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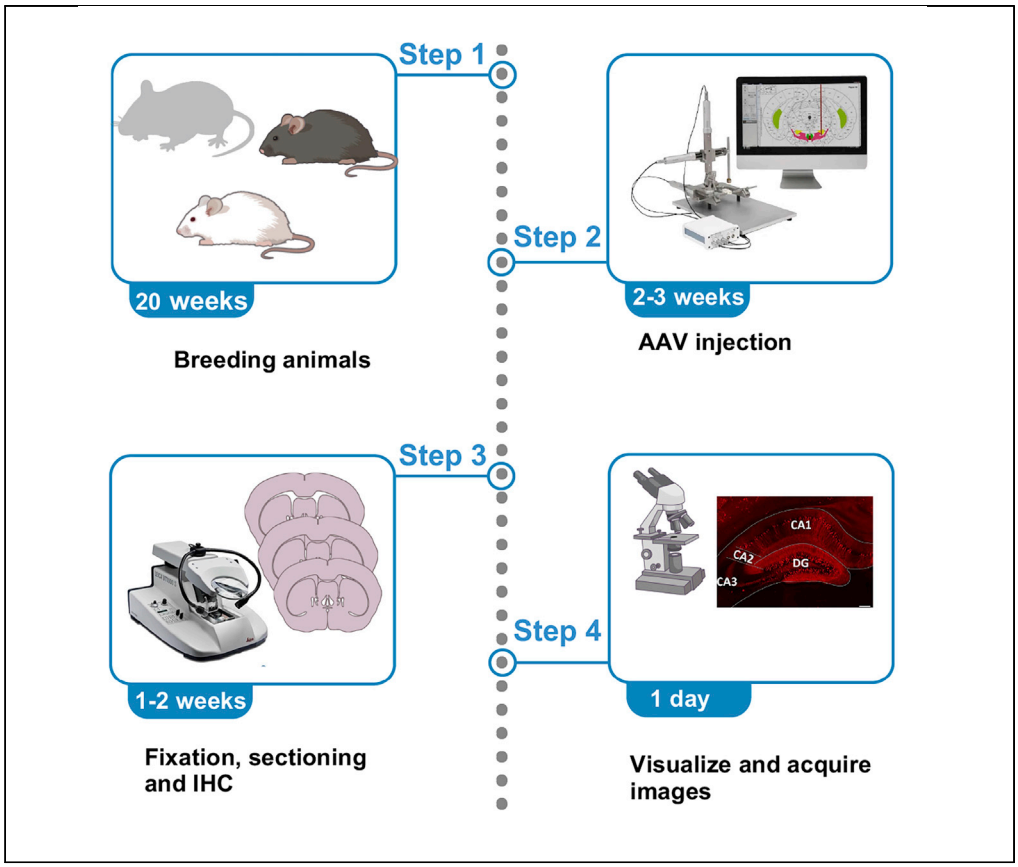
Protocol to map multi-transmitter neurons in the mouse brain using intersectional strategy



Although it is now known that certain neurons can produce, store, and release multiple neurotransmitters, their locations, abundance, and functions remain elusive. We developed intersectional genetic strategies to identify multi-transmitter neurons based on the expression of neurotransmitter-specific genes. Here we present our procedures for whole-brain mapping of GABA/glutamate co-releasing neurons. We also detail our technique for labeling GABA/glutamate neurons in specific brain regions with adeno-associated virus (AAV). Our protocol can be readily extended to other types of multi-transmitter neurons.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Genetic techniques to identify multi-transmitter neurons in the mouse brain

Whole-brain mapping of GABA/glutamate co-releasing neurons

Identifying GABA/glutamate neurons with Cre/Flp-dependent AAV

Applicable to mapping other multi-transmitter neurons

Xu et al., STAR Protocols 3, 101907
December 16, 2022
https://doi.org/10.1016/j.xpro.2022.101907

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Protocol to map multi-transmitter neurons in the mouse brain using intersectional strategy

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https://doi.org/10.1016/j.xpro.2022.101907

SUMMARY

Although it is now known that certain neurons can produce, store, and release multiple neurotransmitters, their locations, abundance, and functions remain elusive. We developed intersectional genetic strategies to identify multi-transmitter neurons based on the expression of neurotransmitter-specific genes. Here we present our procedures for whole-brain mapping of GABA/glutamate co-releasing neurons. We also detail our technique for labeling GABA/glutamate neurons in specific brain regions with adeno-associated virus (AAV). Our protocol can be readily extended to other types of multi-transmitter neurons.

For complete details on the use and execution of this protocol, please refer to Xu et al. (2022).[1]

BEFORE YOU BEGIN

The classical "one neuron, one transmitter" hypothesis posits that each neuron only transmits one type of a neurotransmitter.[2] But it is now clear that many neurons in fact produce and release more than one type of neurotransmitters.[3,4] What is not clear, however, is how abundant these multi-transmitter neurons are and where they are located in the brain. This protocol describes methods using an intersectional strategy to map multi-transmitter neurons based on the expression of genes that specify transmitter type, such as vesicular transporters of neurotransmitters. Specifically, we describe steps for using dual recombinase-dependent reporter mice (Ai65)[5] crossed with Cre and Flp drivers to attain a brain wide distribution of glutamate and GABA co-releasing neurons (Figures 1A and 1B). We also provide a complementary approach to label multi-transmitter neurons in specific brain structures by using Cre and Flp dependent AAV viruses (Figure 1C). This protocol uses glutamate and GABA co-releasing neurons as an example, but it can be readily extended to other types of multi-transmitter neurons.

To perform this protocol, one requires laboratory equipment to conduct genotyping, AAV stereotaxic injection, immunohistochemistry (IHC) and fluorescence imaging. We use a Biorad thermocycler (C1000) for PCR, Leica vibratome (VT1000S) for brain sectioning, Neurostar stereotaxic system for AAV injections and a Keyence microscope (BZ-X700) for acquiring fluorescence images. All these instruments perform satisfactorily but similar equipment can be used depending on availability and preference. Also, we make our AAV preparations in-house. Beckman ultracentrifuge (Optima XE-90) is used in our protocol for AAV purification.

Institutional permissions

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. All animal

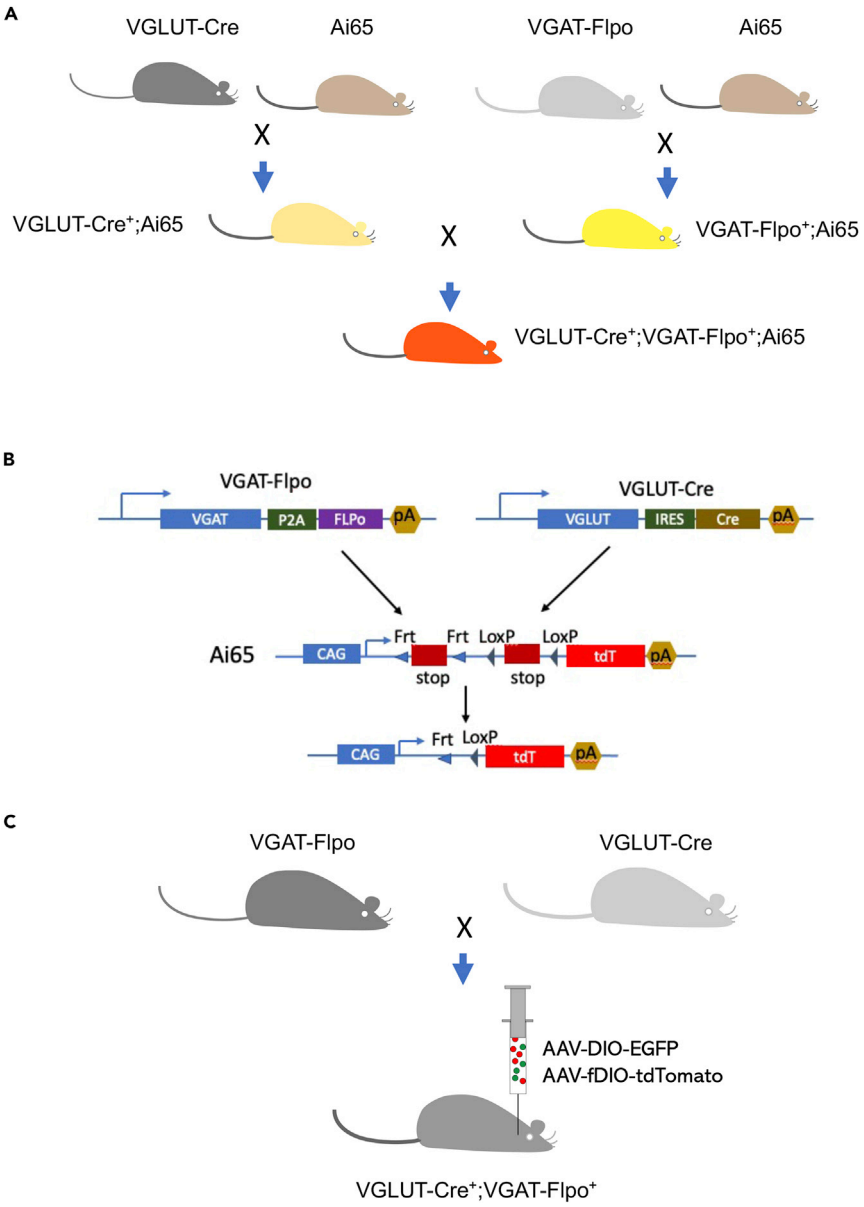

Figure 1. Preparing animals for mapping multi-transmitter neurons

(A) Breeding schemes for generating VGLUT-Cre^{+};VGAT-FlpO^{+};Ai65 triple transgenic mice. Ai65 refers to Ai65^{het} or Ai65^{hom}.

(B) Schematic diagrams of gene expression cassettes in VGAT-FlpO, VGLUT-Cre and Ai65 mice. In Ai65, tdTomato is expressed only when two stop cassettes, LoxP flanked and Frt flanked, are both excised.

(C) Use of Cre dependent AAV (AAV-DIO-EGFP) and Flp dependent AAV (AAV-fDIO-tdTomato) to label glutamate and GABA dual-releasing neurons in VGAT-FlpO and VGLUT-Cre intersectional mice.

procedures were approved by the Institutional Animal Care and Use Committee of Northwestern University. For researchers to perform similar experiments, permission must be obtained from the relevant institutions.

Preparation one AAV productions

Timing: ~2 weeks

Timing: 1–2 weeks (for step 1)

Timing: 1 day (for step 2)

Timing: 30 min to 1 h (for step 3)

This section describes methods to make AAV-in-house. This step can be skipped if researchers prefer to obtain AAV through commercial sources. Our AAV production method is outlined below. It is adapted from previous publications[6–9] and online protocols (https://www.addgene.org/protocols/aav-production-hek293-cells). Briefly AAV was packaged in HEK cells using the triple transfection method. About 3–4 days after transfection, AAV was harvested from cell lysate and also from media, followed by purification over iodixanol gradient ultracentrifugation. Viral titers were measured with a DNA dye-based method we recently developed.[10]

1. Package AAV with desired serotypes using triple transfection method.
   a. Grow the HEK cells to 70%–80% confluence in a cell incubator at 37°C, with 95% humidity and 5% CO2.
   b. Split HEK cells into 10 cell culture dishes (15 cm diameter), wait until cells reach about 80% confluence.
   c. DNA transfection.
      i. Prepare DNA/PEI mixture by adding 105 μg of pΔF6 (Addgene #112867), 52.5 μg of cap/rep helper plasmid, 52.5 μg of AAV vector plasmid, 2 mL of 1.5 M NaCl, 1.1 mL of 1 mg/mL Polyethylenimine (PEI) and 17 mL of H2O.

      Note: We use EndoFree Plasmid Maxi Kit (Qiagen) and Stabl3 *E. coli* strain (ThermoFisher) for making plasmids.

      ii. Mix gently and incubate for 15 min at 20°C–30°C.
      iii. Add 2.0 mL of DNA/PEI mixture to each 15 cm dish.
      iv. Change medium 16 h post-transfection.

      Note: For AAV packaging, an important consideration is AAV serotypes as they have great influence on patterns of viral expression. In CNS serotypes 1, 2, 5, 8, 9 have been most studied. In this project we primarily used AAV serotype 9 (AAV9) for direct labeling and AAV2-retro for retrograde labeling. AAV9 is efficient in almost all brain areas and appears to have rather fast onset of expression therefore it is quite suitable for us. AAV2-retro is efficient in retrograde labeling in most brain regions, with perhaps only a few exceptions.[11] It is therefore also quite appropriate for this project. AAV packaging plasmid expressing Rep/Cap genes can be obtained from Addgene (rAAV2-retro helper #81070; pAAV2/9n #112865).

   d. 3–4 days post-transfection, scrape off cells from the plates, pipette the whole content in the dish into multiple 50 mL Falcon tubes (~ 200 mL from 10 dishes). Perform centrifugation at 1,000 × *g* for 10 min at 4°C to pellet the cell. Transfer supernatant to new tubes.
   e. Resuspend pellet from above with 10 mL of AAV resuspension buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.4).

   Pause point: AAV in resuspension buffer and supernatant from step d can be stored at −80°C indefinitely.

   f. Perform 3 cycles of freeze and thaw, then store the sample on ice.
   g. PEG precipitation of AAV from supernatant collected in step d.
      i. Filter supernatant through a 0.45 μm filtration system (Millipore, S2HVU02RE).

Table 1. Compositions of different iodixanol solutions

	15%	25%	40%	54%
OptiPrep™ 60% (mL)	12.5	20.8	33	45
2 M NaCl (mL)	25	0	0	0
10× DPBS-MK (mL)	5	5	5	5
H2O (mL)	7.5	24.17	5	0
Phenol Red 0.5% (μL)	0	150	0	150
Total volume (mL)	50	50	50	50

ii. Add 3.6 g NaCl and 25 mL 40% PEG ($^1/_4$ volume) per 100 mL of solution from above, keep in ice for 2 h.
iii. Centrifuge at 3000 g for 30 min at 4°C. Discard the supernatant and resuspend the pellet in ~ 5 mL AAV resuspension buffer.

Pause point: Samples can be stored at −80°C indefinitely.

h. Combine samples from **e** and **f** (about 15 mL). Add Benzonase (Sigma E-1014 250 U/μL) to a final concentration of 15 U/mL. Incubate at 37°C for 45 min, then add 10% Sodium Deoxycholate (Sigma D6750) to a final concentration of 0.5%. Incubate at 37°C for another 10 min.
i. Centrifuge at 4°C at 2,000 g for 10 min. Save supernatant (crude AAV lysate).

2. Purify AAV vectors using iodixanol gradient ultracentrifuge.[6]
a. Prepare iodixanol solutions (see Table 1 below).
b. Load crude AAV lysate and iodixanol solutions into Quick Seal tubes (Beckman 25 × 89 mm).
i. Load 15 mL crude lysate into a Quick Seal tube, using either syringe capped with 13 g needle or Pasteur glass pipette.
ii. Underlay with 9 mL of 15% iodixanol solution.
iii. Underlay with 6 mL 25% iodixanol solution.
iv. Underlay with 5 mL 40% iodixanol solution.
v. Underlay with 5 mL 54% iodixanol solution. At this point, the Quick Seal tube should be very close to its full capacity. Use AAV resuspension buffer to top off the tube.
c. Seal the Quick Seal tubes with tube topper.

⚠ CRITICAL: Ultracentrifuge tubes must be balanced to within 0.1 gram. If there is only one AAV sample to prepare, then it is better to bring the volume of the crude lysate to 30 mL and then split the volume into two equal parts to proceed, as this will make tube balancing easier.

d. Centrifuge at 65,000 rpm (~430,000 × *g*) for 90 min in a 70Ti rotor at 18°C.
e. Collect AAV from Quick Seal tubes.
i. At the end of centrifugation, remove Quick Seal tubes from the rotor and secure the tubes on a retort stand.
ii. Insert a 18G needle at top of the tube to release the pressure, then insert a second 18G needle, connected to a 5 mL syringe, at a position about 2 mm below the 40%–54% density junction.
iii. Gently pull back the plunger of the syringe to collect 4–5 mL of fractions, including the junctions at 40% and 54%, along with the lower three-quarters of the 40% gradient.
f. Concentration and buffer exchange.
i. Pre-wet Amicon Ultra-15 100 kDa centrifugal unit (Ultra-15) with AAV formulation buffer (1× DPBS with 35 mL NaCl and 0.001% F68). Spin the Ultra-15 at 3000 × *g* for 5 min, discard the flow-through.

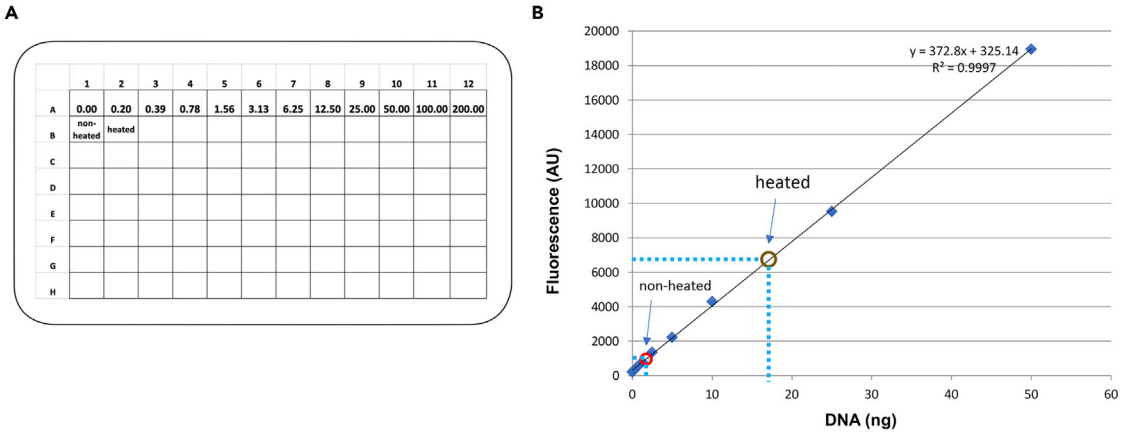

Figure 2. AAV titration with dye-based method

(A) Setting up 96 well plate. Wells in the top row contain DNA standards ranging from 0–200 ng. Two wells in the second row contain non-heated AAV (B1) and heated AAV (B2).

(B) Analysis of data obtained from Cytation 3 plate reader. Standard curve was generated by plotting fluorescence vs. DNA standard. Note that data points for 100 ng and 200 ng DNA were omitted as these two points are slightly off the linear range. Using the linear fit equation, we calculated the DNA quantities in the heated sample and the non-heated sample to be 18.5 ng and 0.5 ng, respectively. Thus, encapsided DNA in 1 μL of the AAV sample is 18 ng, equivalent to ~7.2×10^{12} gc/mL based on Equation 2.

ii. Add AAV solutions collected from Quick Seal tubes to the Ultra-15. Top off the unit with AAV formulation solution, centrifuge at 3000 × *g* until the volume in the Ultra-15 is reduced to less than 1 mL. Repeat this step 3 times, then collect AAV samples from the bottom of Ultra-15.

Note: Our typical yield is 5.0×10^{12} to 5.0×10^{13} gc/mL for 200–500 μL (see AAV titration method below).

iii. Make aliquots of 5–10 μL into Eppendorf tubes and store AAV at −80°C. Thaw AAV on ice before use. Avoid freeze-thaw cycles.

Pause point: Store AAV at −80°C indefinitely. Quantification of AAV can be taken at a later time by using one aliquot of AAV samples from −80°C storage.

⚠ CRITICAL: AAV must be stored in protein low binding tubes to prevent AAV from sticking to regular plastic. We use Eppendorf tubes (22431081) for AAV storage.

3. Measure AAV titer. We use a DNA dye-based method to quantify AAV.[10]

Note: While qPCR method is currently the most popular method for AAV titration, this method is very sensitive to experimental conditions, causing rather big inter-sample and intra-assay variations. DNA dye-based method described here only takes 30 min. It costs very little and most importantly, gives very consistent measurements. More details can be found in our previous paper.[10]

a. Prepare AAV samples for measurement.
   i. Dilute 2 μL of AAV into 18 μL DPBS.
   ii. Transfer 10 μL into a 96 well plate (as non-heated sample).
   iii. Take remaining 10 μL into a PCR tube, incubate at 98°C for 5–10 min and then add the whole content to 96 well plate (as heated sample).

b. Add DNA standard (10 μL of different concentrations) to 96 well plate (Figure 2A).

Note: We use plasmid maxi prep (any will work) to prepare our standard. Each set of standard contains 12-points 1:2 serial dilutions of plasmid DNA ranging from 0 to 20 ng/ul. We recommend making tubes of 500 μL at first and store the standard set in −20°C.

c. Add 90 μL of staining solution (DPBS containing 1:10,000 gelGreen dye) to 96 well plate.

Note: gelGreen dye was obtained from Biotium (Cat 41005). To make staining solution, we simply add ~0.3 μL gelGreen into 3 mL of DPBS. Evidently gelGreen dye is quite stable at 4°C as the dye we initially obtained 3 years ago is still in good condition.

d. Use plate reader (BioTek Cytation 3) to measure fluorescence intensity.

Note: If plate reader is not available, one can also use DNA gel imager,[10] although it will be less quantitative than plate reader.

e. Determine the DNA quantity based on DNA standard curve.
   i. Generate a standard curve using excel or other software (Figure 2B). Perform linear fit of the data points:
      $y = a \times x + b$, where y is fluorescence, x is DNA quantity.
   ii. Calculate DNA quantities for both non-heated and heated samples by equation: $x = (y-b)/a$. Subtract the value of the non-heated sample from the heated sample to derive the quantity of viral DNA.

Note: AAV genome is encapsided and is therefore not accessible to gelGreen dye unless AAV is lysed. Heating AAV at high temperature provides an easy way to lyse the virus. Once the capsids are broken, viral DNA will be released and will bind to gelGreen dye to produce fluorescence. (Purple circle in Figure 2B, as an example). Non-heated samples are also expected to yield some signals due to the presence of non-encapsided viral DNA and perhaps some contamination of HEK cells DNA. But the signals of non-heated samples (Figure 2, red circle as an example) should be much smaller than that of the heated samples.

f. Calculate AAV titer based on the quantity of viral DNA.

$$AAV\ titer\ (gc/ml) = DNA\ (ng) \times 1.82 \times 10^{15}/AAV\ genome\ size \quad \text{(Equation 1)}$$

The details of this equation can be found in our previous study.[10]
For example, if DNA from 1 μL of AAV is measured as 5.0 ng and the AAV is 4.0 kilobases (kb) in length, then the titer of the AAV sample is:

$$5.0 \times 1.82 \times 10^{15}/4000 = 2.28 \times 10^{12} gc/ml$$

To further approximate the calculation, one can assign 4500 bases to AAV genome size and then the Equation 1 will become:

$$AAV\ titer(gc/ml) = DNA(ng) \times 4.05 \times 10^{11} \quad \text{(Equation 2)}$$

Note: Many companies and viral vector cores now offer custom AAV production service. Researchers may also obtain pre-made, ready-to-use AAV preparations from different sources. Thus, for those prefer not to make AAV in-house, they can resort to commercial sources. Of course, making one's own AAV can save significant budget. It also offers quicker turnaround time and more flexibilities to researchers, such as choosing AAV serotypes or choosing special capsids mutants.[12]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-GFP	Abcam	ab5450; RRID: AB_304897
Rabbit anti-RFP	Rockland	600-401-379; RRID: AB_2209751
Alexa Fluor 488 donkey anti-goat IgG	Jackson Immunoresearch	705-545-003; RRID: AB_2340428
Cy3 donkey anti-rabbit IgG	Jackson Immunoresearch	711-165-152; RRID: AB_2307443
Bacterial and virus strains		
Stbl3™ Chemically Competent E. coli (for making plasmid for AAV packaging)	Thermo Fisher	C737303
Biological samples		
HEK 293T/17 (for AAV packaging)	ATCC	CRL-1126
Chemicals, peptides, and recombinant proteins		
DMEM	Corning	10-013-CM
Fetal bovine serum	Corning	35-011-CV
Trypsin-EDTA	Gibco	25300-054
Penicillin-Streptomycin (100×)	Gibco	15140122
Sodium Deoxycholate	Sigma	D6750
Polyethylenimine 25KD (PEI)	Polysciences	2396-2
Polyethylene Glycol 8000 (PEG-8000)	Fisher	BP233-1
Benzonase	Sigma	E-1014
Iodixanol (OptiPrep™ 60%)	Sigma	D1556
GelGreen dye	Fisher Scientific	41005
Phenol Red, 0.5% Solution	Sigma	P0290
EDTA, 500 mM Solution, pH 8.0	Millipore	324504
F68 10%	Gibco	24040-032
10 × DPBS	Gibco	14200075
DPBS	Gibco	14040133
NaCl	Sigma	S9888
MgCl2.6H2O	Sigma	M2670
KCl	Sigma	P3911
Fluoroshield mounting media	EMS	17989
Trizma® base	Sigma	T1503
Normal Donkey Serum	JIR	017-000-121
UltraPure™ 1 M Tris-HCl Buffer, pH 7.5	Thermo Fisher	15567027
NaOH	Sigma	S8045
Paraformaldehyde (PFA)	Sigma	158721
Triton™ X-100	Sigma	X100
Sodium Azide (NaN_3)	Sigma	71289
Taq polymerase	Qiagen	201203
Taq polymerase	Genscript	E00007
Deposited data		
Allen Mouse Brain Reference Atlas		
Allen Mouse Brain *in situ* hybridization Atlas	Allen Institute	https://mouse.brain-map.org/
Allen Mouse Brain Connectivity Atlas Transgenic Characterization	Allen Institute	http://connectivity.brain-map.org/transgenic
Experimental models: Organisms/strains		
Mouse: B6;129S-Slc17a7$^{tm1.1(cre)Hze}$/J VGLUT1-Cre (genotype: Cre+; age: 6–12 weeks; sex: male and female)	The Jackson Laboratory	JAX: 023527
Mouse: Slc17a6tm2.cre/Lowl/J VGLUT2-Cre (genotype: Cre+; age: 6–12 weeks; sex: male and female)	The Jackson Laboratory	JAX: 016963

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: B6;129S-Slc17a8$^{tm1.1(cre)Hze}$/J VGLUT3-Cre (genotype: Cre+; age: 6–12 weeks; sex: male and female)	The Jackson Laboratory	JAX:028534
Mouse: B6.Cg-*Slc32a1*$^{tm1.1(flpo)Hze}$/J VGAT-FlpO (genotype: FlpO+; age: 6–12 weeks; sex: male and female)	The Jackson Laboratory	JAX:029591
Mouse: B6;129S-Gt(ROSA)26Sortm65.1(CAG-tdTomato)Hze/J Ai65(RCFL-tdT)-D or Ai65D (genotype: Ai65^{het} and Ai65^{hom}; age: 6–12 weeks; sex: male and female)	The Jackson Laboratory	JAX: 021875
Oligonucleotides		
Cre 146F 5′-CCTGGAAAATGCTTCTGTCCG	Leneuve et al.[6]	N/A
Cre 537R 5′-CAGGGTGTTATAAGCAATCCC	Leneuve et al.[6]	N/A
Cre IC-F 5′-AACACACACTGGAGGACTGGCTAGG	Leneuve et al.[6]	N/A
Cre IC-R 5′-CAATGGTAGGCTCACTCTGGGAGATGATA	Leneuve et al.[6]	N/A
FlpO 100F 5′-GTGCCGCCGAGCTGACCTACCT	This study	N/A
FlpO 571R 5′-TGCCGCAGTTGATGAATGTGG	This study	N/A
FlpO IC-F 5-CTAGGCCACAGAATTGAAAGATCT	This study	N/A
FlpO IC-R 5′-GTAGGTGGAAATTCTAGCATCATCC	This study	N/A
AI9020 5′-AAGGGAGCTGCAGTGGAGTA	This study	N/A
AI9021 5′-CCGAAAATCTGTGGGAAGTC	This study	N/A
Rosa 5′R 5′-CCAAGTGGGCAGTTTACCGT	This study	N/A
CAG_F 5′-TGGGCAACGTGCTGGTTATT	This study	N/A
hGH_R 5′-TCTTCCCAACTTGCCCCTTG	This study	N/A
TK_F 5′-CTGGCACTCTGTCGATACCC	This study	N/A
5′LoxP_R 5′-GCTTTCATTTATTCATCGCG	This study	N/A
SV40_F 5′-GTCTGGATCCCCATCAAGCTG	This study	N/A
tdT_R 5′-CTTTGATGACCTCCTCGCCC	This study	N/A
Recombinant DNA		
pAAV9-Ef1a-DIO-EGFP -WPRE	Xu et al.[1]	Addgene: 187103
pAAV9-Ef1a-fDIO- tdTomato-WPRE	Xu et al.[1]	Addgene: 187112
pAAV-hSyn-Con/Fon-EYFP-WPRE	Fenno et al.[13]	Addgene: 55650
pAdDeltaF6 (expressing E4, E2a and VA)	James M. Wilson Lab	Addgene:112867
rAAV2-retro (expressing Rep and modified Cap	Tervo et al.[11]	Addgene:81070
pAAV2/9n (expressing Rep/Cap)	James M. Wilson Lab	Addgene:112865
Software and algorithms		
Adobe Photoshop CC	Adobe	
Fiji software	GPL v2, Fiji	http://fiji.sc/Fiji
Other		
Stereotaxic system	NEUROSTAR (Tubingen, Germany)	Neurostar Robot Stereotaxic system
Nanoliter Injector	Drummond Scientific Company (Broomall, PA, USA)	Nanoject III
KEYENCE microscope	KEYENCE CORPORATION (Itasca, IL, USA)	BZ-X700
Vibratome	LEICA BIOSYSTEMS (Deer Park, IL, USA)	VT1000S
Thermocycler	Bio-Rad LABORATORIES (Hercules, CA, USA)	C1000
Ultracentrifuge	Beckman Coulter Manufacturing company (Pasadena, CA, USA)	Optima XE-90
Fluorescence plate reader	BioTek Winooski, VT	Cytation 3
Animal Anesthesia Vaporizers and Accessories	RWD	R5835
7×–90× Binocular Stereo Zoom Microscope on Double Arm Boom Stand	AmScope	SM-4BY
DC Temperature Controller	FHC	40-90-8D
Falcon 50 mL Conical Centrifuge Tubes	Falcon	Cat# 1495949A
Protein low binding tubes (1.5 mL)	Eppendorf	22431081
EndoFree Plasmid Maxi Kit	Qiagen	12362

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Quick Seal tube (25 × 89 mm)	Beckman	342414
Amicon® Ultra-15 Centrifugal Filter Unit	Millipore	UFC910024
96-Well PCR Plate	Thermo Fisher	165305
Stericup Quick Release-HV Sterile Vacuum Filtration System (low protein binding) 0.45 µm	Millipore	S2HVU02RE
Tissue culture dish (150 × 25 mm)	Falcon	353025
Falcon 50 mL Conical Centrifuge Tubes	Falcon	Cat# 1495949A
Protein low binding tubes (1.5 mL)	Eppendorf	22431081
Isothesia (Isoflurane LIQUID)	Henry Schein	NDC 11695-6776-2
Ocular lubricant	PURALUBE VET	NDC 17033-211-38
Dynarex Medi-Cut Stainless surgical blades	Medi-cut	SKU-4131
Curved Dissecting Forceps	Fisher Scientific	S08097
Fisherbrand™ Sharp-Pointed Dissecting Scissors	Fisher Scientific	08-940
Triple Antibiotic- bacitracin zinc, neomycin sulfate, polymyxin-b sulfate, pramoxine hydrochloride ointment	Walgreens	NDC 0363-2900-05
Medbond Tissue Adhesive 2 mL	Medbond	CA1999
1cc Insulin Syringe, 29G × 1/2″	ComfortPoint	26028
Hamilton Syringe 5 µL	Hamilton	7633-01
Hamilton Syringe needle 32GA 1″ PT4	Hamilton	7803-04
Drummond glass micro pipets 3.5″	Drummond Scientific	3-000-203-G/X

MATERIALS AND EQUIPMENT

4% PFA (for brain fixation)

Reagent	Final concentration	Amount
PFA	4%	40 g
10× DPBS	1×	100 mL
ddH2O	N/A	~ 900 mL
Total	**N/A**	**1,000 mL**

Store at 4°C use within one week.

HEK cell culture medium (for AAV production)

Reagent	Final concentration	Amount
DMEM	N/A	890 mL
FBS	10%	100 mL
Penicillin-Streptomycin 100× (10,000 U/mL)	1×, 100 U/mL	10 mL
Total	**N/A**	**1,000 mL**

Store at 4°C, use within 3 months.

PEI solution (for AAV production)

Reagent	Final concentration	Amount
Polyethylenimine 25KD From Polysciences (cat #2396-2)	1 mg/mL	100 mg
H2O	N/A	100 mL
Total	**N/A**	**100 mL**

Preparation note: Dissolve PEI in 80°C hot H2O. Let cool to 20°C–30°C, then neutralize to PH 7.0 with HCl. Filter sterilize (0.22 um).

Aliquot and store in −20°C indefinitely.

40% PEG W/V solution (for AAV production)		
Reagent	Final concentration	Amount
Polyethylene Glycol 8000 (Fisher BP233-1)	40%	400 g
ddH2O	N/A	~600 mL
Total	**N/A**	**1,000 mL**

Preparation note: Add 400 g PEG-8000 in a 1-liter bottle, add ~ 500 mL H2O. Leave the bottle in 55°C water bath for about 1 h to allow PEG to dissolve. Bring final volume to 1000 mL with H2O.
Store at 20°C–30°C, use within 2 years.

AAV resuspension solution (for AAV production)		
Reagent	Final concentration	Amount
NaCl (3.0 M)	150 mM	50 mL
Tris-HCl, pH 8.4 (1.0 M)	50 mM	50 mL
ddH2O	N/A	900 mL
Total	**N/A**	**1,000 mL**

Store up to 1 year in 4°C.

10× DPBS MK solution (for AAV production)		
Reagent	Final concentration	Amount
10 × DPBS	1× DPBS	100 mL
$MgCl_2.6H_2O$	10 mM	2.1 g
KCl	25 mM	1.9 g
ddH2O	N/A	900 mL
Total	**N/A**	**1,000 mL**

Store up to 1 year in 4°C.

AAV formulation buffer (for AAV production)		
Reagent	Final concentration	Amount
NaCl	35 mM	2.05 g
F68 10% (Gibco, 24040-032)	0.001%	0.1 mL
1× DPBS	~ 1×	1,000 mL
Total	**N/A**	**1,000 mL**

Store up to 1 year in 4°C.

Tail lysis buffer (for genotyping)		
Reagent	Final concentration	Amount
NaOH (10.0 M)	25 mM	1.25 mL
EDTA (500 mM)	0.2 mM	0.2 mL
ddH_2O	N/A	499 mL
Total	**N/A**	**500 mL**

Store at 20°C–30°C for one year.

Tail neutralization buffer (for genotyping)		
Reagent	Final concentration	Amount
Tris-HCl (1.0 M) PH5.0	40 mM	20 mL
ddH_2O	N/A	480 mL
Total	**N/A**	**500 mL**

Store at 20°C–30°C for one year.

Recipes of other solution	
Name	Regents
DPBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4
Store at 4°C or 20°C–30°C for one year.	

STEP-BY-STEP METHOD DETAILS

Generate triple transgenic Cre$^+$;Flp$^+$;Ai65 mice

Timing: 20 weeks

Timing: at least 6 weeks (for step 2c)

The aim of this step is to generate triple transgenic mice by crossing together Cre lines, Flp lines and reporter lines. It takes at least two generations to obtain triple transgenic mice that can be used for experiments (Figure 1A). Therefore, a minimum of 5–6 month of time is required to prepare animals. Subsequent steps, including fixation, brain sectioning, IHC and imaging, take relatively shorter times.

1. Obtain mouse lines from Jackson laboratory.

The following are the mouse strains we imported from Jackson laboratory:

VGLUT1-Cre (B6;129S-*Slc17a7*$^{tm1.1(cre)Hze}$/J): JAX 023527.

VGLUT2-Cre (*Slc17a6*$^{tm2(cre)Lowl}$/J): JAX 016963.

VGLUT3-Cre (B6;129S-*Slc17a8*$^{tm1.1(cre)Hze}$/J): JAX 028534.

VGAT-FlpO (B6.Cg-*Slc32a1*$^{tm1.1(flpo)Hze}$/J): JAX 029591.

Ai65 (B6;129S-*Gt(ROSA)26Sor*$^{tm65.1(CAG-tdTomato)Hze}$/J): JAX 021875.

Note: Three vesicular transporters, VGLUT1, VGLUT2 and VGLUT3, are responsible for loading glutamate into synaptic vesicles. They are encoded by *Slc17a7*, *Slc17a6* and *Slc17a8* respectively, with almost mutually exclusive distributions in the brain. Meanwhile, VGAT, which is encoded by *Slc32a1*, is responsible for loading GABA into synaptic vesicles. In this project, we created three sets of Cre-FlpO intersections: VGLUT1-Cre/VGAT-FlpO, VGLUT2-Cre/VGAT-FlpO, and VGLUT3-Cre/VGAT-FlpO.

Note: VGLUT1-Cre,[14] VGLUT2-Cre,[15] and VGLUT3-Cre (Hongkui Zeng - Allen Institute for Brain Science) are all knock-in strains that have an IRES2 sequence and a Cre recombinase sequence inserted immediately downstream of the translational STOP codon of the VGLUT1, 2 and 3. VGAT-FlpO (Hongkui Zeng - Allen Institute for Brain Science) is also a knock-in mouse. It has a T2A sequence and an optimized FLP recombinase (FlpO) inserted into the translational STOP codon of the VGAT gene.

Note: Ai65 is a knock-in line harboring the Rosa-CAG-Frt-STOP-Frt-Lox-STOP-Lox-tdTomato in the Rosa locus.[5] Unless both Cre and Flp recombinase are expressed, the two STOP cassettes prevent transcription of the tdTomato, making the Ai65 line a Cre and Flp dual dependent reporter line (Figure 1B).

Table 2. Chromosome locations of transgenes

	Gene	Chromosome	Transgenic type
VGAT-FlpO	*Slc32a1*	2	knock-in
VGLUT1-Cre	*Slc17a7*	7	knock-in
VGLUT2-Cre	*Slc17a6*	7	knock-in
VGLUT3-Cre	*Slc17a8*	10	knock-in
Ai65	ROSA26	6	knock-in

Note: Before designing an intersectional crossing strategy, it is important to check which chromosome the transgene is located. For knock-in strains, this can be done by searching NCBI (https://www.ncbi.nlm.nih.gov/gene). Here we list the chromosome number of the transgenes studied in this protocol in Table 2. There are 20 chromosomes in mice, thus it is more likely that transgenes from different mouse lines reside on different chromosomes. If this is indeed the case, one should be able to cross lines without much difficulty. However, if transgenes of two mouse lines happen to locate in the same chromosome, then due to the diploid nature of mouse genome, it is not possible to produce progeny that are homozygous for both transgenes. In this scenario, the best outcome for any descendant is to carry one copy of each transgene. Please also note that if transgenic lines are made by conventional pronuclear injection method, either with small, linearized plasmid DNA or with large Bacterial artificial chromosome (BAC) DNA, the integration events are random and as a result the identity of the chromosome containing the transgene are often unknown.

⚠ CRITICAL: Before setting up mice breeding, one needs to find out if there is germline expression of Cre or Flp recombinases. Information about germline expression, if available, are often provided in the Jackson laboratory lab's mouse line database. For the Cre and FlpO drivers listed above, there is no specific note by Jackson lab regarding germline expression. As such, one may choose either male or female to carry the Cre or FlpO recombinase. Should it be known that a certain Cre or FlpO driver can be active in either male or female germlines, researchers may consider using the opposite gender for breeding to avoid transmitting of Cre or Flp mediated genetic alterations throughout all tissue in the progeny.

2. Cross FlpO line and Cre line with Ai65.
   a. Cross VGLUT-Cre mice with Ai65 mice (Figure 1A).
   b. Cross VGAT-FlpO with Ai65 mice (Figure 1A). Step 2b can be done in parallel with step 2a.

Note: Ai65 can be maintained as homozygous (*hom*). Although Ai65het and Ai65hom can both be used for breeding in step 2a and 2b, one should preferably use Ai65hom if available to conserve time and animals.

   c. Wait for pups to be born and to reach weaning age.
   d. Perform mice tail biopsy procedure at weaning and then prepare genomic DNA for PCR.[16]
      i. Cutting less than 0.5 cm of mouse tail into an Eppendorf tube.
      ii. Add 0.2 mL of tail lysis buffer, leave Eppendorf tube overnight in an 85°C oven (shaking is not required). If time is of essence, heat tail samples at 95°C for about 30 min with shaking then move to the next step.
      iii. Add 0.2 mL of tail neutralization buffer. The samples are now ready for PCR.
   e. Perform PCR genotyping.

Note: We use standard PCR conditions and agarose gel-based detection method. The following is an example of reaction set-up based on Qiagen's Taq polymerase and 10× buffer

Table 3. PCR compositions

Component	Amount	Final conc.
Tail DNA	0.5 μL	N/A
10 PCR buffer	1.5 μL	1×
5× Q solution	3.0 μL	1×
Deoxynucleotide Mix (10 mM)	0.3 μL	200 μM
Primer Forward (30 μM)	0.15 μL	0.3 μM
Primer Reverse (30 μM)	0.15 μL	0.3 μM
H2O	9.4 μL	N/A
Taq Polymerase (5 Unit /μL)	0.05 μL	N/A
Total	15 μL	

(#210203) (Table 3). We also use Taq polymerase from Genscript (Cat. No. E00007) and it performs equally well on our tail DNA samples.

Note: Qiagen's 10× PCR buffer contains 15 mM $MgCl_2$, making the final concentration of $MgCl_2$ to be 1.5 mM in the PCR reaction above. For some primers, however, it might be necessary to increase final $MgCl_2$ concentration to 2–2.5 mM. Qiagen's Q solutions can improve suboptimal PCR caused by high degree of secondary structure or high GC-contents in the DNA template. Although Q solution is not required for many PCR reactions, we nevertheless include Q solution in all our PCR reactions.

3. Create triple transgenic Cre^+;Flp^+;Ai65 mice.
   a. Cross VGAT-$FlpO^+$;$Ai65^{het}$ mice with VGLUT-Cre^+;$Ai65^{het}$ mice generated from step 2.
   b. Wait for mouse pups to be born and to reach weaning age.
   c. Perform tail biopsy.
   d. Perform genotyping.

Note: We have used both male and female FlpO and Cre drivers of above lines and we rarely observed germline transmission of stop cassette removal from the Ai65 allele, suggesting that transgene expression (Cre or FlpO) is not active, or very low, in either male or female germlines. With that said, it did occur several times during our study when leaky expression was suspected. Though the molecular mechanism is not known, DNA modifications at the Ai65's stop cassettes, either recombinase dependent or independent, may have caused the leakiness. More discussions are offered in the troubleshooting section.

Note: As expected based on Mendelian inheritance, the ratio for the occurrence of $Ai65^{het}$ mice from pups obtained from this step (step 3) is 2 out of 4. The chance that they are both FlpO positive and Cre positive is $^1/_4$ ($^1/_2$ × $^1/_2$). So overall, the ratio of Cre^+;$FlpO^+$;$Ai65^{het}$ mice is only $^1/_8$. Similarly, the probability of obtaining Cre^+;$FlpO^+$;$Ai65^{hom}$ is $^1/_{16}$ according to Mendelian inheritance. Note that Cre^+;$FlpO^+$;$Ai65^{het}$ and Cre^+;$FlpO^+$;$Ai65^{hom}$ can both be used for experiments, although native signals in the later is stronger due to presence of two copies of the Ai65 transgene.

Note: The most critical parameter for a successful PCR genotyping is the design of primers. For Cre genotyping, we have compared several PCR primer pairs designed by us and by others and we found that a Cre PCR condition established in a previous report[17] was most reliable (Figure 3A). In addition to Cre primers, a pair of internal control primers that recognize mouse *Gabra1* gene were also included (Figure 3A). For FlpO genotyping, a multiplex PCR designed by us produced robust results. In this reaction, the internal control primers recognize mouse *Il2* gene (Figure 3B). Finally, for Ai65 genotyping, we re-designed a PCR strategy by using a 3-primer set instead of a 4-primer set used by the Jackson laboratory. In our design

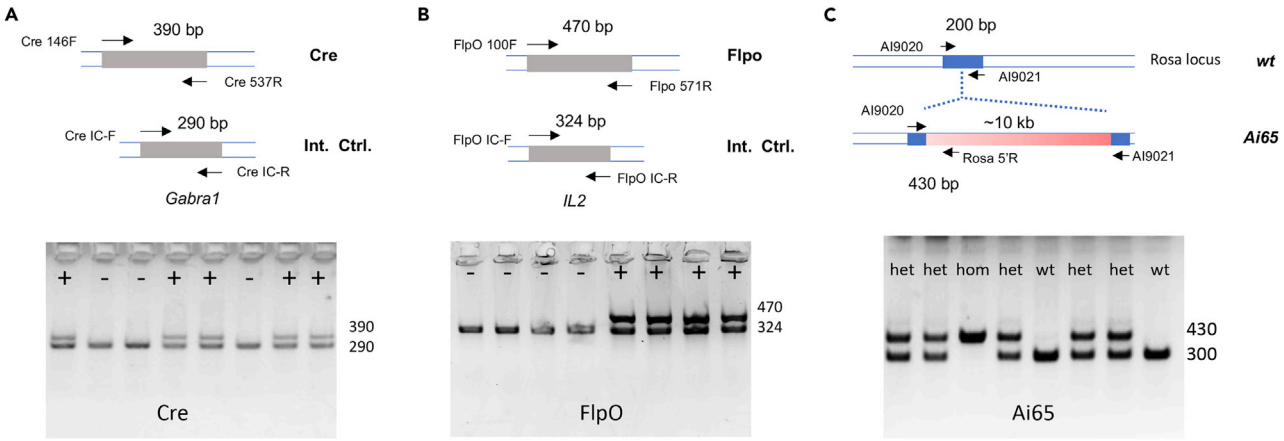

Figure 3. PCR genotyping for Cre, FlpO, and Ai65

(A) Primer locations for Cre (top) and PCR products detected by agarose gel (bottom). The internal control primers recognize mouse *Gabra1* gene.

(B) FlpO primers (top) and PCR gel image (bottom). The internal control primers recognize mouse *IL2* gene.

(C) Ai65 primers (top) and PCR gel image (bottom).

a 5′ reverse primer is employed to amplify 5′ transgene insertion at the rosa locus when it is paired with a 5′ wild type forward primer (AI-9020) to produce a 430 bp band. Since the wild type allele does not contain target DNA for the 5′ reverse primer, only the AI9020 and AI9021 primer pair can produce an amplification which is 300 bp (Figure 3C). Primer sequences are listed in Table 4.

Using cre-dependent and Flp-dependent AAV to map multi-transmitter neurons in Cre/Flp intersectional mice

Timing: 2–3 weeks

Timing: 1–2 h (for step 4)

This section describes the method using AAV to map glutamate and GABA co-expression neurons. It can be readily extended to other types of multi-transmitter neurons.

⚠ CRITICAL: Before the experiments, animal procedures for stereotaxic injection of AAV must be approved by the relevant institutions for animal care and use.

Table 4. PCR primers for Cre FlpO and Ai65 genotyping

Application	Primer name	Sequence	PCR size (bp)	Annealing
Cre	Cre 146-F	5′-CCTGGAAAATGCTTCTGTCCG	Cre: 390	60°C
genotyping	Cre 537-R	5′-CAGGGTGTTATAAGCAATCCC		
	Cre IC-F	5′-AACACACACTGGAGGACTGGCTAGG	Int.: 290	
	Cre IC-R	5′-CAATGGTAGGCTCACTCTGGGAGATGATA		
FlpO PCR	FlpO 100-F	5′-GTGCCGCCGAGCTGACCTACCT	FlpO: 470	65°C
genotyping	FlpO 571-R	5′-TGCCGCAGTTGATGAATGTGG		
	FlpO IC-F	5-CTAGGCCACAGAATTGAAAGATCT	Int.: 320	
	FlpO IC-R	5′-GTAGGTGGAAATTCTAGCATCATCC		
Ai65 PCR	AI9020	5′-AAGGGAGCTGCAGTGGAGTA	*wt:* 300	64°C
genotyping	AI9021	5′-CCGAAAATCTGTGGGAAGTC	*het:* 300, 430	
	Rosa 5′R	5-CCAAGTGGGCAGTTTACCGT	*hom:* 430	

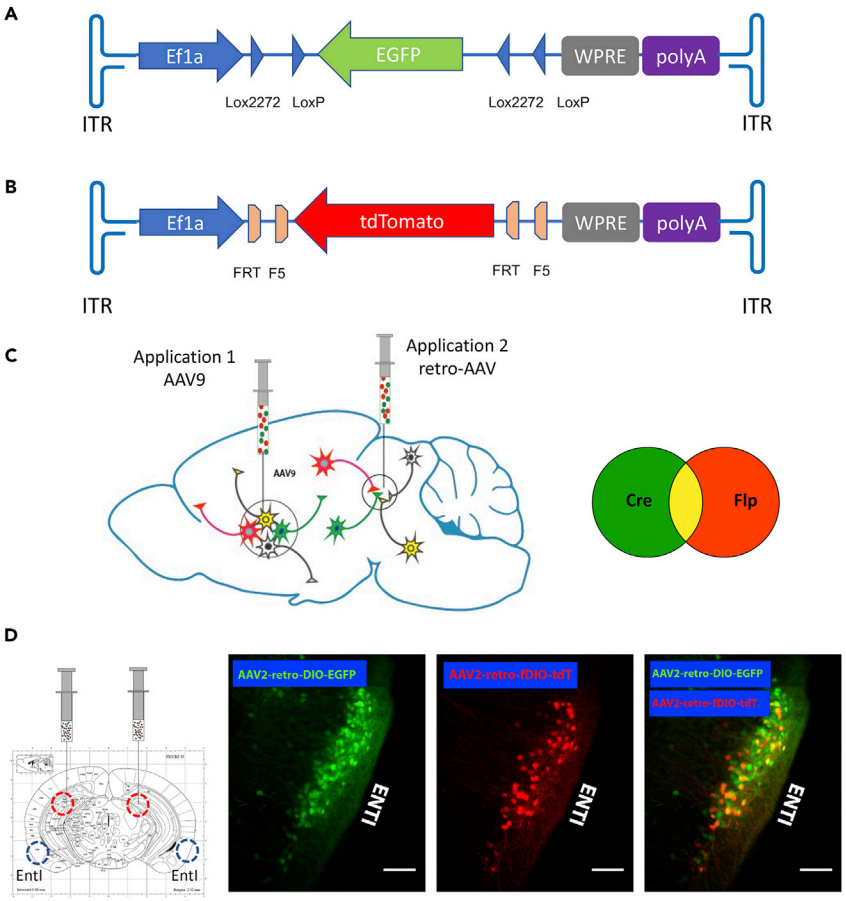

Figure 4. Use Cre-dependent and Flp-dependent AAVs to label glutamate and GABA dual-releasing neurons in VGAT-FlpO and VGLUT-Cre intersectional mice

(A and B) Diagrams of AAV-Ef1a-DIO-EGFP (A) and AAV-Ef1a-fDIO-tdTomato (B).

(C) Co-injecting AAV-Ef1a-DIO-EGFP and AAV-Ef1a-fDIO-tdTomato into $Cre^{+}FlpO^{+}$ mice to label neurons expressing Cre (green) and FlpO (red). $Cre^{+}FlpO^{+}$ double positive cells are identified by expressing both GFP and tdTomato. In application 1, AAVs packaged with AAV9 capsids label cells in the injection sites. In application 2, AAVs packaged with AAV2-retro capsids label cells projecting to the injection sites.

(D) Example of an experiment using AAV2-retro. Two viruses, AAV2-retro-DIO-EGFP and AAV2-retro-fDIO-tdtomato were co-injected into the dentate gyrus (red circles) of a VGLUT2-Cre^{+};VGAT-$FlpO^{+}$ mouse. Labeled neurons were found in the lateral entorhinal cortex (ENTl), with a fraction of them expressing both GFP and tdTomato. Scale bars, 100 μm.

4. Inject AAV to the brains of 6–12 weeks old $Cre^{+}FlpO^{+}$ mice.
   a. Mix equal amount of two viruses, AAV-Ef1a-DIO-EGFP and AAV-Ef1a-fDIO-tdTomato to the concentration of 1–3 × 10^{12} gc/mL of each.

   Note: We constructed two AAV plasmids (Addgene #187112 and Addgene #187103, Figures 4A and 4B) for making AAV-Ef1a-DIO-EGFP and AAV-Ef1a-fDIO-tdTomato.

   b. Perform stereotaxic AAV injection.

   Note: There are excellent protocols that give detailed instructions on how to perform mouse brain stereotaxic AAV injections[18,19] thus we will not repeat their procedures here but instead just give a short outline. Briefly, the whole surgery and injection process includes several steps and usually takes about 1–2 h to complete, depending on experimenter's experience and the number of injections to make in each mouse.

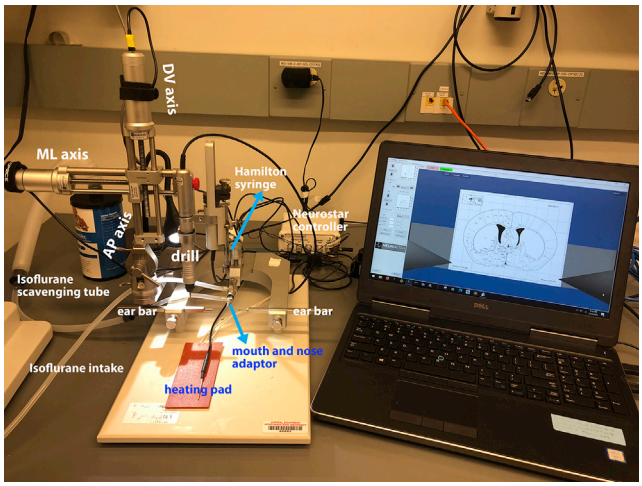

Figure 5. Stereotaxic apparatus

Components of stereotaxic apparatus: ML-Axis, AP-Axis, DV-Axis, drill, syringe, mouth and nose adaptor, mouse heating pad, Isoflurane intake tube and scavenging tube. On the back is the Neurostar controller box connected to a computer.

i. Anesthetize mouse (6 weeks–12 weeks) with ~2% vaporized isoflurane with O2.
ii. Mount the mouse on the stereotaxic frame, providing constant flow of ~1% vaporized isoflurane with O2 through nose cone.
iii. Make scalp incision to expose the skull.
iv. Locate bregma and Lambda points and used these landmarks to set up coordination of the stereotaxic frame.
v. Using software to choose the target sites. Make small holes on the skull above the target sites by using the drill attached to the stereotaxic frame (See Figure 5).
vi. Lower the Hamilton syringe or glass injector (Nanojet III) slowly (5 mm/min or 1 mm/min) to reach the target sites by using the "go to target" function.

Note: Hamilton syringe (Hamilton Company) and Nanojet III injector (Drummond company) are often used for injecting AAV into mouse brain, with the Hamilton syringe been preferred for larger volume (0.1–1.0 μL) per injection site and the Nanojet injector been preferred for smaller volume (30 nl–100 nl) per injection site.

vii. Inject AAV. For injection with Hamilton syringe, we use injection speed of 0.1 μL/mL, controlled by Neurostar system. For injection with Nanojet III injector, we use injecting rate of 3–5 nl/s, for total volume of 30–100 nl.
viii. Wait 5 min then lift Hamilton syringe or Nanojet injector.
ix. Close scalp incision to finish the mouse surgery. Return the mouse to home cage. Provide post-operative care and pain management according to one's approved animal protocol. In accordance with our protocol, we administer carprofen (2–4 mg/kg, subcutaneous injection) to the mouse immediately after surgery and then again after 24 h. We monitor the mouse daily for at least two more days to ensure that the animal is fully recovered.

c. Allow the mouse to recover from surgery and wait for 2–3 weeks for viral mediated gene expression.

Note: Animals used in this study for AAV injections were 6–12 weeks old. The earliest time we checked for viral-mediated expression is 14 days after injection. We could detect expression of fluorescent markers without much difficulty for both AAV9 and AAV2-retro serotypes. However, we generally waited until 3 weeks after AAV injection, in an effort to maintain the consistency in data collection.

Note: As an alternative to using Ai65 reporter mice for whole brain mapping, Cre dependent and Flp dependent AAV vectors can also be used to label co-releasing neurons in localized brain regions (Figure 1C). We and others have used Cre and Flp double-dependent AAV (AAV-Con/Fon-EYFP) developed by Fenno and colleagues for intersectional labeling.[13] Details for using this virus can be found in other papers.[20–22]

Note: This protocol describes another strategy that uses co-injection of two viruses, a Cre-dependent AAV expressing EGFP virus and a Flp/FlpO dependent AAV expressing tdTomato, for intersectional mapping (Figure 4). We constructed two AAV plasmids (Addgene #187112 and Addgene #187103, Figures 4A and 4B) for this purpose and demonstrated two applications.[1] The first application is to directly label Cre and FlpO expressing cells in the viral injection sites. For this we packaged AAV-Ef1a-DIO-EGFP and AAV-Ef1a-fDIO-tdTomato with AAV9 capsids and co-injected 1:1 mixture of the two AAVs ($\sim$ 1.0–3.0 $\times$ 10^{12} gc/ml of each AAV) to the Cre/FlpO intersectional mice (Figure 4C, application 1). The second application is to label Cre and FlpO expressing cells that project to certain brain targets. For this application we packaged AAVs with AAV2-retro capsids[11] and co-injected two retro-AAVs to the target sites. As retro-AAVs are transported retrogradely from axon terminal and pre-synapses back to neuronal bodies, Cre and FlpO expressing neurons projecting to the target sites can be tracked (Figure 4C, application 2). For both applications, we showed that co-injection of two viruses worked efficiently,[1] because a single injection can label three cell populations simultaneously, the Cre positive, the Flp positive and the Cre and Flp double positive neurons (Figure 4C). An example is provided in Figure 4D. Here we co-injected AAV2-retro-DIO-EGFP and AAV2-retro-fDIO-tdtomato into the dentate gyrus of a VGLUT2-Cre^{+};VGAT-$FlpO^{+}$ mouse. We found GFP labeled neurons and tdTomato labeled neurons in the lateral entorhinal cortex (ENTl) (Figure 4D, image panels). A fraction of neurons expressed both GFP and tdTomato (Figure 4D, rightmost panel), suggesting that they could be glutamate and GABA co-releasing neurons sending terminals to dentate gyrus.

Note: Stereotaxic AAV injection has become a standard technique in neuroscience. We used Neurostar stereotaxic system which is a computer controlled motorized stereotaxic device (Figure 5). It is easier to make movements of the injectors and the drills by using Neurostar system compared with manual stereotaxic devices. Also, the Neurostar system uses algorithms to offset the errors caused by imperfect head mounting, making the injections more precise and more consistent than conventional stereotaxic devices.

Brain fixation, sectioning, and IHC

Timing: 1–2 weeks

Timing: about 2 days (for step 5)

Timing: 2 h (for step 6)

Timing: $\sim$1 week (for step 7)

Timing: 1 day (for step 7a)

Timing: 1 day (for step 7b)

Timing: 1 day (for step 7c)

Timing: 1 day (for step 7d)

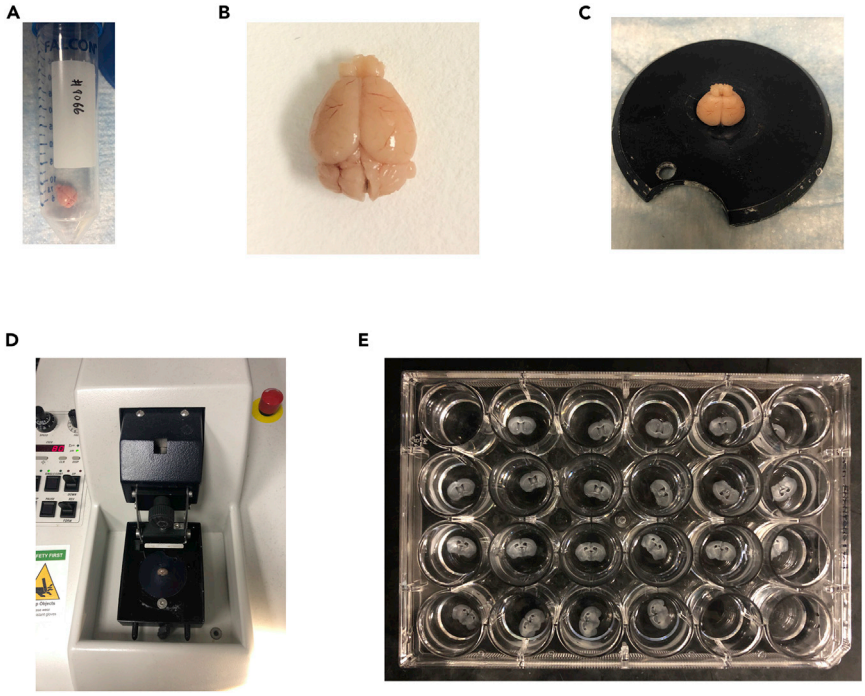

Figure 6. Brain fixation and sectioning

(A) The brain is stored in 4% PFA immediately after dissection.

(B) The brain is ready for sectioning two days after PFA fixation.

(C) After making a cut at the inferior colliculi with a razor blade, glue down the brain to the sample plate.

(D) Place sample plate in the cutting chamber and section brain at 80 μm thickness.

(E) Collect brain slices into 24 well plates.

Timing: 1 day (for step 7e)

Timing: 1–2 h (for step 7f)

In this step, brains are fixed, sectioned and immunohistochemistry is performed to fluorescently label the targeted cells.

5. Perform PFA Fixation.
   a. Perform euthanasia.
   b. PFA fixation. Quickly open the skull, remove the whole brain and place it into a 50 mL Falcon tube. Wash brain once with DPBS and then add 5–10 mL of 4% PFA for fixation (Figure 6A)
   c. Fix brain with 4% PFA for 2 days in 4°C with slow shaking. After two days, the brain is ready for vibratome sectioning (Figure 6B).

Note: Fixation methods. We have tested two different fixation methods. This first method is to perform whole body transcardiac perfusion of 4% PFA. Following perfusion, brain was removed and allowed to stay in 4% PFA overnight for post fixation and before sectioning with vibratome. In the second method, we euthanized animal then quickly removed the brain and soaked it 4% PFA at 4 degree for 2 days and then performed sectioning with vibratome. We were surprised that two fixation methods yielded similar imaging qualities for us. Since transcardiac perfusion method takes much longer time, produces more PFA waste and post more safety hazard concerns to lab workers, we normally don't use this method.

Note: Fixation time. Two days after 4% PFA fixation for at 4°C, brains will get hard and become quite easy for vibratome sectioning (50–100 μm). We also tried 24-hr FPA fixation. At this time, the brains were a bit softer, and they were slightly more difficult to cut thin sections, compared with the brains fixed for two days. The longest fixation we tested was 7 days. Though we found that staining and imaging results were still fine, it is advisable not to wait for this long as over fixation can cause poor antibody staining and jeopardize tissue integrity.

6. Perform brain sectioning with vibratome.
   a. Wash brain with DPBS (10 min each time for 3 times) after 4% PFA fixation.
   b. Place the brain on its ventral side and use razor blade to make a perpendicular cut at the inferior colliculi and then glue the brain down to the sample platform with superglue (Figure 6C).
   c. Place the sample platform into the vibratome cutting chamber, add enough DPBS buffer to submerge the whole brain (Figure 6D).
   d. Section brain with vibratome at frequency of 60 Hz and 0.4 mm. s^{-1}, with 80 μm thickness.
   e. Collect brain sections into 24 well plate with each well containing one brain slice (Figure 6E). For one whole brain, we typically collect 70–80 slices, in 3–4 24-well plates.
7. Perform IHC in the 24 well plates. This ensures that serially sectioned slices always remain in the correct order.

Note: It is not always required to perform IHC because native fluorescent protein signals from the Ai65 reporter or from AAV reporters can be bright enough for direct visualization of the labeled cells. However, we found that it is beneficial to perform IHC as fluorescent dyes such as Alexa dyes, Cy3 and Cy5 offer higher photostability and brightness compared to endogenous fluorescent proteins, making the imaging process easier and allowing samples to be stored for longer time.

a. Blocking.

Add ~ 1 mL of blocking buffer (DPBS containing 0.5% Triton X-100, 5% donkey serum) to each well. Place the 24 well plate on a low-speed orbital shaker and incubate at 4°C for overnight.

b. Stain with primary antibody.
   i. Remove blocking buffer.
   ii. Add ~ 1 mL of primary antibody solution (DPBS containing 0.5% Triton X-100).
   iii. Incubate at 4°C overnight.

Note: For this study, we used two primary antibodies: Goat anti-GFP (1:500; Abcam AB5450) and rabbit anti-RFP (1:500; Rockland 600-401-379).

c. Washing.

Wash once with washing buffer (DPBS) for 1 h at 20°C–30°C. Wash second time at 4°C overnight.

d. Stain with secondary antibody.
   i. Remove washing solution.
   ii. Add ~ 1 mL of secondary antibody solution (DPBS containing 0.5% Triton X-100) and incubate at 4°C overnight.

Note: In this study, we used two secondary antibodies: Alexa Fluor 488 donkey anti-goat (1:200; Jackson 705-545-003) and Cy3 donkey anti-rabbit IgG (1:200; Jackson 11-165-152).

e. Washing.

Wash once with washing buffer (DPBS) for 1 h at 20°C–30°C. Wash second time at 4°C for overnight.

f. Mounting.

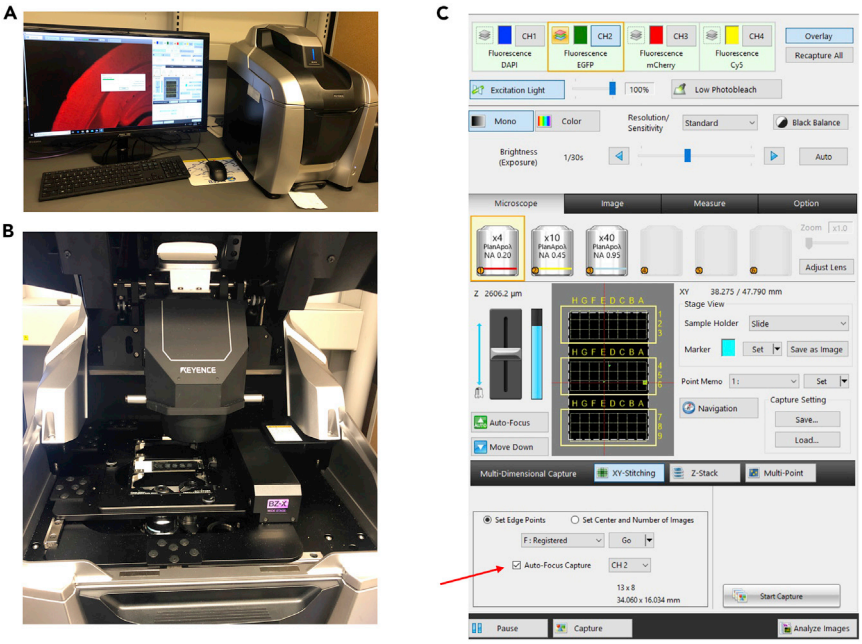

Figure 7. Keyence microscope (BZ-X700) system

(A) Scanning in process. Dark room is not required.

(B) Picture of the slide holder that was built inside of the box.

(C) Control panel of the software. Red arrow points to the Auto-Focus capture check box.

Brains sections were mounted on glass slides (25 × 50 mm) with fluoroshield mounting medium with DAPI (EMS 17989). About 8 brain slices can be mounted on one glass slide (Figure 8A).

Note: Antibody can be re-used many times. To prevent microbial proliferation in the antibody solutions, we add sodium azide to a final concentration of 0.02%–0.03% in our antibody cocktail.

Note: The protocol we used here was modified from a protocol we previously developed for staining retina tissues, in which we used relatively longer blocking, antibody staining, and washing times. For brain tissues, it is possible to shorten the whole staining process to within 2–3 days although we haven't tested it ourselves.

Visualizing and acquiring images

Timing: 1 day

This step is to acquire brain images. We used a Keyence microscope (BZ-X700) to acquire fluorescence images (Figure 7). This is an all-in-one fluorescence imaging microscope with ability to capture, at once, high resolution images of an entire brain slice, or even multiple brain slices. The computer-controlled automatic scanning process obviates the need to constantly change imaging areas manually, making the scanning of whole brain much easier. The steps for image acquisition are summarized below.

8. Prepare for image acquisition.
   a. Turn on microscope then turn on computer.

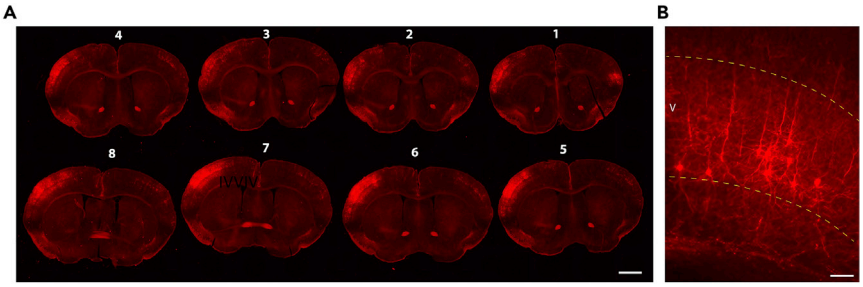

Figure 8. Sample slide showing dual glutamate-GABA neurons in a mouse brain

(A) Distribution of glutamate GABA co-releasing neuron in a VGLUT2-Cre^{+};VGAT-FlpO^{+}Ai65^{het} mouse is presented. Strong labeling was found in primary somatosensory area and piriform area. The original image was taken with 4× objective. The dimension of stitched file is 30,000 × 10,000 pixels. Scale bar: 1.5 mm.

(B) Image taken with 10× objective showing layer 5 neurons at piriform area. Scale bar: 50 μm.

b. Open the BZ-X700 acquisition software, choose the correct sample holder then load sample slides onto the holder (Figure 7B).
c. Select appropriate objective from the acquisition software control panel (Figure 7C). Start with 4× objective for the whole brain scanning. 10× objective can be used later for higher magnification imaging.
d. Choose appropriate fluorescence channels. If more than one channel is needed select "multi-color" button on the upper left of the screen.
e. Focus sample and define scanning range.
    i. Focus the brain samples by click the Auto-Focus button. Fine manual focus function is also available if required.
    ii. Define scanning range by marking one by one the 4 outside corners of the area to be scanned with software using the "set edge points" functions that can be found at bottom sections of the control panel.

9. Image acquisition and data saving.
    a. Start the imaging acquisition by clicking the "Start capture" button. With the 4× objective, it usually takes about 20–30 min to scan one slide (75 mm × 25 mm).
    b. When finished, execute image stitching function and export final images as Tiff format for further analysis.

⚠ CRITICAL: Make sure that Auto-Focus capture function is on as this allows the microscope to automatically adjust focus during acquisition.

EXPECTED OUTCOMES

It has been established that multi-transmitter neurons exist in the brain. The questions we try to address are what is their abundance and where are they located. At least for glutamate and GABA co-releasing neurons, we found them to be distributed in more than 50 brain structures, which is much more than we anticipated.[1] Note that image data can be utilized for a more quantitative approach by using software such as image J to count cells. Meanwhile the intersectional protocol can be applied to map other types of co-releasing neurons.

Figure 8A shows an image of a sample slide scanned with 4× objective. The sample contains series of 8 brain slices taken from a VGLUT2-Cre^{+}VGAT-FlpO^{+}Ai65 mouse, (Figure 8A). These 8 slices correspond to Bregma ~0.64 mm to Bregma 0 of the Anterior-Posterior (*AP*) axis. In these slices, tdTomato-labeled neurons were observed in primary somatosensory area and piriform area. In contrast, we observed almost no labeling in Caudoputamen (CP) and Nucleus accumbens. Interestingly there is an imbalance of labeling between the left and right hemisphere. The biological mechanism underlying such imbalance is unknown but is certainly worthy of further study. Figure 8B shows

an image taken with 10× objective. Individual cells in layer 5 of the piriform area can be distinguished.

LIMITATIONS

The major limitation of the protocol is the time-consuming process for generating multi-transgenic mice through rounds of breeding. It takes months, if not a year, to obtain animals suitable for experiments. But once animals are ready, the remaining steps of the protocol can go much quicker.

The second limitation of the protocol is the requirement of large amounts of labor to perform animal care, genotyping, brain fixation, sectioning, staining and imaging.

TROUBLESHOOTING

Problem 1

No Immunofluorescence signal is observed (steps 8 and 9).

Potential solution

This could be due to a genotype error (step 3). Make sure that genotype is correct. When performing PCR genotyping, it is important to always include positive and negative controls. For the experiments using Cre and Flp dependent AAV virus, failing to observe fluorescence can be caused by insufficient amount of AAV and/or insufficient time for expression (step 4). We recommend testing different experimental conditions including varying AAV doses and time courses of expression.

Problem 2

Immunofluorescence signal is weak or high background is observed (steps 8 and 9).

Potential solution

Although antibody can be reused for many times, if the immunofluorescence signal deteriorates noticeably, switch to freshly diluted antibody. Make sure that antibodies, both primary and secondary, are used at optimal concentration ranges (step 7).

Problem 3

Leaky expression and germline expression are found (steps 8 and 9).

Ai65 was designed as a dual Cre/Flp-dependent reporter to express tdTomato in cells where both Cre and Flp are present. In all the animals we have examined in this study, we did not detect leaky expression in either Ai65 itself or Ai65 crossed with VGLUT-Cre drivers. But over the course of the study, it occurred several times that substantial leaky expression was found in Cre^{-};Flp^{+};Ai65 mice, suggesting that Cre-dependent STOP was somewhat leaky in these mice. There could be two possible causes for the leaky expression: 1) Sporadic germline expression of Cre in the breeders. This can cause the removal of LoxP flanked STOP cassette in germline of breeders, which can then be transmitted to progeny. 2) Rare Cre-independent recombination within the STOP sequence during and after development. Notably, three identical SV40 polyA sequences were used to construct the LoxP-flanked STOP cassette in Ai65 mice (Figure 9A). Such repetitive sequences can render instability of the region, making the STOP sequence prone to spontaneous recombination and thus demonstrating leakiness. In comparison, the FRT-flanked STOP contains three different kinds of polyA sequences: a synthetic polyA sequence, a polyA sequence from the human growth hormone gene, and a polyA sequence from the Herpes Simplex Virus TK gene (Figure 9A), which likely makes the FRT-STOP more stable and less prone to erroneous recombination.

Potential solution

If leaky expression is suspected (steps 8 and 9), one can check the integrity of the FRT-flanked STOP and LoxP-flanked STOP by PCR. We have designed three PCR primer pairs (Table 5) to inspect the three junctions of the two STOP sequences (Figure 9A). If both STOP sequences are intact, the

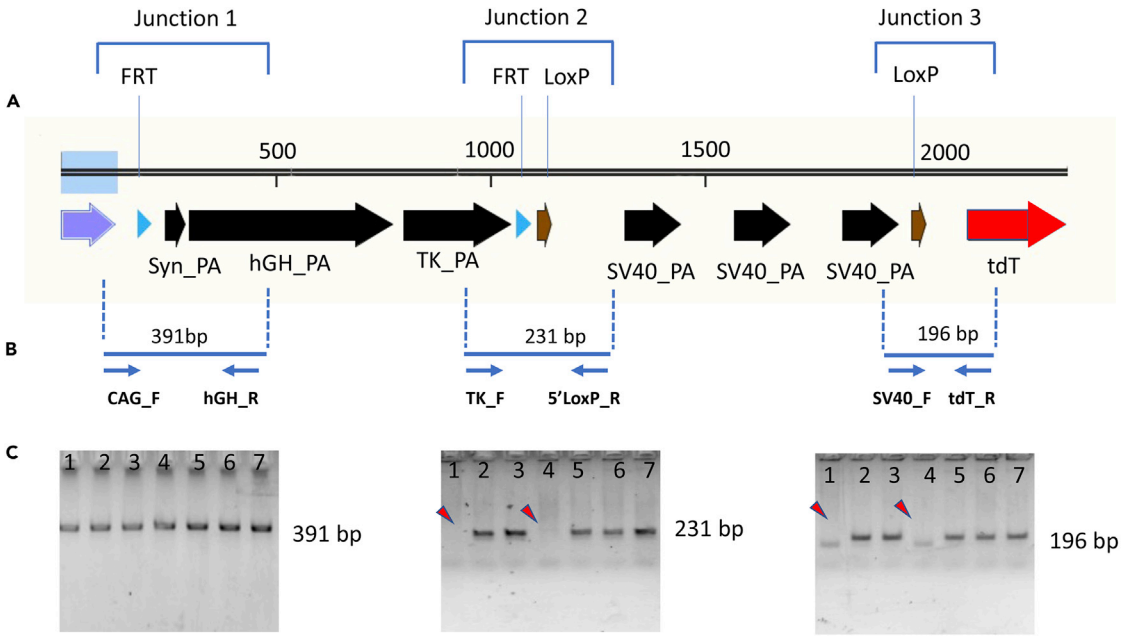

Figure 9. FRT-STOP-FRT and LoxP-STOP-LoxP of Ai65 design

(A) FRT-STOP-FRT and LoxP-STOP-LoxP in Ai65 mice.

(B) Primer locations at 3 junctions of the two Ai65 STOPS.

(C) PCR gel images. Sample #1 and # 4 did not yield correct bands in assays for Junction 2 and Junction 3 (red arrows), indicating that LoxP-flanked STOPs were not intact in the two samples.

samples should produce correct PCR products in all three reactions (Figure 9C). Failing to yield correct PCR products in any of the 3 assays would indicate that at least one STOP is defective and thus Ai65 mice must be replaced.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yongling Zhu (yongling-zhu@northwestern.edu).

Materials availability

This study did not generate new unique reagents or materials. All of the reagents described here are commercially available through the indicated vendors. There are no unique reagents or restrictions to availability of reagents.

Data and code availability

This study did not generate new unique code.

Table 5. PCR primers for testing integrity of STOP cassettes in Ai65 mice

Application	Primer name	Sequence	PCR size (bp)	Annealing
Junction 1	CAG_F	5′-TGGGCAACGTGCTGGTTATT	391	60°C
	hGH_-R	5′-TCTTCCCAACTTGCCCCTTG		
Junction 2	TK_F	5′-CTGGCACTCTGTCGATACCC	231	60°C
	5′LoxP_R	5′-GCTTTCATTTATTCATCGCG		
Junction 3	SV40_F	5′-GTCTGGATCCCCATCAAGCTG	196	60°C
	tdT_R	5′-CTTTGATGACCTCCTCGCCC		

ACKNOWLEDGMENTS

This work was supported by NIH grants R01 EY030169 (Y.Z.), R01 EY018204 (Y.Z.), Whitehall Foundation Grant (Y.Z., 2017-05-20), R01 MH099114 (A.C.), R01 EY032506 (Y.Z. and A.C.), and Research to Prevent Blindness.

AUTHOR CONTRIBUTIONS

J.X., A.C., and Y.Z. conceived of the research, developed protocols, and wrote the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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