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

(Floating) **XD**

Injection

1. Mount isoflurane anesthetized rat on a stereotaxic frame.

2. Fill mineral oil into a Drummond glass capillary of which tip is 10-20 µm

3. Set the capillary onto the Nanoject II injector and fill 0.1-1.0% CTB solution about 1.0 µl.

4. Advance the capillary to the target brain region and wait for a while (> 5 min).

5. Inject total 0.1-0.5 µl CTB solution by applying 33-65 TTL pulses at 0.2 Hz (4.6 nl / impulse).

6. Wait for a while and retract the capillary gently.

7. Suture skins on the skull and house the rat normally for 2-4 days.

Perfusion and Sectioning

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

9. Section the brain via vibrating microtome into 50 to 60-µm-thick slices and harvest them in 24-well plates containing PBS.

Antibody application (Room temperature, Light shielding)

10. PBS wash.

11 10% NDS in PBS-X, 30 min on shaker

12. 1st antibody in PBS-XD, overnight on shaker

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

14. 2nd antibody solution, 2 hr on shaker

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

16. PBS wash

Counterstaining with fluorescent Nissl

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

18. PBS wash (quick × 1, 10 min × 2)

Mounting

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

Coverslipping

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

Observation

21. Observe with epifluorescence or confocal microscopy.

**Solutions**

**CTB solution**

0.1-1.0% CTB in 50 mM PBS (0.5%)

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

20% Triton X-100 8.25 ml final 0.3%

in PBS 541.75 ml

**PBS-XD** (150 ml)

normal donkey serum 1.5 ml (final 1%)

sodium azide 30 mg (final 0.02%)

in PBS-X 148.5 ml

**10% NDS in PBS-X** (5 ml)

normal donkey serum 0.45 ml (final 10%)

in PBS-XD 4.55ml

**1st antibody**

Go anti-CTB (List Biological Cat# 703, RRID:AB\_10013220) 1:10k dil.

in PBS-XD

**2nd antibody** (1:1000 dilution, 2 µg/ml final)

Donkey anti-goat IgG Alexa 488 (A-11055)

Or

Donkey anti-goat IgG Alexa 633 (A-21082)

in PBS-XD

**Diluted NeuroTrace Solution** ( NeuroTrace Blue, N-21479)

Confocal microscopy (1:150 dilution with PBS)

Epifluorescence microscopy (1:300 dilution with PBS)

**Reagents**

C9903 (Sigma): 2MG, USD499.00

703 (List): 0.1 mg, USD700.00

A-11055 (Thermo Fisher Scientific): 0.5 ml, 71,300 HUF

A-21082 (Thermo Fisher Scientific): 0.5 ml, USD196.00

N-21479 (Thermo Fisher Scientific): 1 ml, 320 USD

Normal donkey Serum IHR-8135 (ImmunoBioScience): 20 ml, $60,00