Protocol:

Selective Targeting of Interneuron Progenitor Cells within the MGE with a Barcoded Retroviral Library

Overview:

This protocol is extracted from the **Figure 1: "Targeted Infection of MGE Progenitor Cells with a Retroviral Library"** of the paper: "Clonally Related Forebrain Interneurons Disperse Broadly across Both Functional Areas and Structural Boundaries.", Mayer, Christian et al. The aim was to replicate their methodology as accurately as possible. For the steps where they referenced methodologies from previous studies, we sought out these original sources to obtain a comprehensive understanding of the procedures. Therefore it does not only replicate the steps detailed in the primary paper but also integrates and cross-references the protocols from the earlier publications they cited. All the papers used to construct this protocol are indicated when relevant and referenced at the end. Whenever standardised protocols, not mentioned in the original paper or its references, were used, we sourced the most up-to-date publicly available relevant protocols from verified institutes or research laboratories. As a result, any assumption or additional information we inferred based on our scientific reasoning will be written in blue font.

We recommend to use or adapt the following protocol if you are interested in single cell-specific labelling and identification through fluorescence, *see Figure 1 from the original paper below*. We also strongly recommend adhering to established SOPs and guidelines for safe and responsible conduct in the laboratory. It is imperative to follow all biosafety protocols meticulously, especially when handling viral materials. This includes using appropriate personal protective equipment (PPE), working in designated biosafety areas, and employing proper sterilisation and waste disposal methods. Always consult your institution's safety guidelines and seek advice from biosafety professionals when necessary.

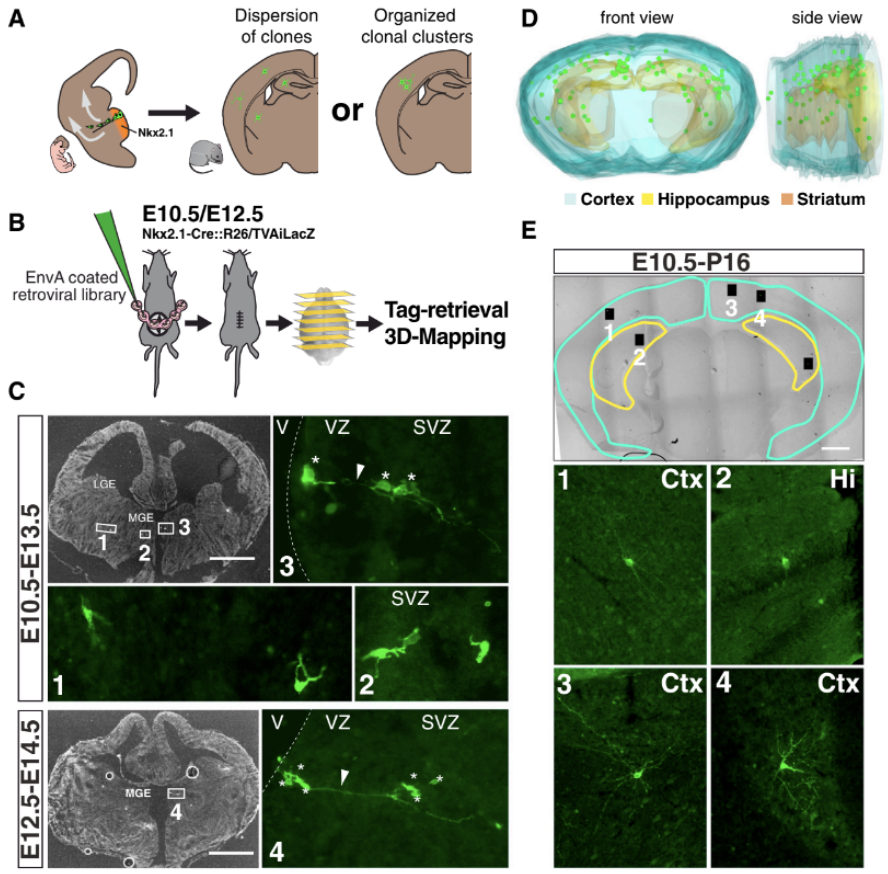


Figure 1. Targeted Infection of MGE Progenitor Cells with a Retroviral Library

(A) A diagram summarizing the experimental design of this study. Orange depicts the area of Nkx2.1-Cre expression.

(B) The experimental strategy.

(C) Overview images (black-and-white) and high-magnification images (green) of sections of embryonic mouse brains 2 to 3 days after injection of a retroviral library at E10.5 or E12.5. Radial clusters of cells expressing GFP (green, asterisks) were found in the VZ and SVZ of the MGE, but not the CGE, LGE, or cortex. Several cells with short processes (white asterisks) are arrayed along a radial process (white arrows).

(D) 3D reconstruction illustrating the distribution of GFP-expressing neurons with a barcode in the forebrain of a P16 mouse that was infected with a retroviral library at E10.5.

(E) Representative high-magnification images and their location within a 25-μm coronal brain section in the cortex (cyan tracing) and hippocampus (yellow tracing). Ctx, cortex; Hi, hippocampus. Scale bars, 500 μm.

See also Figure S1.

Table of contents:

I. Preparation of mouse colonies:

A. Aim

Conditionally express TVA in mitotic cells of the MGE by crossing an MGE-specific transgenic Cre driver line[1] with R26-TVAiLacZ[2] mice. Sourced and adapted from Purdue University's: "Mouse Breeding Colony Management"[3] document.

B. Diagram of Procedure

C. Materials

- ☐ Nkx2.1-Cre/+ Mouse strain: Purchase from Jackson Laboratory (Product ID: 008661).
- ☐ R26LSL-TVAiLacZ/+ Mouse strain: Purchase from Jackson Laboratory (Product ID: 017626).

D. Experimental Procedure

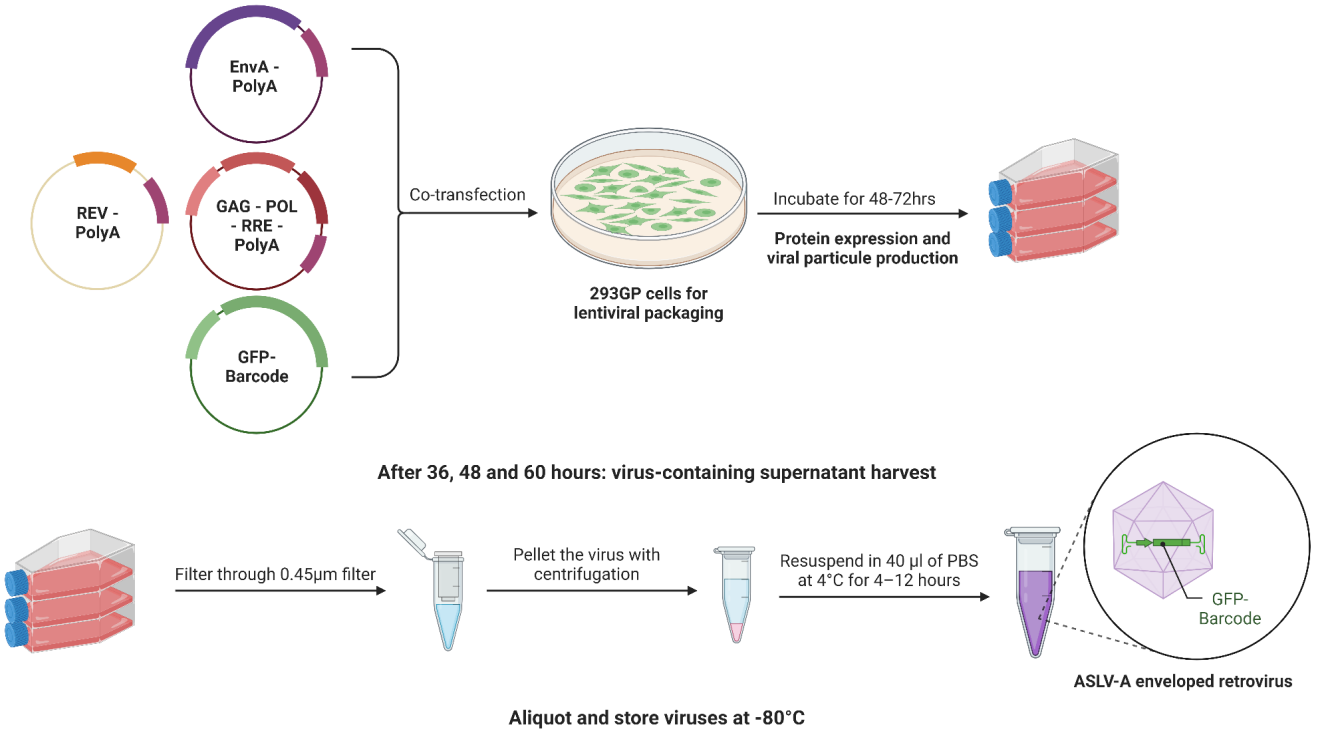
1. Obtain Nkx2.1-Cre mice and R26-TVAiLacZ mice from the appropriate sources.
2. Set up breeding pairs of Nkx2.1-Cre mice and R26-TVAiLacZ mice.
3. Allow the mice to breed overnight.
4. Check for the presence of a vaginal plug the next morning to confirm successful mating.
5. Define embryonic day E0.5 as noon of the day a vaginal plug is detected after overnight mating.
6. Separate the male and female mice into individual cages.
7. Monitor the pregnant female mice for signs of pregnancy, such as weight gain and abdominal distension.

II. Preparation of the retroviral library:

A. Aim

To produce ASLV-A (EnvA) pseudotyped retroviral vectors encoding GFP and containing approximately 10^5 random 24-bp oligonucleotide tags (barcodes) for targeted infection in mitotic cells of the MGE. Sourced and adapted from "A method for rapid gain-of-function studies in the mouse embryonic nervous system", Gaiano et al., methods[4].

B. Diagram of Procedure



C. Materials

- ☐ 293-derived packaging cell line (293GP cells) from Takara Bio (Cat: 632180).
- ☐ **Transfer plasmid:** retroviral construct encoding GFP with barcodes from AddGene (Cat:140024).
- ☐ **Packaging plasmids :** GAG-POL-RRE-PolyA (Cat:12251) and REV-PolyA (Cat:12253)
- ☐ **Envelope plasmid:** ASLV-A envelope glycoprotein (EnvA) from AddGene (Cat:102987).
  Note: this plasmid encodes for Lentivirus expressing eGFP and (EnvA-B19 Rabies Glycoprotein) fusion protein. However, we assumed that the presence of a fused eGFP would not hinder future fluorescence imaging since retroviruses are mostly degraded. However, in case of eGFP hindering we suggest buying custom-design plasmids from your supplier of choice.
- ☐ Calcium phosphate transfection reagents from Thermo Fisher (Cat: K278001).
- ☐ Lipofectamine 2000 Transfection Reagent from Thermo Fisher (Cat: 11668019).

- ☐ Opti-MEM medium from Thermo Fisher (Cat: 31985062).
- ☐ 175 cm^2 flasks or 15 cm dishes from Thermo Fisher (Cat: 159926).
- ☐ 0.45 µm filter from Thermo Fisher (Cat: F2502-3).
- ☐ SW28 rotor from Beckman Coulter (Cat: 342207).
- ☐ Phosphate-buffered saline (PBS) from Thermo Fisher (Cat:10010023).
- ☐ Biohazard Safety Level 2 containment facilities from Thermo Fisher (Cat: 1323TS).

D. Experimental Procedure

1. Use the biohazard Safety Level 2 containment facilities.
2. Transfect 293GP cells with 30 µg of the GFP-barcode retroviral construct, 35 µg of the packaging constructs and 35 µg of the ASLV-A EnvA construct per plate at 90% confluence:

   Note: here we assume 30 µg and 35 µg as those were the amounts used in "A method for rapid gain-of-function studies in the mouse embryonic nervous system", Gaiano et al. as this is the paper referenced in "Clonally Related Forebrain Interneurons Disperse Broadly across Both Functional Areas and Structural Boundaries.", Mayer, Christian et al. when detailing the retroviral library preparation. However, we do not know if this is the definitive amount they used, other amounts may have to be considered.

   a. Prepare the transfection mixture by diluting 30 µg of the GFP-barcode retroviral construct, 35 µg of the packaging constructs and 35 µg of the ASLV-A EnvA construct in a total volume of 500 µL of Opti-MEM medium. Mix gently by pipetting up and down.
   b. Prepare a separate tube with 500 µL of Opti-MEM medium.
   c. Add 10 µL of Lipofectamine 2000 reagent to the tube containing Opti-MEM medium. Mix gently by pipetting up and down.
   d. Incubate the diluted Lipofectamine 2000 reagent for 5 minutes at room temperature.
   e. Combine the diluted DNA construct mixture (step a) with the diluted Lipofectamine 2000 reagent (step d). Mix gently by pipetting up and down.
   f. Incubate the transfection mixture for 20 minutes at room temperature to allow the formation of DNA-Lipofectamine complexes.
   g. While the transfection mixture is incubating, prepare the 293GP cells. Ensure that the cells are at 90% confluence in a suitable culture dish or plate.
   h. Remove the culture medium from the 293GP cells and wash them once with 1 mL of Opti-MEM medium.
   i. Add 2 mL of Opti-MEM medium to the 293GP cells.
   j. Add the transfection mixture (step f) dropwise to the cells, ensuring even distribution.
   k. Incubate the cells at 37°C in a 5% CO2 incubator for 6 hours.
   l. After the incubation period, carefully remove the transfection mixture from the cells.
   m. Add 10 mL of complete growth medium to the cells.

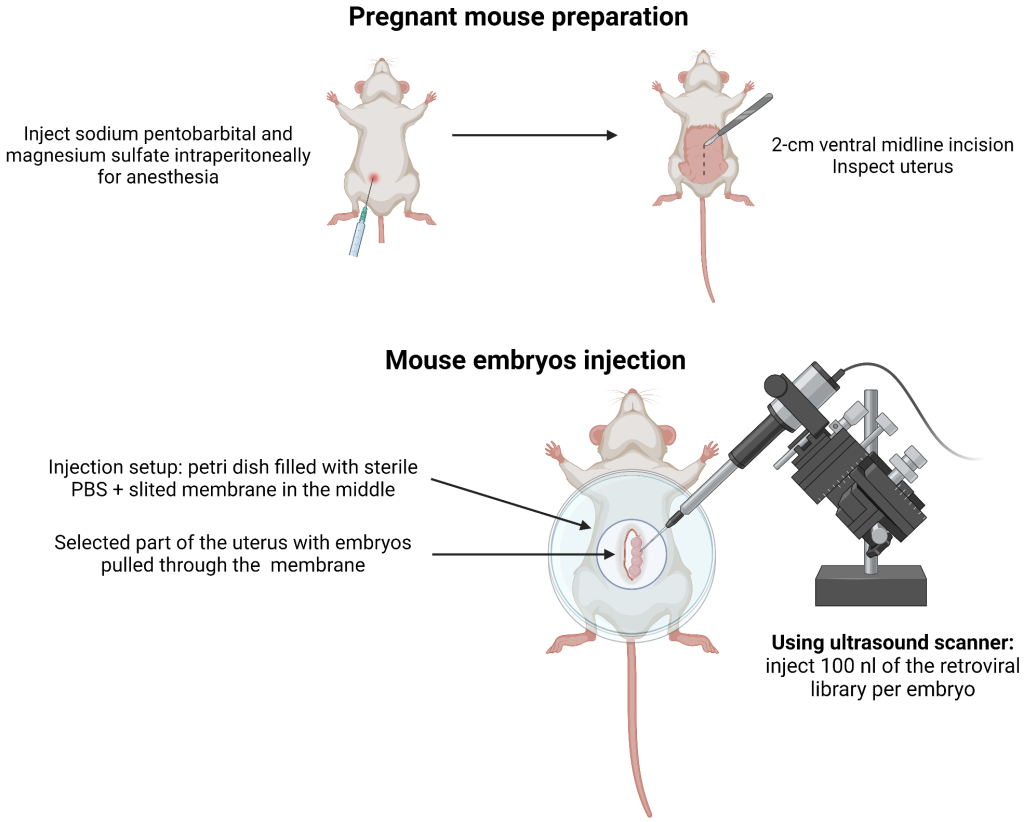
   n. Incubate the cells at 37°C in a 5% CO2 incubator for 48-72 hours to allow for protein expression and viral particle production.
3. Harvest virus-containing supernatant after 36, 48, and 60 hours.
4. Pool and filter harvests through a 0.45μm filter.
5. Pellet the virus for 1.5 hours at 25,000 rpm at 4°C in an SW28 rotor.
6. Resuspend pellets in 40 μl of PBS at 4°C for 4–12 hours.
7. Aliquot and store viruses at −80°C.

III. In utero survival surgery and injection of retroviral vectors:

A. Aim

To inject a retroviral library into the lateral ventricles of transgenic E10.5–E12.5 embryos, the beginning of the peak phase of interneuron neurogenesis in this region. Sourced and adapted from "Alteration of limb and brain patterning in early mouse embryos by ultrasound-guided injection of Shh-expressing cells", Liu et al., methods[5].

B. Diagram of Procedure



C. Materials

- ☐ Mouse Strains: Nkx2.1-Cre from Jackson Laboratory (Product ID: 008661); R26-TVAiLacZ micefrom Jackson Laboratory (Product ID: 017626).
- ☐ Retroviral Vectors: Prepared in 293GP cells, stored at -80°C.
- ☐ Anaesthetics:
  - ☐ Sodium pentobarbital: Purchase from Sigma-Aldrich (SKU P-013-1ML); available at 1.0 mg/mL; dilute to 0.6 mg/10 g body weight using saline.

- [ ] Magnesium sulphate: Purchase from Sigma-Aldrich (SKU M2643-500G); dilute to 1.4 mg/10 g body weight.

- [ ] Surgical Equipment: Scissors, forceps, suturing materials.
- [ ] Ultrasound Backscatter Microscope (UBM): Vevo 770 High-Resolution In Vivo Micro-Imaging System.
- [ ] Injection Equipment:
  - [ ] Injection needles from glass microcapillary pipettes (outer diameter = 1 mm, inner diameter = 0.5 mm, length = 100 mm; Sutter Instruments), pulled to produce a long taper (Flaming/Brown micropipette puller; Sutter Instruments) and broken under a microscope at an outer diameter of 60–70 mm (inner diameter 40–50 mm). Bevelled to an angle of 20° to produce a sharp tip (Micropipette Beveler EG-40; Narishige).
  - [ ] Needles held in a micropipette holder (MPH310; World Precision Instruments) and connected with 1-mm (inner diameter) tubing to an oil-hydraulic manual microsyringe pump (MO-10, Narishige) to draw the cell suspension into the needle.
  - [ ] The micropipette holder attached to a 3-axis micromanipulator (MO-155, Narishige), used to position the needle tip using UBM image-guidance during the injection procedure.
- [ ] Injection setup:
  - [ ] Two-level holding stage
  - [ ] Petri dishes (100 mm diameter, 25 mm deep, from Sigma Aldrich Cat: P5731) were modified by punching a 25 mm diameter hole in the middle and then stretching a thin, 35 mm diameter rubber membrane over the hole.
- [ ] PBS with calcium- and magnesium-chloride, Sigma, St. Louis, MO (Cat: D1283)

D. Experimental Procedure

1. Weight the mouse.
2. Mix sodium pentobarbital (0.6 mg/10 g body weight) with magnesium sulphate (1.4 mg/10 g body weight).
3. Inject this mixture intraperitoneally into the timed-pregnant (E9.5–E12.5) mouse.
4. Shave the abdomen of the anaesthetised mouse.
5. Make a 2-cm ventral midline incision through the skin and peritoneal muscle to expose the uterus.
6. Gently pull out the uterus, allowing both sides of the uterine horn to be examined and one side selected for injection.
7. Place the pregnant mouse in the injection setup.
8. Cut a 15–20 mm slit in the rubber membrane in order to access the uterus after the Petri dish is mounted over the pregnant mouse.
9. Fill the petri dish with sterile phosphate-buffered saline (PBS with calcium- and magnesium-chloride, Sigma, St. Louis, MO).
10. Pull through the slit in the membrane the selected part of the uterus containing one or two embryos for injection through the uterine wall into selected embryonic target regions with UBM-guidance.

11. Using the ultrasound scanner Vevo 770 High-Resolution In Vivo Micro-Imaging System (FujiFilm VisualSonics): inject approximately 100 nl of the retroviral library (titer of ca. 1-5 * 10^8 cfu/ml) per embryo with an oil-hydraulic manual microsyringe pump (MO-10, Narishige).
12. Push back the injected embryos into the abdominal cavity, and pull one or two new embryos into the PBS.
13. After injecting all selected embryos, suture the mouse and allow it to recover in a warming/ humidifying chamber.
14. Monitor and care for the mouse post-surgery.

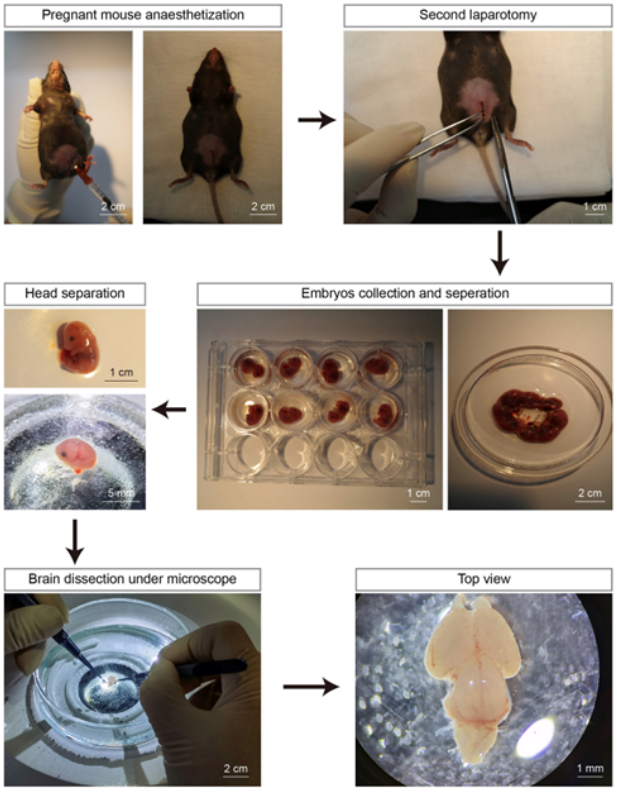
IV. Brain section collection and imaging:

A. Aim

To collect and image brain sections from embryonic mouse brains for further GFP-positive cells identification and analysis. Sourced and adapted from the supplementary files of "Clonally Related Forebrain Interneurons Disperse Broadly across Both Functional Areas and Structural Boundaries", Mayer et al.[6] as well as "Frozen tissue preparation for high-resolution multiplex histological analyses of human brain specimens", Shao et al.[7], and "Protocol for analysis of senescent neuronal stem cells in genetic-modified embryonic mice using in utero electroporation technique"[8] from STAR protocols.

B. Diagram of Procedure

1.



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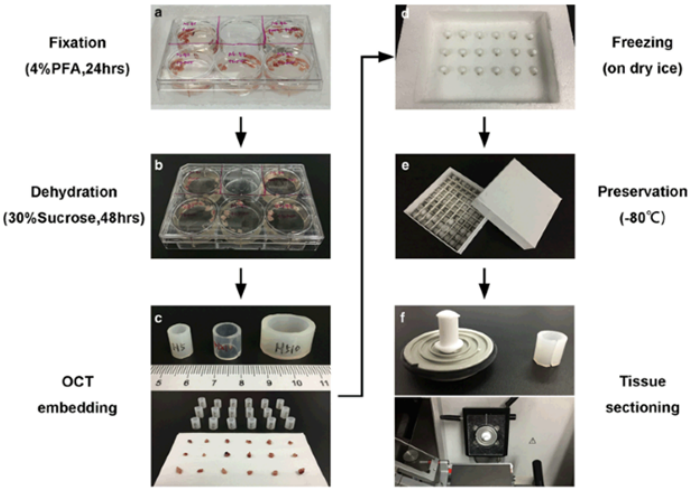

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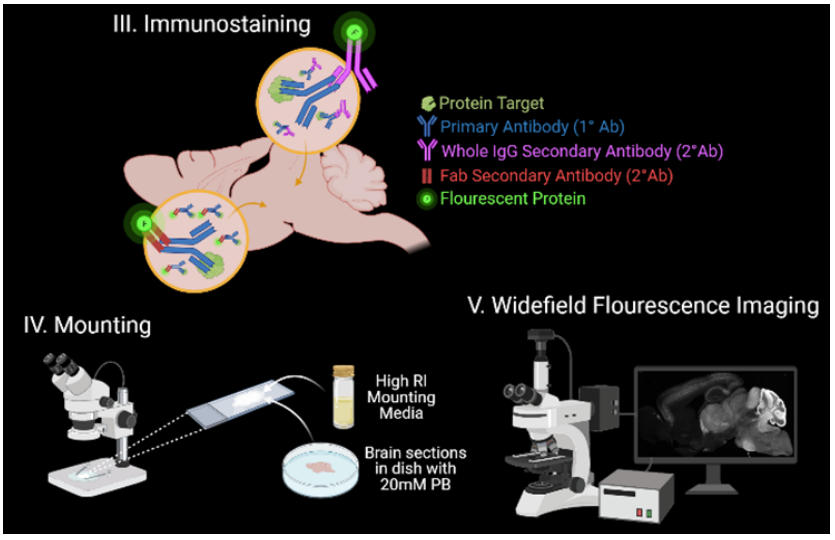


C. Materials

- [ ] Pregnant mice, with embryonic mouse brains 2 to 3 days after injection of a retroviral library at E10.5 or E12.5 **OR** with P16 mouse that was infected with a retroviral library at E10.5.
- [ ] PBS and 4% paraformaldehyde (PFA) (from Sigma, Cat: 158123) for perfusion.
- [ ] OCT Compound for embedding (From Carlroth, Cat: 6478.1).
- [ ] Cryostat (e.g., CM3050 S, Leica).
- [ ] PET-Membrane FrameSlides (From MMI, Cat: 50102).
- [ ] Anti-GFP-Alexa488-conjugated antibodies (1:1000 dilution) (from Thermo Fisher, Cat: A-21311).
- [ ] Fluoromount-G for mounting (from Thermo Fisher, Cat: 00-4958-02).
- [ ] Fluorescence microscope (e.g., AXIO, Zeiss) with a digital camera (CoolSNAP KINO, Photometrics).

D. Experimental Procedure

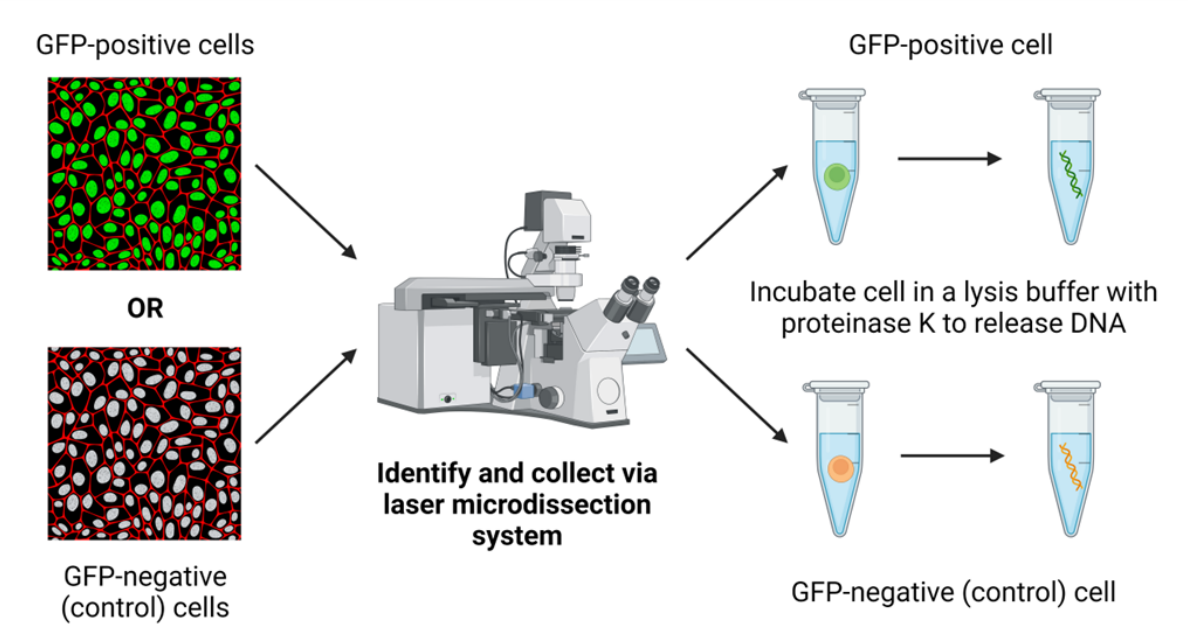
1. Perfuse mice transcardially with PBS followed by 4% PFA.
2. Remove and embed brains in OCT, then section at 25 µm on a cryostat.
3. Collect sections on PET-Membrane FrameSlides.
4. Stain sections with anti-GFP-Alexa488-conjugated antibodies.
5. Mount sections with Fluoromount-G.
6. Image stained cells at 20x resolution and entire sections at 10x, tiling images using microscope software for 3D reconstructions.

V. Sample collection:

A. Aim

Collect GFP-positive cells.

B. Diagram of Procedure



C. Materials

- [ ] Laser Microdissection System (LMD6000, Leica).
- [ ] Lysis buffer from Thermo Fisher, cat EO0491.
- [ ] Proteinase K (1:1000 concentration; QIAGEN, cat: 19157).
- [ ] Microcentrifuge tubes (20 ml capacity, from Sigma, cat: 57188-U).

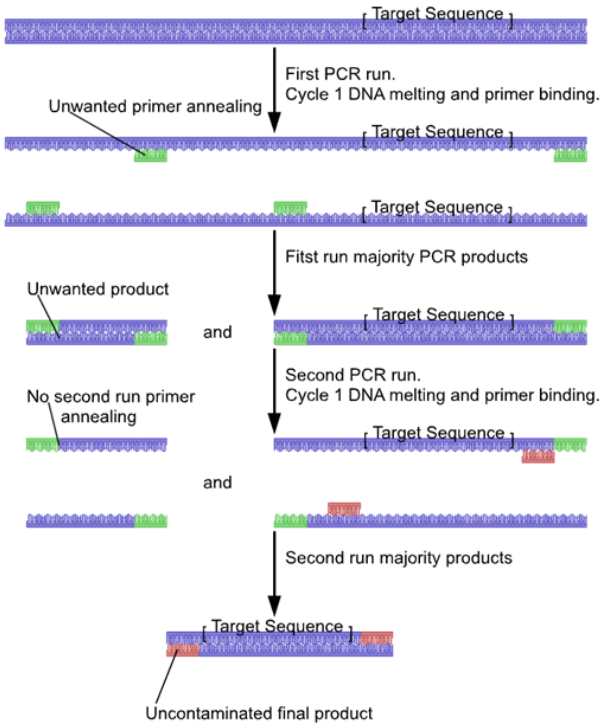
D. Experimental Procedure

1. Identify GFP-positive cells under the laser microdissection system.
2. Adjust the system settings to target and isolate individual GFP-positive cells.
3. Collect each dissected cell into a microcentrifuge tube containing 20 ml of lysis buffer.
4. Add proteinase K to the lysis buffer at a 1:1,000 ratio.
5. Optionally, collect adjacent GFP-negative tissue as a control in a separate tube.
6. Store the collected samples at appropriate conditions for downstream processing.

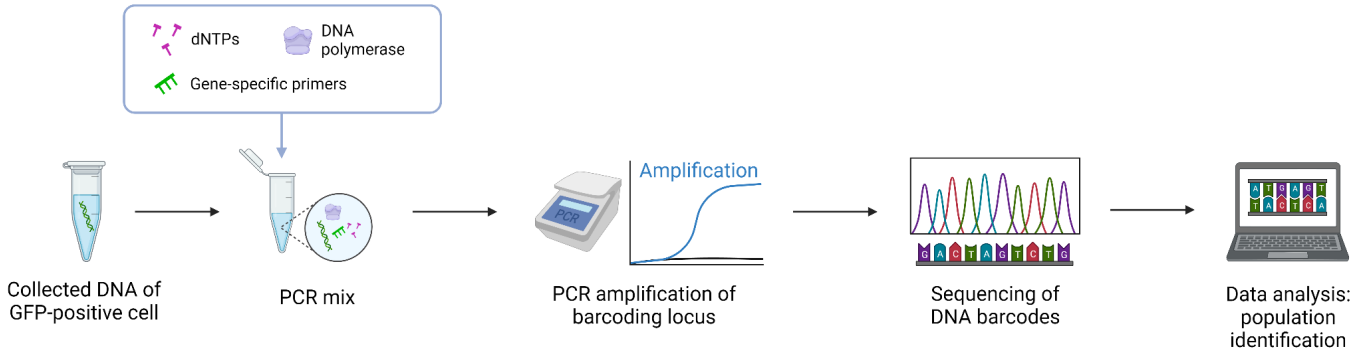
VI. Barcode sequencing:

A. Aim

To lyse cells and perform viral barcode sequencing through a two-step nested PCR. Using two-step nested PCR reduces non-specific amplification of the DNA template (see workflow[9] below) thereby allowing for more accurate barcode identification.



B. Diagram of Procedure



C. Materials

- ☐ Lysis buffer with Proteinase K, from Thermo Fisher, Cat, EO0491.
- ☐ PCR reagents (Taq polymerase, dNTPs, MgCl2, PCR buffer, kit from Thermo Fisher, Cat: 10572014).
- ☐ Primers (from IDT):

First PCR:

- ☐ SBR161-o1 : GACAACCACTACCTGAGCACCCAGT
- ☐ SBR126-o2 : GGCTCGTACTCTATAGGCTTCAGCTGGTGA

Nested PCR:

- ☐ SBR160-n1 : ATCACATGGTCCTGCTGGAGTTCGTGA
- ☐ SBR128-n2 : ATTGTTGAGTCAAAACTAGAGCCTGGACCA

- ☐ Thermal cycler (from Applied Biosystems, Cat: A37834).
- ☐ Gel electrophoresis equipment (from Invitrogen, Cat: G8300).
- ☐ Gel Band Purification Kit (from Sigma, Cat: GE28-9034-70).

D. Experimental Procedure

1. Incubate cells in a lysis buffer with Proteinase K to lyse the cells and release DNA.
2. First PCR: Mix DNA with primers SBR161-o1 and SBR126-o2. Run 40 cycles at 60°C annealing temperature, 35s elongation.
3. Nested PCR: Use the first PCR product with primers SBR160-n1 and SBR128-n2. Run 30 cycles with the same temperature and elongation time.
4. Perform gel electrophoresis to visualise PCR products. Purify using Gel Band Purification Kit.
5. Sequence the purified PCR products.

VII. Barcode recovery from silenced vectors:

A. Aim

To recover barcodes from retrovirally infected cells that did not express GFP, maintaining spatial information of barcodes.

B. Materials

- ☐ Laser Capture Microdissection (LCM) system by Leica LMD6.
- ☐ Polyethylene terephthalate membrane slides by Thermo Fisher, Cat: LCM0522.
- ☐ Plasmid isolation kit by Thermo Fisher, Cat: K0502.
- ☐ Sequencing equipment by Ion Torrent, Cat: A41431.

C. Experimental Procedure

1. Collect large tissue pieces from the same polyethylene terephthalate membrane slides using LCM.
2. Process tissues collected from different sections and different brain structures separately to preserve spatial barcode information.
3. Isolate plasmids from single bacterial colonies and sequence individual barcodes.
4. Sequence a minimum of six colonies per sample to estimate barcode numbers.
5. If multiple barcodes are found, sequence up to 16 colonies per sample.
6. **Exclude barcodes found in more than one brain or hemisphere to avoid contamination or overrepresentation.**

References:

[1] Xu, Qing et al. “Fate mapping Nkx2.1-lineage cells in the mouse telencephalon.” *The Journal of comparative neurology* vol. 506,1 (2008): 16-29. https://doi:10.1002/cne.21529

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Select: Mouse Breeding Colonies Management: https://www.purdue.edu/research/oevprp/regulatory-affairs/animal-research/docs/Mouse%20Breeding%20Colony%20Management%20Document.pdf

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