

## Pre-embedding immunoperoxidase technique at the EM level

- 1) Perfuse-fix the animal, cut brain in a vibratome (60  $\mu$ m-thick sections) and store sections in 0.1 M PB (pH 7.4) at 4°C.
- 2) Wash sections in TBS (30 min).
- 3) Blocking solution: 10% Normal Goat Serum (NGS) in TBS (1 hour).
- 4) Primary antibodies (final protein concentration of 2-5  $\mu$ g/ml) diluted in TBS with 2% NGS (overnight at 4°C).
- 5) Wash sections in TBS (3 x 15 min).
- 6) Secondary antibodies: biotinylated IgG (1:200 dilution) in TBS with 2% NGS (2 hours, RT).
- 7) Wash sections in TBS (2 x 15 min).
- 8) ABC complex in TBS (90 min). Prepare solution 30 min before use.
- 9) Wash sections in TBS (2 x 15 min).
- 10) Wash sections in TB (2 x 15 min).
- 11) Incubate sections in 3-3'-diaminobenzidine tetrahydrochloride (DAB) 0.05% in TB, pH 7.4 (5 min).
- 12) Add 0.01% H<sub>2</sub>O<sub>2</sub> in distilled water. Develop the reaction for several minutes.
- 13) Stop reaction in TB.
- 14) Wash sections in TB (4 x 10 min).
- 15) Wash sections in 0.1M PB (15 min).
- 16) Osmication: 1% osmium tetroxide in 0.1M PB (30 min).
- 17) Wash sections in 0.1M PB (5 x 8 min).
- 18) Wash sections in distilled water (5 min)
- 19) Uranyl acetate: 1% in distilled water (30 min).
- 20) Dehydration: 50%, 70%, 90%, 96%, 100% I and 100% II (10 min each).
- 21) Propylene oxide (2 x 10 min).
- 21) Flat-embedding in epoxy resin (Durcupan) (10 g A + 10 g B + 0.3 g D + 0.3 g C).
- 22) Polymerization in oven at 55-60°C.