**UMass SCGE Protocol for mTmG reporter mice- protocol 2**

Mice injections:

* Take the RNP aliquot from the freezer and keep on ice until ready to use.

Let the RNP to warm at room temperature before injection (this can be done by loading the syringe first, while preparing the mice for the surgery).

* The mice are anesthetized with Fentanyl/Midazolam/Dexmedetomidine (0.1/5.0/0.25 mg/kg) via IP injection at a volume of 0.1 mL/10 g body mass.
* IS injection coordinate are: ML +/- 2.0 mm

AP +1.0 mm

DV -3.0 mm

* Speed of injections is: 500 nL/min (to a 2 uL total injection) and wait 2 min post injection. Perform injections on both sides of the brain.
* Administer the following reversal agents & analgesics with injection volumes of 0.1 mL/10 g body mass:
  + - Flumazenil/Atipamezole 0.5/5 mg/kg via IP injection (anesthetic reversal agents).
    - Buprenorphine 0.3 mg/kg via subcutaneous injection (analgesics).
* The mice are sacrificed 14 days post injection. (Our in-house tests thus far have gone for 7 days post-injection, but 14 days should be fine too.)

Mice dissections:

* Inject the mice with Fatal-Plus (Vortech Pharmaceuticals) dilution (0.1 ml/10 g body mass dose) via IP injection
  + Fatal-Plus stock solution is diluted 1:10 in 0.9% sterile saline
* Perfuse the mice with 1XPBS pH 7.4
* Collect the brains
* Fix in cold 4% PFA for 18 hours at 4oC. (Important, no ethanol)
* Transfer the brains into 1XPBS pH 7.4
* Section the brains with Vibratome at 40 µm.
* Stain floating slices using IHC for GFP

4% Paraformaldehyde preparation:

1. Weigh paraformaldehyde in hood (4g per 100mL needed)
2. Warm milliQ water (1/3 of total volume needed)
3. Add a few (4-5) drops of 10M NaOH to warm milliQ water
4. Add paraformaldehyde to warm, basic water. Cover and stir on stir plate at 50°C until dissolved (in hood)
5. Add 10X PBS so that the final solution will be 1X PBS
6. Measure pH and adjust to about 7.0 with HCl
7. Filter with a 0.22μM filter and store at 4°C for up to 2 weeks

Immunohistochemistry for GFP

*Following Vectastain Elite ABC Kit*

1. Cut 40 µm sections with Vibratome and store floating in 1X PBS pH 7.4 (24 well plate) at 4⁰C until use.
2. Wash each well with 500µL 3% hydrogen peroxide for **exactly** 3 minutes. (stock is 30%)

\*Dilute wells with 1X PBS after timer goes off before starting to remove hydrogen peroxide.

1. Wash with 1XPBS 2 times for 5 minutes
2. Prepare 1.5% goat serum blocking solution: add 3 drops (150µL) of Normal Goat Serum **(Vector labs #S-1000)** to 10mL of 1X PBS.
   1. Add **500µL per well** and block sections for at least 1 hour at room temperature. (It is better to do 3-4 hours of blocking if you have time)
3. Wash with 1X PBS 3 times for 5 minutes. **Leave blocker on (-) controls**
4. Prepare primary antibody **(anti-GFP Thermo/Invitrogen G10362)**, add **500µL per well** and incubate at room temperature for 5 minutes and overnight at 4⁰C:

Normal Goat Serum (3 drops or 150µL in 10 mL 1X PBS)

1:1,000 dilution GFP primary antibody

1. Wash with 1X PBS 2 times for 5 minutes
2. Prepare secondary antibody, add **500µL per well** and incubate at room temperature for 10 minutes

Normal Goat Serum (3 drops or 150µL in 10 mL 1X PBS)

Biotinylated anti-rabbit IgG secondary antibody **(Vector Labs Goat Anti-Rabbit IgG Biotinylated Cat. #BA-1000)** (1 drop or 50µL in 10mL)

1. Wash with 1X PBS 2 times for 5 minutes
2. Prepare ABC Reagent **(Vector Labs VECTASTAIN Elite ABC Kit, Perixodase standard cat. #PK-6100)**: Add 2 drops of Reagent A to 5mL 1X PBS, then add 2 drops of Reagent B. Gently mix and **let stand for 30 minutes before use.**
3. Add **500µL** **per well** ABC Reagent and incubate on shaker at room temperature for 5 minutes.
4. Wash with 1X PBS 2 times for 5 minutes
5. Stain with 1XDAB **(Thermo Metal Enhanced DAB Substrate Kit cat. #34065)** for exactly 2 minutes at room temperature, **500µL per well.**

10X DAB (stock) in Stable Peroxide Buffer (NOT PBS)

\*Dilute wells with 1X PBS before starting to remove DAB to avoid over staining later wells.

1. Wash with 1X PBS 3 times for 5 minutes
2. Mount on slides with 1X PBS and 0.5% gelatin in 30% alcohol.
3. Dry overnight or until completely dry, coverslip using Cytoseal.

\*All incubations and 5 minute washes should be on rocker at room temperature unless stated otherwise.

*Materials:*

* *Normal goat serum: Vector labs S-1000*
* *Rabbit Anti-GFP Recombinant Monoclonal Antibody: Thermo/Invitrogen G10362*
* *Goat Anti-Rabbit IgG Biotinylated: Vector Labs BA-1000*
* *VECTASTAIN Elite ABC Kit, Perixodase (standard): Vector Labs PK-6100*
* *Metal Enhanced DAB Substrate Kit: ThermoFisher 34065*
* *Gelatin: Sigma G-1890, Type A from Porcine Skin*