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Manipulating gene expression in projection-specific neuronal populations using combinatorial viral approaches

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Abstract

The mammalian brain contains tremendous structural and genetic complexity that is vital for its function. The elucidation of gene expression profiles in the brain, coupled with the development of large-scale connectivity maps and emerging viral vector-based approaches for target-selective gene manipulation, now allow for detailed dissection of gene-circuit interfaces. This protocol details how to perform combinatorial viral injections to manipulate gene expression in subsets of neurons interconnecting two brain regions. This method utilizes stereotaxic injection of a retrograde transducing CAV2-Cre virus into one brain region, combined with injection of a locally transducing Cre-dependent AAV virus into another brain region. This technique is widely applicable to the genetic dissection of neural circuitry, as it enables selective expression of candidate genes, dominant-negatives, fluorescent reporters, or genetic tools within heterogeneous populations of neurons based upon their projection targets.

Keywords

AAV; CAV; viral vector; neural circuit; stereotaxic surgery

INTRODUCTION

The human brain contains an estimated 100 billion neurons that collectively generate trillions of synaptic connections (Hubel, 1979; Lent et al., 2012). In tandem to this structural complexity, the human genome contains over 20,000 protein-encoding genes (Consortium, 2004), many with heterogeneous expression patterns in the brain (Hawrylycz et al., 2012). Merging these two independently complex landscapes to map the genetic influence on brain connectivity and function represents one of the greatest challenges for modern neuroscience. Studies of genetic model organisms such as zebrafish (*Danio rerio*), fruit flies (*Drosophila melanogaster*), and nematodes (*Caenorhabditis elegans*) have significantly advanced our

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INTERNET RESOURCES

Jackson Labs <http://jaxmice.jax.org/>

GENSAT <http://www.gensat.org>

Allen Institute for Brain Science <http://www.brain-map.org/>

AIBS Connectivity Atlas: <http://connectivity.brain-map.org/>

understanding of how genes control the evolutionarily conserved processes of neurogenesis, neural fate specification, axon guidance, synaptogenesis, and neuronal survival (Hadjieconomou et al., 2011; Luo et al., 2008; Robles and Baier, 2012; Wang and Jin, 2011). To better understand the intersection between genes and neural circuits at the systems level in the mammalian nervous system, the house mouse (*Mus musculus*) has emerged as an unparalleled model organism based on the tractability of genetic manipulation.

The genetic toolbox available to interrogate circuit function in mice has expanded exponentially over the past two decades (Luo et al., 2008). The possibility of functionally testing a gene of interest in a select brain region is now a reality in modern neuroscience. In this protocol, we will outline the essential steps to manipulate gene expression in a target-selective manner. The focus will be on using combinatorial viral approaches to fluorescently label, functionally alter, or genetically perturb specific projections connecting two brain regions.

The use of viral vectors in neurobiological research has expanded due to their increasing flexibility and the ability to deliver viral particles to precise brain regions. Use of adeno-associated virus (AAV) in particular has become widespread due to its ease of production, low tropism, stable expression, and non-toxic nature (Scammell et al., 2003; Slack and Miller, 1996). AAV viral vectors in which transgene expression is dependent on Cre recombinase are widely available, allowing for conditional, cell-selective gene expression (Schnutgen et al., 2003). When Cre-dependent AAV viral vectors are injected into a mouse brain expressing *Cre* in a discrete subset of neurons (a Cre driver mouse line), then Cre-mediated recombination of the transgene will facilitate expression of the viral constructs in a restricted manner.

Current Cre driver mouse lines allow for regionally restricted and cell-type specific manipulation of gene expression; however, they are often insufficient to isolate projection-specific neuronal populations. To precisely define genetic control over circuit function development of methodologies allowing for specific transgene expression in subset of neurons based strictly upon their projection targets is critical. This protocol outlines a method that combines the use of locally transducing Cre-dependent AAV viruses with the retrograde transducing Canine Adenovirus 2 expressing *Cre* (CAV2-Cre). CAV2 is a desirable means of retrograde *Cre* delivery, as CAV viral vectors are largely restricted to transduction of neurons, permit stable expression, and have little to no toxicity (Hnasko et al., 2006; Kremer et al., 2000; Soudais et al., 2001). Dual viral injections of AAV and CAV2-Cre into interconnected regions will selectively label the subset of neurons projecting from one region to another. A wide array of constructs can be cloned into an AAV vector, enabling the conditional expression of candidate genes, dominant negatives, fluorescent reporters (calcium, pH, or voltage sensors), and optogenetic or pharmacogenetic tools.

The Basic Protocol outlined below provides a general template for planning and executing a combinatorial virus experiment, which can be adapted to address a wide range of experimental hypotheses. Detailed instructions on virus production, stereotaxic surgery, and post-hoc sectioning of brain tissue are provided in the support protocols.

Importantly, though this protocol describes experiments performed on mice, many of the experiments described are equally suited for use in rats (Witten et al., 2011). It should also be noted that although we exclusively describe the use of AAV1/2 and CAV2 here, in principle other combinations of local and retrograde viral vectors can be used, including lentivirus (Ahmed et al., 2004), herpes-simplex virus (Lo and Anderson, 2011), pseudorabies virus (Card et al., 2011), or rabies virus (Osakada et al., 2011).

BASIC PROTOCOL

Using combinatorial viral strategies to study gene-circuit interfaces

Studies using electrolytic, physical, and chemical lesions have identified critical functions for many different brain regions. In addition, knockout animals and pharmacological agonists and antagonists with varying degrees of specificity have been used to probe gene and protein function. These methods typically fail to discriminate between neurons with heterogeneous projections to multiple targets. For example, midbrain dopamine neurons, once thought to be a homogeneous population of neurons, have been demonstrated to be quite diverse, projecting to a diversity of downstream targets (Lammel et al., 2013). Therefore, it is advantageous to functionally test for genetic requirements in neurons based upon their projection specificity. As detailed below, this can be achieved by performing dual viral injections in two brain regions.

Before beginning this protocol, a researcher must determine which brain regions express a gene of interest and establish the connectivity of those regions. Many resources are available to help researchers identify such connections. In addition to data in the primary literature from specific mapping studies, a large scale “connectomics” project is currently underway at the Allen Institute for Brain Science (AIBS). This project aims to systematically map all the interconnections in the mouse brain. Their online resource (<http://connectivity.brain-map.org/>) provides a valuable starting point for identifying intriguing circuit connections, but once a potential projection is identified this data should be confirmed and expanded upon by the researcher, as outlined here:

Materials

Mouse brain atlas

Fluorogold (available from Fluorochrome, LLC)

Wild-type mouse, fluorescent reporter mouse, or Cre driver mouse

Adeno-associated virus encoding reporter or gene of interest (AAV; see support protocol 2)

Canine associated virus-2 encoding Cre recombinase (CAV2-Cre; see support protocol 3)

Establish coordinates for injections: *After identifying the brain regions to be studied, coordinates for stereotaxic injections must be optimized as follows:*

- 1 Determine initial coordinates (anterior-posterior, dorsal-ventral, and medial-lateral) by locating the injection target in a brain atlas. There are several high quality mouse brain atlases available, including the widely-used print atlas by Paxinos and Franklin (Paxinos and Franklin, 2013) or the atlas generated by the AIBS, available both in print (Dong, 2008) and free online (<http://www.brain-map.org/>).
- 2 During stereotaxic surgery (see support protocol 1), inject 0.2 μ l of Fluorogold or another appropriate dye at the determined coordinates. As injection accuracy can vary, it is suggested that 2–4 animals are injected using the same coordinates.
- 3 Perfuse the animal 24 hours following the injection, according to approved procedures. Though Fluorogold will eventually be transported retrogradely and spread to neurons throughout the brain (see below), cells surrounding the injection site will be strongly labeled and easily identifiable at this time point.
- 4 Section brain tissue (see support protocol 4), collecting sections surrounding the region of injection. Note that stereotaxic injection does not always lead to a spherical diffusion. For example, white matter tracts in the brain can act as barriers to alter the diffusion pattern, so it is important to sample across a large region ($\pm$ 1 mm from the injection site) to reveal the complete diffusion pattern.
- 5 If the location of the injection does not match the intended coordinates, adjust the coordinates accordingly and repeat with additional animals until consistent, accurate injections are achieved.
- 6 Once coordinates are determined, perform test injections using viral vectors, adjusting titer and injection volume to cover the desired region. A good starting point is 0.5 μ l of virus at a titer of 1×10^9 particles/ μ l. Allow a longer time between injection and perfusion (10–14 days) to ensure full expression of the virus.

Identify circuit connections

- 7 *Perform anterograde tracing studies:* An anterograde tracing study will determine the projection targets of a specific brain region. Three alternate methods are presented here. All methods utilize stereotaxic injection, described in support protocol 1, AAV virus production, described in support protocol 2, and tissue sectioning, described in support protocol 4.
 - A. Stereotaxic injection of a non-conditional AAV encoding a fluorescent protein. When stereotaxically injected into a region of interest, an AAV encoding a fluorescent marker (i.e. GFP) will transduce neurons surrounding the injection site. The cell bodies and processes of these neurons will be filled with the fluorescent protein, allowing for visualization of projection targets. After stereotaxic injection (see support protocol 1), allow approximately 2 weeks for full expression.

Perfuse the animal and section the brain tissue (see support protocol 4); projection targets of the region of interest will contain fluorescently labeled fibers. Specificity can be increased by reducing the injection volume and/or virus titer to limit the number of cells transduced by the virus.

- B.** Stereotaxic injection of AAV-Cre into a genetic fluorescent reporter mouse. Several mouse lines are available containing a “floxed-stop” fluorescent reporter, such as GFP or TdTomato at the Rosa26 genome locus (Madisen et al., 2010). In these animals, reporter expression occurs only in the presence of Cre recombinase. Stereotaxic injection of AAV-Cre into a region of interest will turn on the reporter in the neurons of that region, allowing for visualization of processes following brain tissue sectioning, as described above. We recommend the Rosa26-fs-TdTomato line (Gt(Rosa)26Sor^{<tm14}, or Ai14), available from Jackson Laboratories) because of its high fluorescence intensity (Madisen et al., 2010).
- C.** Stereotaxic injection of a conditional AAV into a specific Cre driver line. In order to identify the projection targets of a specific population of neurons, a mouse line expressing Cre recombinase under a cell-type specific promoter can be used (see section on Internet Resources). If such a line is available, stereotaxic injection of a conditional AAV vector will result in reporter expression only in Cre-expressing neurons near the injection site. One configuration for conditional expression that has proved successful is the double-floxed inverted open reading frame, termed DIO or FLEX (Schnutgen et al., 2003). Projection fibers containing the fluorescent reporter can be visualized as described above.

8 *Perform retrograde tracing study:* Unlike the anterograde tracing study underway at the AIBS, no large-scale map of retrograde connections is available. Two potential methods are suggested for simple labeling of neurons that project to a given target. CAV2 production is described in support protocol 3.

- A.** CAV2-Cre injection in a reporter mouse line (recommended method): Stereotaxically inject CAV2-Cre into a fluorescent reporter mouse line, such as Ai14 (Madisen et al., 2010). CAV2-Cre will be taken up by synaptic terminals at the injection site and will be retrogradely transported to cell bodies, turning on reporter expression in those cells. Projection inputs can be identified by sectioning of the brain tissue and screening for fluorescently labeled cell bodies.
- B.** Fluorogold injection in a wild-type mouse: Fluorogold is a retrogradely transported dye that will label neurons projecting to the site of injection. Stereotaxically inject 0.2 µl of 4% Fluorogold in saline into the target region. Perfuse animals 7–10 days following injection and

section the brain tissue. Following sectioning, Fluorogold can be visualized under UV illumination.

Perform dual viral injections

- 9 Once accurate coordinates and injection parameters have been verified, dual injection surgeries can be performed on a cohort of animals intended for behavioral or electrophysiological studies (allow 2–3 weeks following surgery for recovery and full viral expression before experiments are performed). Three classes of experiments are presented here, each of which alters gene expression in neurons projecting from one specific brain region to another. All three rely on injection of CAV2-Cre into the projection target region, but each utilizes a different Cre-dependent AAV injected into the projection origin region. Each experiment should include appropriate controls, typically animals expressing a control virus such as GFP.
 - A. Expression of an artificial regulator of cellular activity: Light activated ion channels and pumps, such as channelrhodopsin, halorhodopsin, and archaerhodopsin (Fenno et al., 2011), can be used to induce or inhibit action potential firing in neurons in vivo or in vitro. Receptors Activated Solely by Selective Ligands (RASSLs) (Coward et al., 1998) and Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) (Armbruster et al., 2007) are ligand-activated receptors that can also be used to activate or inhibit cellular activity in vivo or in vitro by administration of a specific agonist. Such artificial regulators of neural activity can be used to probe the consequences of increasing or decreasing activity within a given circuit element.
 - B. Loss-of-function through expression of a conditional dominant-negative or gain-of-function through conditional gene overexpression: Considerable insight into gene function can be gained through conditional, projection-specific expression of a dominant-negative to reduce protein function, or through conditional gain-of-function through gene overexpression.
 - C. Rescue of gene knockout by re-expression: Minimal gene sufficiency can be tested in select neural projections utilizing conventional knockout mice together with a combinatorial virus approach. Starting with a null genetic background, a conditional AAV encoding the gene to be rescued is injected into a brain region of interest and CAV2-Cre is injected into a projection target, allowing for selective reconstruction of gene sufficiency within a circuit (Parker et al., 2011).

Post-hoc histology

- 10 Following behavioral or other experimentation, all animals should be perfused and histology should be performed in order to confirm targeting (see support protocol 4). Criteria should be established (i.e. a certain number or percentage of

cells transduced) in order to enable an unbiased determination of whether targeting was sufficiently accurate to include a given animal in behavioral analysis.

SUPPORT PROTOCOL 1

Stereotaxic surgery for viral delivery to brain

This support protocol details how to stereotaxically inject virus, fluorescent tracer, or dye to a brain region of interest. This technique can be applied to single or multiple injections.

Materials

Wild-type mouse, fluorescent reporter mouse, or Cre driver mouse

Sterile cotton swabs

Sterile water

Sterile 0.9% saline

Topical anesthetic

Betadine

Sutures or surgical adhesive

Desired virus, dye, or tracer

Stereotaxic equipment (e.g. from Kopf):

Stereotaxic Alignment Instrument

Centering Scope 40×

Stereotaxic Alignment Indicator

Stereotaxic Drill

Syringe Holder

Electric razor

Microsyringe pump (e.g. from World Precision Instruments)

5 µl injection syringe (e.g. from Hamilton)

Surgical tools:

Forceps

Scalpel

Skin Clamps

Protocol steps: *All surgical procedures should be performed only in accordance with a protocol approved by your Institutional Animal Care Use Committee, which might require different methods for anesthesia, surgical procedures, pain management, and post-surgical care and follow-up. Make sure all surgical equipment is sterilized prior to use.*

1. Under anesthesia (typically induced by isofluorane), place front teeth of mouse in bite bar in the stereotaxic frame and secure the skull in place using the earbars, ensuring the tick marks on each earbar are equal. Skull should not shift under moderate pressure.
2. Remove hair from top of the head using an electric razor.
3. Apply topical anesthetic with a cotton swab; after 5 minutes remove with a clean swab and rinse skin with sterile water.
4. Apply Betadine sterilizing solution with a cotton swab; rinse with sterile water and repeat 2 more times.
5. Using a scalpel, cut the skin on the top of the head down the midline and expose the skull. Use skin clamps to keep skin flaps pulled apart.
6. Using the microscope attachment, locate bregma and adjust the stereotaxic apparatus to center bregma underneath the crosshairs of the microscope. Zero the values of the x, y, and z arms of the stereotaxic device.
7. Use the stereotaxic device to measure the distance between bregma and lambda (Figure 1). Move halfway between bregma and lambda, and use the alignment indicator attachment to level the skull in the left-right and anterior-posterior directions.
8. Return the stereotaxic frame to the zero position and confirm bregma is still centered under the microscope. Adjust if necessary.
9. Re-measure the distance between lambda and bregma.
10. Adjust the anterior-posterior (A-P) coordinate based on the lambda to bregma distance. The “standard” mouse has a lambda-bregma distance of 4.21 mm; therefore, calculate an adjustment factor (F) by dividing the measured lambda-bregma distance (in mm) by 4.21 (Figure 2). Multiply F by the chosen A-P coordinate to calculate the adjusted value. There is no need to adjust the medial-lateral or dorsal-ventral coordinates.
11. Drill holes at the appropriate coordinates, taking care not to lower the drill into the brain tissue. Apply pressure with a cotton swab to control any minor bleeding.
12. Load a syringe with the first virus or tracer, and place the syringe in the syringe holder on the stereotaxic arm.
13. Touch the tip of the syringe to bregma, and zero the z coordinate.
14. Expel a small amount of liquid from the syringe to verify that no air bubbles are present.
15. If necessary, clear drilled holes of dried blood using sterile saline and a cotton swab. Slowly lower the syringe through the drilled hole to a distance 0.5 mm deeper than the dorsal-ventral injection coordinate and wait two minutes.

16. Begin the injection. The syringe pump should be set to a rate of 250 nl/min. Total volume injected should be determined empirically, but is typically 250–750 nl. While the syringe is injecting, slowly raise the syringe 0.5 mm.
17. Wait an additional 10 minutes, then slowly remove the syringe.
18. Expel a small amount of liquid from the syringe to clear blood and/or cerebrospinal fluid from the tip.
19. Repeat steps 13–18 for additional injections. If possible, use a separate syringe for each virus. If a single syringe is to be used with more than one virus, rinse thoroughly with sterile water in between injections.
20. Close the incision using sutures or medical adhesive and perform post-surgical follow-up care as indicated by your IUCAC protocol.

SUPPORT PROTOCOL 2

Adeno-associated virus production

Adeno-associated virus (AAV) has become widespread in neurobiological studies due to its ease of use, stable expression, and low tropism (Scammell et al., 2003). Production of AAV requires a shuttle vector containing your construct of interest and a packaging vector encoding additional components necessary for virus production (Gregorevic et al., 2004). Many shuttle vectors already contain the sequences required for Cre-dependent gene expression (Schnutgen et al., 2003). The primary limitation of AAV is its small capacity. The typical size limitation for a cDNA to be cloned into a conditional AAV shuttle vector is approximately 2.3 Kb (assuming a promoter of approximately 1 Kb, such as the CAG promoter (Judge and Chamberlain, 2005).

Note: The packaging vector is time-consuming and labor-intensive to produce. We recommend outsourcing the amplification and purification of the packaging vector to a plasmid manufacturing company, such as Nature Technology.

Materials

HEK293T/17 cells, low passage
HEK culture media (see recipe)
10 cm tissue culture dishes
15 cm tissue culture dishes
0.25% Trypsin-EDTA
50 ml conical tubes
AAV shuttle vector with desired construct
pDG-1 packaging vector
2M CaCl₂, sterile
2× HEPES solution (see recipe)

Phosphate mix (see recipe)
Pasteur pipette
Serum-free HEK media (see recipe)
Dry ice
95% ethanol
25 × 89 mm large ultracentrifuge tubes (e.g. from Beckman)
40% sucrose in PBS, sterile
CsCl solution (see recipes)
13 × 51 mm heat-seal ultracentrifuge tubes (e.g. from Beckman)
1.5 ml tubes
6× DNA loading buffer/dye
1% agarose gel
Ethidium bromide or equivalent
1×HBSS (Hanks' Balanced Salt Solution)
10,000 molecular weight cutoff dialysis cassettes, 0.5–3 ml capacity (e.g. from Pierce)
5 ml syringe and 20 gauge needle
0.2 µm pore syringe filter
Parafilm
Incubator for cell culture (37°C, 5% CO₂)
Biosafety cabinet
Water bath (37°C)
Hemocytometer
Clinical centrifuge
Sealer for heat-seal tubes
Ultracentrifuge
SW-27 rotor (or equivalent)
VTi-65 rotor (or equivalent)
Electrophoresis equipment for agarose gel
UV box

Preparation of HEK293T/17 cells (~1 week prior to Day 1): *All steps should be performed in a tissue culture hood until cells have been harvested.*

- 1 Thaw frozen cell stock ($\sim 5 \times 10^6$ cells) in a 37°C water bath for approximately 5 minutes. Add to a 10 cm culture dish along with 10 ml warm HEK culture media, rock gently to disperse and place in incubator.
- 2 The next day, aspirate media from plate and immediately add 5 ml warm Trypsin-EDTA. Incubate 2 minutes at 37°C.
- 3 Aspirate Trypsin-EDTA and immediately add 10 ml of warm culture media to the plate. Triturate cells until dispersed and transfer to a 15 cm culture dish with an additional 10 ml of culture media (20 ml total).
- 4 Grow cells in incubator 2–4 days until confluent. Prepare five 15 cm dishes with 20 ml of warm culture media. Use EDTA-Trypsin (as described in steps 2–3) to resuspend cells, and divide cells evenly among the 15 cm dishes. Rock gently to disperse and return to incubator. Cells should be confluent in 2–4 days.

Day 1

- 5 Resuspend cells (as described in steps 2–3) and count density using a hemocytometer. Plate cells at a density of $\sim 3.7 \times 10^6$ cells/10cm dish in 10 ml HEK culture media. The steps below assume a total of fifty 10 cm dishes, but this number can be adjusted depending on desired yield.

Day 2: Transfection

- 6 Transfect in the early afternoon. For each 10 cm plate, mix together in a 50 ml conical tube:
 - 50 μ l 2M CaCl₂
 - 10 μ g AAV vector
 - 20 μ g pDG1 shuttle vector
 - ddH₂O to 400 μ l
- 7 In a separate tube, for each 10 cm plate mix together:
 - 396 μ l 2× HEPES solution
 - 4 μ l *phosphate mix*

NOTE: Test transfections should be performed prior to beginning the protocol to determine optimal amount of phosphate mix for peak transfection efficiency. Up to 8 μ l/plate may be required.
- 8 Add DNA solution dropwise to phosphate solution while gently vortexing. Solution should be cloudy with fine particles (no large clumps).
- 9 Incubate precipitation for 10–15 minutes at room temperature. Vortex on high briefly to break up any large particles, then add 800 μ l gently to each plate of cells. Be careful to avoid dislodging cells. Gently rock plate to spread the precipitation. Fine particles should be visible under the microscope. Return cells to incubator.

Day 3

- 10 First thing in the morning, use a vacuum with sterile Pasteur pipette to remove the media from each plate and replace with 10 ml warm serum-free HEK media. Take care to avoid dislodging cells.
- 11 Incubate for 48 additional hours.

Day 5: Harvest and purify viral vector

- 12 Cells should look round but still attached; culture media may be yellow. Using a pipette-aid to dislodge cells with repeated pipetting, harvest cells from all plates in a total of 120 ml media. Divide the harvested cells and media evenly into 4 50 ml conical tubes.
- 13 Liberate viral vector through repeated freeze-thaw cycles. Freeze tube completely in a bath of dry ice and ethanol; solution may turn from red to yellow/orange. Place tube into 37°C water bath and thaw completely. Repeat for a total of 3-freeze/thaw cycles.
- 14 Spin tubes in a clinical centrifuge at 2000 rpm for 30 minutes.
- 15 Prepare 6 large ultracentrifuge tubes by adding 15 ml of 40% sucrose to the bottom of each tube.
- 16 Collect the supernatant from the spin and divide evenly between the 6 ultracentrifuge tubes (approximately 22 ml/tube).
- 17 Spin at 27000 rpm overnight at 4°C (SW-27 rotor, or equivalent).

Day 6

- 18 Discard the supernatant. Resuspend and combine pellets in a total of 5 ml of CsCl solution. Use a Pasteur pipette to dislodge and break up pellets until no chunks remain; repeated pipetting will be required. Keep tubes on ice during resuspension.
- 19 Transfer resuspension solution to a heat-seal tube and fill with additional CsCl if needed. Seal tube and spin at 50000 rpm overnight at 4°C (VTi65 rotor or equivalent).

Day 7

- 20 Use razor blade to carefully remove top of heat seal tube. Use a pipette to remove 1 ml fractions, starting from the top, and place each fraction in a 1.5 ml tube.
- 21 Remove 1 µl from each fraction and add to a separate thin-walled tube containing 1 µl 6× DNA loading buffer and 4 µl H₂O. Boil tubes for 3 minutes and place immediately on ice

- 22 Run samples in a 1% agarose gel and stain with ethidium bromide or equivalent dye. Samples from fractions containing viral particles should contain a faint smear of DNA. Only these fractions should be used in the following step.
- 23 Repeat steps 19–22 using the virus-containing fractions. When adding the fractions to a new heat-seal tube, bring the volume up to 5 ml with additional CsCl solution.

Day 8

- 24 Fill a 2–3 liter bucket with 1.5 L 1×HBSS. Place a dialysis cassette in the bucket and hydrate for at least 10 minutes.
- 25 Identify the virus-containing fractions from the second CsCl spin (as in step 22). Using a 20 gauge needle attached to a 5 ml syringe, load these fractions into the hydrated dialysis cassette according to manufacturer's instructions. Place back in the HBSS-filled bucket and dialyze for 2–4 hours at 4°C.
- 26 Exchange the 1×HBSS with fresh 1×HBSS (1.5L) and dialyze overnight at 4°C

Day 9

- 27 Carefully remove solution from dialysis cassette using a syringe and needle, and filter using a 0.2 µm pore syringe filter into a 15 ml conical tube.
- 28 Load a large ultracentrifuge tube with 15 ml 40% sucrose. Add the filtered virus solution on top, and fill the remaining volume with 1×HBSS. Spin at 27000 rpm overnight at 4°C (SW-27 rotor, or equivalent).

Day 10

- 29 Carefully decant off and discard supernatant. Place tube on ice.
- 30 Resuspend pellet in 100 µl 1×HBSS: add HBSS to tube, cover with parafilm, and vortex. Let sit for 5–10 minutes on ice, then vortex again. Repeat several times until pellet is resuspended. Transfer to 1.5 ml tube. This is your viral suspension.
- 31 To harvest any residual particles, repeat previous step using a second 100 µl of 1×HBSS. Transfer to a separate 1.5 ml tube labeled “wash”.
- 32 Approximate the titer of the resuspended virus and the wash by running a DNA gel as described in steps 21–22. Compare to viruses of known titer. If viral particles are abundant, dilute the resuspended virus with the wash solution. Further dilution should be done with 1×HBSS.
- 33 Aliquot virus and store at –80°C.

SUPPORT PROTOCOL 3

Production of CAV2-Cre

CAV2-Cre allows for the retrograde transduction and expression of *Cre* recombinase (Hnasko et al., 2006). Expression of *Cre* is stable and no adverse effects on cell viability

following long-term transduction have been reported (Hnasko et al., 2006). Detailed descriptions of the initial cloning and production of CAV2 viral vectors can be found elsewhere (Kremer et al., 2000). Here we will describe the basic method of CAV2-Cre amplification and purification. CAV2-Cre is replication incompetent due to the lack of a critical *E1* gene from the viral genome (E1-deleted viral vector) (Kremer et al., 2000). The vector can be amplified using dog kidney (DK) cells with stable expression of the *E1* gene (DK-ZEO).

Materials

- DK-ZEO cells
- 10 cm tissue culture dish
- 15 cm tissue culture dish
- DK-ZEO culture media (see recipe)
- 0.25% Trypsin-EDTA
- CAV2-Cre viral vector stock
- Cell scraper
- 50 ml conical tubes
- Dry ice
- 95% ethanol
- CsCl solutions (see recipes)
- Pasteur pipettes
- 25 × 89 mm large ultracentrifuge tubes (e.g. from Beckman)
- 5 ml syringe and 20 gauge needle
- 14 × 89 mm small ultracentrifuge tubes (e.g. from Beckman)
- PD-10 Columns (e.g. from GE Life Sciences)
- PBS w/CaCl₂ and MgCl₂ (see recipe)
- 1.5 ml tubes
- Viral lysis solution (see recipe)
- Water bath (37°C, 56°C)
- Incubator for cell culture (37°C, 5% CO₂)
- Biosafety cabinet
- Clinical centrifuge
- Ultracentrifuge
- SW-27 rotor (or equivalent)

SW-41 rotor (or equivalent)

Spectrophotometer

Preparation of DK-ZEO cells (~ 2 weeks)

- 1 Thaw frozen cell stock ($\sim 5 \times 10^6$ cells) at 37°C for approximately 5 minutes. Add to a 10 cm culture dish along with 10 ml culture media and place in incubator.
- 2 The next day, aspirate media from plate and immediately add 5 ml Trypsin-EDTA. Incubate 5–10 minutes at 37°C.
- 3 Aspirate Trypsin and immediately add 10 ml of culture media to the plate. Triturate cells until dispersed and transfer to a 15 cm culture dish with an additional 10 ml of culture media. Rock gently to disperse and place in incubator.
- 4 Grow cells in incubator 2–4 days until confluent. Propagate cells until you have forty 15 cm dishes of DK-ZEO cells at ~70–80% confluence. Recommended propagation procedure: Split original 15 cm dish of cells to four 15 cm dishes, then when confluent split four 15 cm dishes into ten 15 cm dishes, and then ten 15 cm dishes into forty 15 cm dishes.

Day 1: Viral transduction of DK-ZEO cells and viral amplification: *To effectively transduce DK-ZEO cells requires 50–100 viral particles per cell. Each 15 cm dish will contain $\sim 7.7 \times 10^7$ cells, requiring a total of $\sim 3.85\text{--}7.7 \times 10^9$ viral particles.*

- 5 Add viral particles to 520 ml of culture media and mix gently
- 6 Aspirate culture media from plates of cells that have grown to confluence. Add 13 ml of the culture media plus viral vector mix to each of the forty 15 cm dishes.
- 7 Return cultures to 37°C incubator at 5% CO₂. Amplification of the E1-deleted CAV2-Cre vector requires approximately 48 hours of incubation.

Day 3: Harvest and purify viral vector

- 8 Use a cell scraper to dislodge cells adhered to the plate and transfer media plus cells to a 50 ml conical tube. Distribute media plus cells from all 40 plates between twelve 50 ml conical tubes.
- 9 Spin cells at 1160×g in a clinical centrifuge for 5 minutes. Remove and save 10 ml of media and aspirate the remaining media from the conical tubes. Resuspend cells in 10 ml of media to yield a final volume of ~14 ml in one 50 ml conical tube.
- 10 Liberate viral vector through repeated freeze-thaw cycles. Freeze tube completely in a bath of dry ice and ethanol; solution may turn from red to yellow/orange. Place tube into 37°C water bath and thaw completely. Repeat for a total of 3-freeze/thaw cycles.

- 11 Spin cells, liberated viral vector, and media at 3220×g for 10 minutes.
- 12 Add 10 ml CsCl (specific density 1.25 g/ml) to a large ultracentrifuge tube. Carefully add 10 ml CsCl (specific density 1.40 g/ml) to the bottom of the tube (underneath the 1.25 g/ml CsCl solution) using a Pasteur pipette. Add supernatant from Step 11 to the top by gently pipetting down the side of the tube. Fill remaining volume with PBS. Spin sample for 2 hours and 10 minutes at 27,000 rpm (SW-27 rotor or equivalent).
- 13 Using a 20-gauge needle attached to a 5 ml syringe, puncture the centrifuge tube just below the viral band (opaque band at the interface of the 1.25 and 1.4 g/ml CsCl solutions). Pull the band by directing the beveled edge of needle just below the bottom of the band. You should get ~2 ml of solution containing the viral band.
- 14 Add approximately 10 ml CsCl (specific density 1.34 g/ml) to a small ultracentrifuge tube. Carefully add viral vector from Step 13 to the top by gently pipetting down the side of the tube. Spin sample for 18 hours at 35,000 rpm (SW-41 rotor or equivalent).

Note: the 14 × 29 ml tube has a volume of 12 ml. In order to ensure that your final volume is 12 ml, subtract the total volume of viral vector from Step 13 from 12 and add that volume of CsCl solution to the centrifuge tube.

Day 4

- 15 Repeat Step 13.
- 16 Equilibrate PD-10 column with PBS containing CaCl_2 and MgCl_2 , according to manufacturer's instructions. Label nine 1.5 ml tubes. Add viral vector from Step 15 to the column and collect 1 ml of flow-through into each of the first two tubes. Add 3.5 ml of PBS containing CaCl_2 and MgCl_2 to the column and collect seven 0.5 ml fractions in the remaining tubes.
- 17 Dilute 30 μl of each fraction with 270 μl lysis solution in a fresh tube and incubate in a 56°C water bath for 10 minutes. Using a spectrophotometer, measure the OD_{260} of each fraction and calculate the number of particles using the following formula: $\text{OD}_{260} \times 10 \times 1.1 \times 10^{12}$.
- 18 Fractions 4–7 typically contain the majority of the CAV2-Cre viral vector. These fractions can be pooled, and aliquoted (20 μl each), and stored at –80°C. Approximately 500 μl should be reserved for subsequent amplifications.

SUPPORT PROTOCOL 4

Brain sectioning

Sectioning of brain tissue for post-hoc histology is critical not only for the initial optimization of injection coordinates, but also to ensure consistency among all animals used

for behavioral or other experiments. Many methods for cutting and mounting of brain sections are available; we present one simple method here.

Note: Immunohistochemistry can be performed as desired on floating sections (following step 9) to stain for a cell-specific marker or other proteins. Immunohistochemistry is not typically required for visualization of fluorescent reporters such as GFP or TdTomato unless the signal is quite dim.

Materials

4% paraformaldehyde (PFA) in PBS, pH 7.4 (see recipe)

Small glass or plastic jar with a secure lid

30% sucrose in PBS

Freezing media (e.g. O.C.T. from TissueTek)

Disposable embedding molds

Dry ice

95% ethanol

Rounded spatula

1× PBS

24- or 48-well plate

Brain atlas

Paintbrush

Glass slides

Mounting media (e.g. Fluoromount)

Cryostat

Protocol Steps

1. Following perfusion, remove brain from skull and submerge in small jar in 4% PFA overnight at 4°C.
2. Decant PFA and replace with 30% sucrose. Maintain at 4°C with gentle rocking or rotation until brain sinks to bottom of container (typically 1–2 days).
3. Fill embedding mold $\frac{3}{4}$ full with freezing media. Add brain and use spatula or forceps to orient with olfactory bulbs facing down.
4. Freeze in a shallow bath of dry ice/ethanol, taking care to ensure that brain remains in upright orientation. Ethanol should not come over the top of the cup.
5. Section immediately or store at –80°C indefinitely. If stored at –80°C, let equilibrate to –20°C before sectioning.

6. Peel apart walls of embedding mold to release frozen block. Using additional freezing media, freeze block (olfactory bulbs facing up) to chuck in cryostat.
7. Once block is firmly frozen to cryostat, begin taking 50 μ m thick sections, referencing the brain atlas to adjust position of brain to ensure even orientation along all 3 axes.
8. Continue taking sections until the region of interest approaches. Take 30 μ m thick sections through the region of interest, picking up each section with a wet paintbrush and depositing it into one well of a 24- or 48-well plate filled with PBS.
9. If whole brain sectioning is desired, for example to map unknown projection targets, cut 30 μ m sections through the entire brain, collecting approximately every 3rd slice.
10. Using a paintbrush, mount the slices onto glass slides. Cover with preferred mounting media and a coverslip. Allow to dry completely before imaging.

REAGENTS AND SOLUTIONS

HEK and DK-ZEO culture media

500 ml Dulbecco's modified Eagle's medium (DMEM)

50 ml Fetal Bovine Serum (FBS) for HEK and 10 ml for DK-ZEO

5 ml 200 mM L-Glutamine

5 ml 100 $\times$ Penicillin/Streptomycin

Add FBS, Glutamine, and Pen/Strep to DMEM bottle under sterile conditions. Invert to mix.

Serum-free HEK culture media

As above, but without the fetal bovine serum.

HEPES solution

8.18 g NaCl

5.96 g HEPES

ddH₂O to 500 ml, pH to 7.05

Mix until dissolved. Filter sterilize.

Phosphate Mix

4.95 ml 1M NaH₂PO₄

10.05 ml 1M Na₂HPO₄

85 ml ddH₂O

Mix until dissolved. Filter sterilize. 1 M stock solutions of phosphates may need to be heated in order to dissolve.

CsCl (d = 1.37 g/ml)

50.9 g CsCl

100 ml ddH₂O**CsCl (d = 1.25 g/ml)**

45.0 g CsCl

100 ml ddH₂O**CsCl (d = 1.40 g/ml)**

56.0 g CsCl

100 ml ddH₂O

In a graduated cylinder, add CsCl and top off with water to the exact volume (mix to dissolve). Weigh 1 ml of solution and titrate with water or CsCl until density is equal to 1.37, 1.25, or 1.40.

Viral lysis solution

0.1% SDS

10 mM Tris-HCl, pH 7.4

4% Paraformaldehyde (PFA), pH 7.4

4 g paraformaldehyde

100 ml PBS

2 pellets NaOH

HCl

Combine paraformaldehyde, water, and NaOH. Stir over gentle heat to dissolve. Adjust pH to 7.4 using HCl.

COMMENTARY**Background Information**

The diversity of circuit function in the mammalian brain requires approaches enabling the genetic dissection of neuronal function based upon their anatomical connectivity. Brain structures that were once thought to be highly homogenous are now being found to exhibit incredible heterogeneity, both at the genetic and anatomical level. Therefore, it is critical to develop techniques to advance the genetic dissection of complex brain circuits. The protocol outlined here allows for the selective expression of genetic tools to explore subsets of neurons.

The fundamental basis of dual viral approaches is based upon the groundbreaking discovery and development of the Cre-LoxP system for homologous recombination (Sternberg and Hamilton, 1981; Sternberg et al., 1981a; Sternberg et al., 1981b), along with the use of

single viral vectors to express genes of interest in the central nervous system (Slack and Miller, 1996). Combining these techniques enables the use of one Cre-expressing virus to activate another Cre-dependent virus, which is widely applicable to the selective expression of recombinant genetic tools to study gene-circuit interactions. For example, dual viral expression allows for the precise expression of genetically-encoded calcium indicators, voltage sensors, pH sensors, and recombinant proteins, both wild type and dominant negative versions.

These technical advances in viral vector development are paralleled by our increasing knowledge of the complex genetic and anatomical connectivity of the brain. First performed solely by individual investigators, the large-scale initiatives at the AIBS and GENSAT provide broadly useful resources for the neuroscience community. These brain atlas projects detail the gene expression profile of all known protein-encoding genes in the mouse genome, along with large numbers of genes for other species including humans and non-human primates (Bernard et al., 2012; Hawrylycz et al., 2012; Lein et al., 2007; Zeng et al., 2012). A complementary dataset, also being completed at the AIBS, is a mouse brain connectivity atlas mapping all the interconnections within the brain. Taking advantage of these genetic and anatomical projects, together with the viral approaches in this protocol, will lead to greater insight into the genetic basis of the development and function of the brain.

Critical Parameters and Troubleshooting

Combinatorial viral approaches require virus to be delivered to multiple locations, and mistargeting to one brain region can negate even perfect targeting to a second region. Therefore, it is important to firmly establish the accuracy and precision of the injection coordinates. Subtle discrepancies between atlas coordinates and the actual results can occur, so optimization is required for each set of injection coordinates. Furthermore, differences can also occur due to the age, sex, and the specific inbred strain used for the experiments.

Anticipated Results

The ideal result would be targeting 100% of the neurons that project from one brain region to another. However, as discussed, there are many factors that prevent the realization of this ideal. We have found that we can reproducibly transduce ~90% of the neurons in a single brain region. If a second brain region can also be targeted with ~90% efficiency, then the theoretical limit on viral co-expression is ~81% ($90\% \times 90\%$). For certain applications, this amount of transduction efficiency may be sufficient to observe an effect. However, loss of function may require greater levels of inactivation to observe a phenotype. Greater coverage can be achieved by increasing the number of injection sites, but this can lead to incorporation of targeted regions outside the area of interest.

Time Considerations

The initial mapping experiments to validate each injection coordinate can take from several weeks to several months, depending upon the number of rounds required to produce a high level of targeting accuracy and precision. For example, if using an AAV with a strong promoter (i.e. chicken β -actin or CMV), strong viral expression of a fluorescent marker can

be observed after 10 days. Therefore, considering the time to perfuse and possibly stain for marker expression, it will typically take 2–3 weeks to test for expression. If targeting is not correct or if there is a high degree of variability in viral transduction, additional rounds of viral targeting must take place to optimize the coordinates.

Upon completing optimization procedures, combinatorial viral injections can be performed. Depending upon the constructs and application, viral expression can be observed in as little as 10 days. However, for studying functional circuitry, it may be advantageous to wait longer, 2–3 weeks, for maximal viral expression to occur.

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Figure 1.

Combinatorial viral delivery to study pathway specific gene function. (A) Illustration of combined viral injections of a conditional AAV-FLEX construct (red) into a region containing a neuronal population of interest and a CAV2-Cre viral vector (blue) into a projection target of the neurons of interest. (B) Illustration of local (red) AAV-FLEX viral vector transduction and retrograde (blue) CAV2-Cre transduction. (C) Demonstration of combinatorial viral-mediated gene expression. Injection of CAV2-Cre into a target region of interest (blue) of a Cre-dependent reporter line (Ai14) illustrates coverage area of viral vector injection and the number of neurons retrogradely transduced. Injection of AAV-FLEX containing conditional expression cassette of interest locally transduces neurons at the

sight of injection; however, only neurons with combined CAV2-Cre and AAV-FLEX will express the transgene (red).

Figure 2.

Illustration demonstrating the identification of Bregma and Lambda on a mouse skull. Following incision, the scalp is held open using skin clamps, allowing access to the top of the skull. Bregma can be seen as the intersection of the midline rostral-caudal fissure and the second major medial-lateral fissure. Lambda can be identified as the most rostral medial-lateral fissure. Stereotaxic x and y coordinates are set to zero at Bregma. Moving from midline to the left is in the negative x direction and positive from midline to the right. Similarly, y coordinates are negative caudal to Bregma and positive rostral to Bregma. Stereotaxic targeting of the y coordinate can be improved using a Bregma-Lambda correction factor (F), taken as the distance between Bregma and Lambda and dividing by 4.21 (the distance from Bregma to Lambda in a commonly used mouse brain atlas).