**Fluoro-Gold (Millipore) retrograde tracing with Fluoro Nissl counter** (Floating) **XG**

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 4% FG solution about 1.0 µl.

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

5. Inject total 0.15-0.3 µl FG 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 one week.

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 12-well plates containing PBS.

Antibody application (Room temperature, Light shielding)

10. PBS wash.

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

12. 1st antibody in PBS-XG, 48 h on shaker

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

14. 2nd antibody solution, 2 h 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**

**PBS-X** (total 500 ml)

20% Triton X-100 12.5 ml final 0.5%

in PBS 487.5 ml

**PBS-XG** (approx. 150 ml)

normal goat serum 1.5 ml (final 1%)

sodium azide 30 mg (final 0.02%)

in PBS-X 148.5 ml

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

normal goat serum 0.5 ml (final 10%)

in PBS-X 4.5 ml

**1st antibody**

Rb anti-Fluoro-Gold (Millipore Cat# AB153-I, RRID:AB\_2632408) 1:2000 dil.

in PBS-XG

**2nd antibody solution**

Go anti-Rb IgG Alexa 633 (A-21071): (1:500 dilution, 4 µg/ml final) (stock as 2 mg/ml)

in PBS-XG

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

Confocal microscopy (1:150 dilution with PBS)

Epifluorescence microscopy (1:300 dilution with PBS)

**Reagents**

AB153-I (Millipore):

A-21071 (Invitrogen): 0.5 ml, 360 USD

N-21479 (Invitrogen): 1 ml, 320 USD

Normal Goat Serum G9023-10ML (Sigma): 10 ml, 29 USD