

Histological Processing

Histology core, Gensat Project (November, 2007)

Mice breeding and genotyping

BAC transgenic founders, or F1 females from female founders, are caged each individually with 2 CD-1 (Charles River) females (or one male) to generate timed pregnant animals and early postnatal animals. Female mice are kept in a room with light/dark cycle of 12h to synchronize estrus, and both plugs and births are checked daily. The day of impregnation is designated E0.

Timed pregnant animals are sacrificed as described below on day E15.5 to examine brain structures and spinal cord in embryos. Transgenic (heterozygote) progeny is identified by genotyping for the presence of EGFP. PCR is performed as described in the Transgenic Core Protocol.

Animals in the second litter are genotyped 5 days after birth for the presence of EGFP. The day of birth is designated P0. Transgenic progeny are sacrificed and processed for histological analysis on P7. Some of these animals are kept until adulthood (42 days) and sacrificed then for histological studies.

Histological analysis

1. Neonatal and adult animals are anesthetized with 50 mg/ml Nembutal (P7 :0.05 cc; adult: 0.1 cc; pregnant adult: 0.2 cc).
2. Anesthetized adult and P7 animals are perfused with 4% paraformaldehyde.
3. The brains and spinal cords are removed and post-fixed for 1h at 4°C. Embryos are removed from deeply anesthetized dams by laparoscopy, placed in ice cold PBS and fixed by immersion in 4% paraformaldehyde for 16h at 4°C.
4. The brains and spines from adult/P7 animals and whole E15 embryos are then placed in sucrose solution at 4°C according to the following:

Adult and P7 -5% sucrose for 1 hour
-15% sucrose overnight
-30% sucrose overnight

E15.5 : - 5% sucrose for 1 hour (after rinsing twice with PBS)

-15% sucrose overnight

5. Adult and P7 brains are embedded in “Neg50” Frozen Section Medium (Thermo Fisher Scientific) and embryos are embedded in O.C.T. Compound (Tissue Tec, Sakura). The embedded tissue is stored at -80°C, and is transferred to -20°C the day before sectioning.
6. Serial, sagittal sections of brain are cut at 20 microns with a Leica cryostat and directly mounted onto slides. Spinal cord coronal sections are cut at a thickness of approximately 16 microns. In E15.5 mice, the brain and cord are cut in situ. Sections are taken through the entire head for the brain and through the entire body for the spinal cords.

Adult sections are chosen to approximate the series presented in the Paxinos and Franklin atlas² with sections 1-10 matching atlas sections 129, 126, 123, 120, 117, 114, 111, 108, 105 and 102, respectively. P7 sections are cut to approximate sections from the Valverde atlas³ with sections 1-11 matching Valverde figures 25-35, respectively. E15.5 sections are chosen to approximate those of the Schambra atlas⁴ with sections 1-10 corresponding to Schambra GD 16, figures 1-10, respectively. Transverse spinal cord sections are cut through the cervical, thoracic and lumbar regions for all ages.

Sections are either used directly for confocal imaging or immunostained with antibodies against EGFP.

EGFP Immunolabelling

1. Slides taken from a -20°C freezer are allowed to dry ~30 minutes before washing them 4 times in PBS for 4 min.
2. Slides are postfixed in ice-cold methanol for 5 min at 4°C on a shaker, and then washed twice in PBS for 5 min.
3. Slides are quenched in 0.3% H₂O₂ for 20 min for adult and P7 tissue, and 30 min for E15.5 tissue. They are then washed 4 times in PBS for 4 min.
4. Slides are blocked for 30 min at room temperature in 10% nonfat milk in PBS/TritonX100 0.2%, adding 4 drops/ml of Avidin blocking solution (Avidin/Biotin Blocking Kit SP-2001, Vector Labs).
5. Slides are incubated with primary antibody (anti-GFP, rabbit IgG from Molecular Probes, Cat # A11122; 1:10,000 dilution) overnight at 4°C. Each slide is incubated with ~300µl of primary antibody solution

(diluted with 1% normal goat serum in PBS/TritonX100 0.2%), adding 4 drops/ml of diluent solution of Biotin (Avidin/Biotin Blocking Kit SP-2001, Vector Labs) and cover-slipped with a 12-545M 24x60-1 coverglass..

The rest of the staining is performed at room temperature either manually or on an automated immunostainer.

Manual immunostaining

- 6.** Slides are washed with PBS/Tritonx100 0.2%, 4 times for 4 min.
- 7.** Slides are incubated with secondary antibody (Biotin-SP-conjugated AffiniPure Goat Anti-Rabbit IgG, Jackson Immunoresearch Code Number 111-065-144; 1:500 dilution) for 30 min at room temperature.
- 8.** Slides are washed with PBS/TritonX100 0.2%, 4 times for 4 min.
- 9.** Next they are incubated with Streptavidin-HRP (TSA BIOTIN system, NEL-700 NEN Life Science Products) diluted 1:100 with 1% NGS in PBS/TritonX100 0.2% for 30 min, and then washed with PBS/TritonX100 0.2%, 4 times for 4 min.
- 10.** Slides are then incubated with TSA (**T**yramide **S**ignal **A**mplification Biotin System, NEL-700 NEN Life Science Products) diluted 1:100 with amplification diluent in the kit for 10 min, followed by 3 washes with PBS/TritonX100 0.2% for 5 min
- 11.** Finally, slides are incubated with ABC (ABC Kit Elite PK-6100, Standard Vector Labs) for 30 min (ABC needs to be made 30 min before use; 1 drop of each A and B is added for every 2.5 ml of PBS), and washed with PBS 3 times for 5 min
- 12.** DAB (Diaminobenzidine Tablet Sets, Sigma, Catalog # D4418) staining: 1 "GOLD" and 1 "SILVER" tablet are combined in 15 ml DH₂O. Slides are incubated for 1-10 minutes depending on appearance.

Automated immunostaining

In this case, the rest of the staining is performed at room temperature on an automated immunostainer (DAKO Autostainer - Universal Staining System), following the incubations and washes from the manual protocol.