 10 minutes.
23. Prepare DAB reagent\*:
  - 0.01% H<sub>2</sub>O<sub>2</sub>
  - 0.5 mg/ml diaminobenzidine
  - 0.03% NiCl<sub>2</sub>
  - 0.05 M Tris (pH=7.2)
24. Add DAB reagent. Watch under the microscope to see how long the reaction should proceed, usually less than 10 minutes.
  - Develop ~4-6 plates at a time.
25. Terminate the reaction by adding Tap water, 2 ml.
26. Remove and rinse once with tap water.
27. Mount with glycerol plastine and a coverslip.

**4% Paraformaldehyde in PBS** (in fume hood), 100 mls

Heat <90 mls H<sub>2</sub>O to 60–70°C.

Add 4 g PFA (powder, cold room floor).

Add 5N NaOH dropwise to get into solution if necessary.

Add 10X PBS to 1X final.

pH after cooling (p =7.4). Keep at 4°C for up to 1 month.

**\*DAB reagent (made fresh):**

For 20 ml:

1) In one tube, add 6.66 ml of 30% H<sub>2</sub>O<sub>2</sub> to 10 ml of water.

2) In a separate tube, add 1 ml of 1M Tris (pH=7.2) to 9 ml H<sub>2</sub>O. Add 0.2 ml of 3% NiCl<sub>2</sub>.

3) Weigh 10 mg of DAB into a separate tube.

4) Immediately before use, add Tris/NiCl<sub>2</sub> solution to DAB (10 ml added to 10 mg), then add 10 ml of H<sub>2</sub>O<sub>2</sub> solution. Mix by pipeting, then add to cells.