**ChAT (Millipore), GAD (Frontier), and Glutamate (ImmunoStar) immunostaining with Fluoro Nissl counter** (Floating) **PBX-XD**

Animals

1. If needed, 150 ug colchicine should be injected into lateral ventricle or targeted tissue two days before perfusion to enhance GAD and glutamate signals in the soma.

Perfusion, Agarose-embedding, Sectioning

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

3. Remove the dura. If possible, the arachnoid membrane, the pia also.

4. Embed the brain into the 4% electrophoresis quality agarose.

5. Section the brain with vibrating blade microtome into 40-µm-thick slices and harvest them in 12 well plates containing PBS

Antibody application (Room temperature, Light shielding)

6. 10% NDS in PBS-X, 30 min on shaker

7. 1st antibody mixture in PBS-XD, 2 overnight on shaker.

8. PBS-X wash (quick × 1, 10 min × 2)

9. 2nd antibody mixture in PBS-XD, 2 hr on shaker

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

11. PBS wash

Counterstaining with fluorescent Nissl

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

13. PBS wash (quick × 1, 10 min × 2)

Mounting

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

Coverslipping

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

Observation

16. Observe with epifluorescence or confocal microscopy.

**Solutions**

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

20% Triton X-100 2.5 ml final 0.1%

in PBS 497.5 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 mix solution**

Go anti-ChAT (Millipore Cat# AB144P, RRID: AB\_2079751) 1:500 dil.

Rb anti-GAD67/65 (Frontier Institute Cat# GAD-Rb, RRID:AB\_2571698) 1:200 dil.

Ms anti-Glutamate (ImmunoStar Cat# 22523, RRID:AB\_572244), 1:500 – 4k, (1:1000)

in PBS-XD

**2nd antibody mix solution**

Do anti-Go IgG Alexa 488 (A-11055): (1:1000 dilution, 2 µg/ml final)

Do anti-Rb IgG Alexa 555 (A-31572): (1:1000 dilution, 2 µg/ml final)

Do anti-Ms IgG Alexa 647 (A-31571): (1:1000 dilution, 2 µg/ml final)

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**

AB144P (EMD Millipore): 50 µg, 700 USD

GAD-Rb-Af260 (frontier science): 20 µg, \37,800, 50 µg, \70,200

22523 (ImmunoStar): 100 µl, 440 USD

A-11055 (Thermo Fisher Scientific): 0.5 ml, 79,4000 HUF

A-31571 (Thermo Fisher Scientific) 0.5 ml, 79,400 HUF

A-31572 (Thermo Fisher Scientific) 0.5 ml, 79,400 HUF

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

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