**BDA anterograde tracing & cFos immunostaining**

**with Fluoro Nissl counter** (Floating)

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 10% BDA solution about 1.0 µl.

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

5. Inject total 0.3 µl BDA by applying 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 40 to 50-µm-thick slices and harvest them in 24-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, ON on shaker

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

14. 2nd antibody and Streptavidin 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 550 ml)

20% Triton X-100 8.25 ml final 0.3%

in PBS 541.75 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 0.45 ml (final 10%)

in PBS-X 4.55ml

**1st antibody** (1:1000 dilution)

cFos (rabbit anti-cFos, ABE457)

in PBS-XG

**2nd antibody and Streptavidin solution**

Goat anti-rabbit IgG Alexa 488 (A-11034): (1:500 dilution, 4 µg/ml final) (stock as 2 mg/ml)

Streptavidin-Alexa 555 (S-21381): (1:500 dilution, 2 µg/ml final) (stock as 1 mg/ml)

(1:400-1000 dilution)

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**

ABE457 (Millipore): 100 µg, 269 USD

A-11034 (Invitrogen): 0.5 ml, 360 USD

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

S-21381 (Invitrogen): 1 mg, 320 USD

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