**CTB (List) retrograde tracing with Fluoro Nissl counter**

(Floating) **XCR**

Injections

1. Anesthetize C57BL/6 mice (20–40 g) with ketamine/xylazine cocktail (80/10 mg/kg, i.p.) and place them under a dissection microscope.

2. Fill a microneedle syringe (NANOFIL; WPI) with 1% CTB solution in 50 mM PBS and inject in total 5 µl of the solution into the whisker pads subcutaneously.

3. Two to Four days survival.

Perfusion, Cryo-embedding, Sectioning

4. Transcardially perfuse with ice-cold saline followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2–7.3). Postfix overnight with the same fixative.

5. Infiltrate with sucrose gradient (10–30%).

6. Cryo-embed in cryomold (Tissue-Tek) with O.C.T. compound (dry ice/acetone).

7. Section the brain via cryostat into 20-µm-thick slices and harvest them into 24-well culture plates containing anti-freeze solution.

8. PBS wash.

Antibody application (Room temperature, Light shielding)

9. 10% NRS in PBS-XCR, 30 min on shaker

10. 1st antibody in PBS- XCR, ON on shaker

11. PBS-X wash (quick × 1, 10 min × 2)

12. 2nd antibody in PBS-XCR, 2 h on shaker

13. PBS-X wash (quick × 1, 10 min × 2)

14. Streptavidin-Alexa633 solution, 2 h on shaker

15. PBS wash (quick × 1, 10 min × 2)

Counterstaining with fluorescent Nissl

16. Diluted NeuroTrace (Blue), 40-60 min.

17. PBS wash (quick × 1, 10 min × 2)

Mounting

18. Mount on gelatin-coated glass slides and Air-dry (30 min)

Coverslipping

19. Coverslip with 50% (v/v) glycerol/2.5% (w/v) DABCO in PBS.

Observation

20. Observe with epifluorescence or confocal microscopy.

**Solutions**

**CTB solution**

1.0% CTB in 50 mM PBS

**PBS-X** (in total 550 ml)

20% Triton X-100 8.25 ml final 0.3%

in PBS 541.75 ml

**PBS-XCR** (150 m)

lambda-carrageenan 0.18 g (final 0.12%)

normal rabbit serum 1.5 ml (final 1%)

sodium azide 30 mg (final 0.02%)

in PBS-X 148.5 ml

**10% NRS in PBS-XCR** (5 ml)

normal rabbit serum 0.45 ml (final 10%)

in PBS-XCR 4.55ml

**1st antibody** (1:10000 dil.)

Go anti-CTB (List Biological Cat# 703, RRID:AB\_10013220)

in PBS-XCR

**2nd antibody** (1:400 dil., 3.75 µg/ml final)

Biotinylated Rb anti Go IgG (BA-5000; Vector Laboratory)

in PBS-XCR

**Streptavidin solution** (1:400 dil., 2.5 µg/ml final)

Streptavidin-Alexa Fluor 633 (S21375; Thermos Fisher Scientific)

in PBS-X

**Diluted NeuroTrace Solution** ( NeuroTrace Blue, N-21479, Thermo Fisher Scientific)

Confocal microscopy (1:150 dilution with PBS)

Epifluorescence microscopy (1:300 dilution with PBS)

**Reagents**

Normal rabbit Serum (S-5000, Vector Laboratory)