**Glutamate (ImmunoStar) immunostaining with Fluoro Nissl counter (Floating) Donkey**

Animals

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

Perfusion, Agarose-embedding, Sectioning

2. Transcardially perfuse with saline followed by **4% paraformaldehyde, 0.5% 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, 30 min on shaker

7. 1st antibody in PBS-D, 1 overnight on shaker

8. PBS wash (quick × 1, 10 min × 2)

9. 2nd antibody in PBS-D, 2 hr.

10. PBS 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-D** (150 ml)

normal donkey serum 1.5 ml (final 1%)

sodium azide 30 mg (final 0.02%)

in PBS 148.5 ml

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

normal donkey serum 0.45 ml (final 10%)

in PBS-D 4.55ml

**1st antibody**

Ms anti-Glutamate (ImmunoStar Cat# 22523, RRID:AB\_572244), 1:4000

in PBS-D

**2nd antibody** (one of them)

Do anti-Ms IgG Alexa 555 (A-31570) 1:1k dil., 2 µg/ml final,

Do anti-Ms IgG Alexa 594 (A-21203) 1:1k dil., 2 µg/ml final, or

Do anti-Ms IgG Alexa 647 (A-31571) 1:1k dil., 2 µg/ml final

in PBS-D

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

Confocal microscopy (1:150 dilution with PBS)

Epifluorescence microscopy (1:300 dilution with PBS)

**Reagents**

22523 (ImmunoStar): 100 µl, 440 USD

A-21203 (Thermo Fisher Scientific): 0.5 ml, 74,200 HUF

A-31570 (Thermo Fisher Scientific) 0.5 ml, 71,900 HUF

A-31571 (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

**References**