Cre Activated and Inactivated Recombinant Adeno-Associated Viral Vectors for Neuronal Anatomical Tracing or Activity Manipulation

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Recombinant adeno-associated viruses (rAAVs) transcriptionally activated by Cre recombinase (Cre-On) are powerful tools for determining the anatomy and function of genetically defined neuronal types in transgenic Cre driver mice. Here we describe how rAAVs transcriptionally inactivated by Cre (Cre-Off) can be used in conjunction with Cre-On rAAVs or genomic Cre-reporter alleles to study brain circuits. Intracranial injection of Cre-On/Cre-Off rAAVs into spatially intermingled Cre^+ and Cre^- neurons allows these populations to be differentially labeled or manipulated within individual animals. This comparison helps define the unique properties of Cre^+ neurons, highlighting the specialized role they play in their constituent brain circuits. This protocol touches on the conceptual and experimental background of Cre-Off rAAV systems, including caveats and methods of validation. © 2015 by John Wiley & Sons, Inc.

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INTRODUCTION

Understanding brain circuits necessitates mapping how the cellular diversity within a given brain region contributes to both local and long-range synaptic connectivity. Traditional anterograde or retrograde anatomical tracers such as biotinylated dextran amine or the B subunit of cholera toxin allow mapping of long-range axonal projections between brain regions, but are agnostic to cell type. In contrast, intracranial injection of DNA-based recombinant adeno-associated viruses (rAAVs) transcriptionally activated by Cre recombinase (Cre-On) can be used in conjunction with Cre driver transgenic mice to label or manipulate defined cell types in particular brain regions. This widely applicable technique has provided striking insight into the physiological and synaptic properties of individual cell types and their contribution to behavior (Cardin et al., 2009; Kravitz et al., 2010; Kozorovitskiy et al., 2012). However, defining how Cre^+ neurons uniquely function within their constituent brain circuits requires methods for comparison to neighboring Cre^- neurons. To this end, we developed rAAVs that are transcriptionally inactivated by Cre (Cre-Off) and can be used in conjunction with Cre-On rAAVs and Cre reporter alleles to express different transgenes within intermingled Cre^+ and

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Cre$^-$ populations (Saunders et al., 2012). These Cre-Off rAAVs vectors, which carry a number of popular fluorophore and optogenetic constructs, are available from Addgene (*http://www.addgene.org/Bernardo_Sabatini/*). The goal of this unit is to provide a conceptual overview of Cre-sensitive rAAVs, explain methodological caveats, and provide methods to validate Cre-Off rAAV activity. This unit is designed to function as an extension of previous Current Protocols which provide step-by-step instructions for rAAV packaging (*UNIT 4.17*) and intracranial rAAV injection (*UNIT 1.20*).

Controlling rAAV Transcription through Cre-Lox Recombination

Designing and interpreting experiments with Cre-sensitive rAAVs requires a basic understanding of rAAV transduction and mechanisms of Cre-mediated recombination. AAVs are parvoviruses, built from linear, single-stranded (ss) DNA genomes packaged in icosahedral capsids (Fig. 1.24.1A; Cotmore and Tattersall, 2013). AAVs enter cells through capsid-glycan interactions on the cell surface (Murlidharan et al., 2014). After transport to the nucleus, ss-genomes self-prime using inverted terminal repeat (ITR) sequences and endogenous DNA polymerase to become double stranded (ds). ds-AAV genomes can remain linear, circular, or undergo ITR-dependent recombination to form circular concatemers (Fig. 1.24.1A; Yang et al., 1999; Yan et al., 2005). The circularized multimers appear to be responsible for long-term episomal expression (Vincent-Lacaze et al., 1999). AAVs can also integrate into the host genome (Deyle and Russell, 2009), favoring regions prone to instability and DNA repair (Miller et al., 2004; Inagaki et al., 2007). Integration can also be site-specific through an engineered targeting sequence and homologous recombination (Donsante et al., 2007; Lisowski et al., 2012; Wang et al., 2012; Barzel et al., 2015). Since the ITRs are the only endogenous AAV sequences necessary for expression, rAAVs are built simply by replacing the two endogenous genes *rep* and *cap* with an expression cassette, typically consisting of a promoter, transgene, and polyadenylation signal (Fig. 1.24.1A).

The structure of ds-rAAV genomes can be manipulated in the nucleus by site-specific recombinases like Cre, which invert or excise DNA flanked by a pair of 34 bp locus of x-over (lox) sites (Fig. 1.24.1B). Lox sites consist of two palindromic recognition regions (13 bp) surrounding a nonpalindromic spacer (8 bp). The spacer region confers directionality to the lox sites, and the relative orientation of lox sites determines how Cre will affect the intervening DNA. If lox sites are oriented in the same direction, the flanked DNA will be excised, leaving behind a single lox site in the original orientation. If lox sites are oppositely oriented, both the flanked DNA and the lox sites will be recombined in the inverted, anti-sense orientation. Recombination is normally reversible, with reaction efficiency dependent on both the intervening distance (still present at megabase distances; Zheng et al., 2000) and sequence similarity of the paired lox sites (Siegel et al., 2001).

To achieve efficient, Cre-conditional transgene expression, Cre-mediated recombination must be engineered to be irreversible. One strategy to achieve permanent Cre-On expression is to excise a stop cassette between the promoter and transgene using similarly oriented loxP sites (Kuhlman and Huang, 2008). While reversible in theory, the chance of the excised DNA, Cre, and remaining loxP site interacting in the nucleus is low. A second strategy uses two pairs of recombination-incompatible lox sites to engineer an irreversible Cre-mediated inversion. The so called Flip-Excision (FLEx) system, using loxP and lox2272 (Fig. 1.24.1B), was designed as a somatic reporter for cell-specific Cre activity. In the presence of Cre, an anti-sense reporter gene was inverted into the sense orientation with respect to the promoter, allowing functional reporter mRNA to be transcribed (Fig. 1.24.2A; Schnütgen et al., 2003). While both the stop-cassette and FLEx systems have been applied to rAAVs, the smaller sequence requirement and lack of leaky transcription have made the rAAV FLEx system a widely adopted choice to achieve Cre-On expression in vivo (Atasoy et al., 2008). FLEx rAAVs are also referred

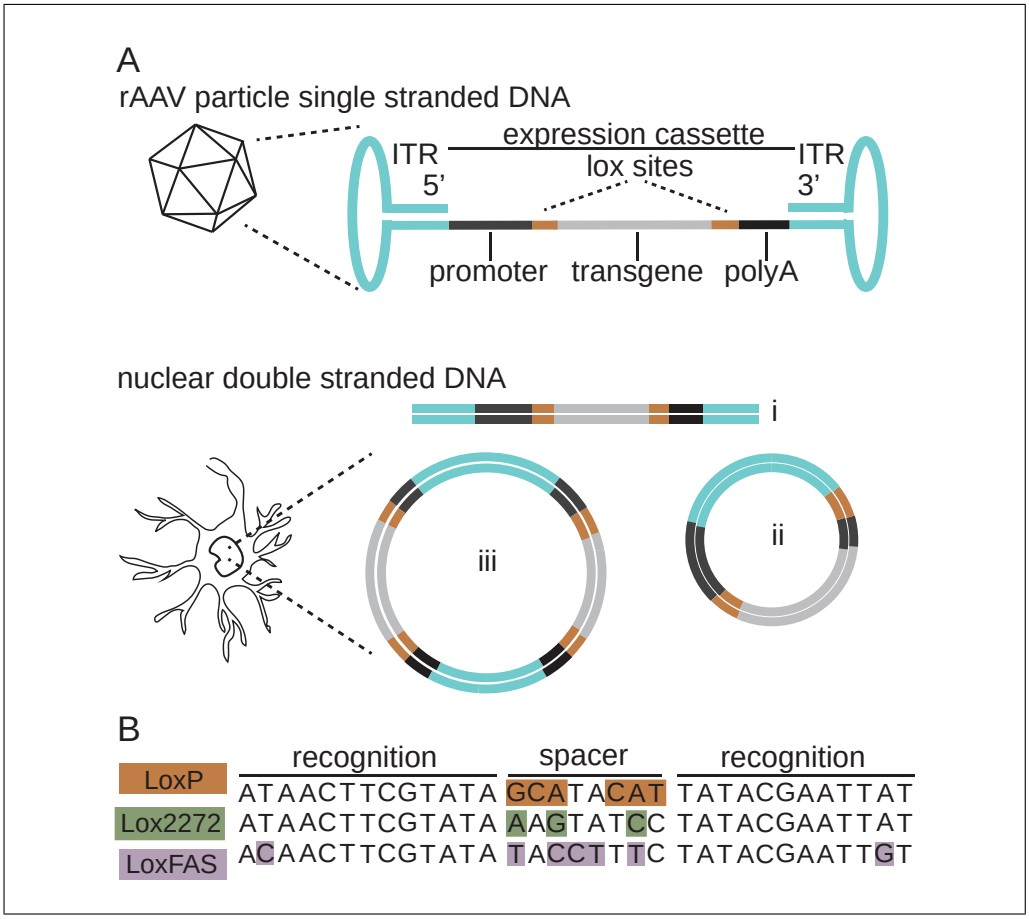

Figure 1.24.1 rAAV genome organization and manipulation by Cre through incompatible lox sites. (**A**) Top, rAAV particles contain a single stranded (ss) DNA genome in which the endogenous *rep* and *cap* genes are replaced by an expression cassette containing a promoter, transgene, and polyadenylation signal. Lox sites situated within this cassette confer Cre-sensitive expression. The only required endogenous rAAV sequences are the inverted terminal repeats (ITRs), which self-prime in the nucleus to polymerize into a double-stranded (ds) form. Bottom, ds-rAAV genomes can remain linear (i) or circularize remaining independent (ii) or undergo ITR-mediated recombination into multimeric concatamers (iii). Cre-mediated recombination may also produce other forms of multimeric ds-rAAV genomes. (**B**) Sequence comparison of recombination incompatible lox sites used in Cre-On and Cre-Off rAAVs. Lox sites (34 bp) contain two palindromic recognition sequences (13 bp) and a nonpalindromic spacer region (8 bp) that confers directionality and compatibility to recombination. Recombination occurs between lox site pairs in two basic steps. First, two Cre molecules bind each of the recognition sequences on a single lox site. Second, two pairs of Cre-bound lox sites form a complex, catalyzing DNA synapsis and subsequent strand exchange within the spacer region. The efficiency of strand exchange depends on spacer region sequence similarity. LoxP, lox2272, and loxFAS recombine efficiently with like-pairs but not unlike-pairs (Siegel et al., 2001). Differences in the lox sequences are highlighted.

to as Double-floxed Inverted Orientation (DIO), a naming convention which refers to the starting orientation of the transgene with respect to the promoter.

Development of Versatile Cre-Off rAAVs

The FLEx system can also be used to generate Cre-Off rAAVs by simply starting the transgene in the sense orientation (Double-floxed Orientation, DO) with respect to the promoter (Fig. 1.24.2A). DO rAAVs achieve efficient Cre-Off expression (Saunders et al., 2012). However, when co-injected with Cre-On DIO rAAVs, DO and DIO rAAVs unexpectedly interact, producing a profound reduction in DIO transgene expression (Fig. 1.24.3A). Similarly, we observed that both DO and DIO rAAVs can inhibit

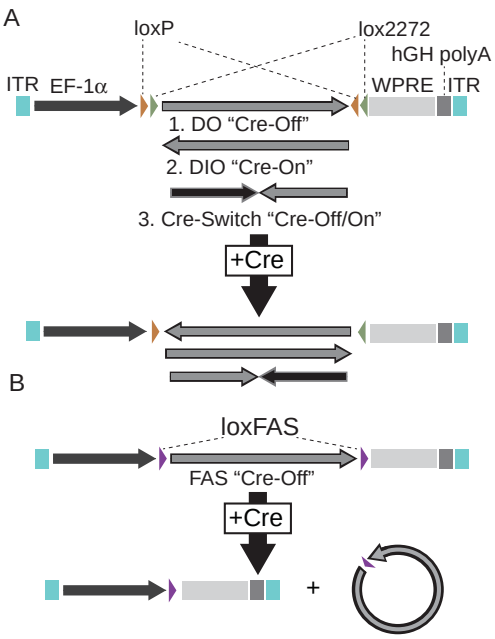

Figure 1.24.2 Cre-On/Off transgene expression in DIO, DO, and FAS rAAVs. (**A**) Oppositely oriented loxP (orange triangles) and lox2272 (green triangles) sites permit Cre-mediated recombination and inversion of the flanked transgene with respect to the EF-1α promoter. Downstream sequences stabilize the mRNA (woodchuck polyresponse element; WPRE) and trigger polyadenylation (human growth hormone polyadenylation; hGH polyA). After recombination, the transgene is flanked by one loxP and one lox2272 site, which do not recombine efficiently, effectively locking the transgene into position. The starting orientation of the transgene determines the Cre dependence of expression. The double-floxed orientation (DO, 1) configuration, in which the open reading frame (ORF) of the transgene begins in the functional orientation with respect to the promoter, maintains expression only in cells lacking Cre (Cre-Off). In the opposite starting orientation, the double-floxed inverted (DIO, 2) ORF must be recombined to be functional, and expression is achieved only in Cre expressing cells (Cre-On). A single transgene containing two ORFs oriented oppositely with respect to each other and separated by stop codons (3) switches expression between the two ORFs depending on Cre expression. For Cre-Switch transgenes, the first, forward-oriented ORF is expressed in Cre^- cells whereas the second, inverted ORF is activated in Cre^+ cells. (ITR, inverted terminal repeats; cyan). (**B**) Cre-Off control of transgene expression can also be achieved by Cre-based excision of the ORF using alternative loxFAS sites. loxFAS sites (purple triangles) flank the ORF and are oriented in the same direction such that the flanked sequence is excised by Cre. Figure and legend adapted from Saunders et al. (2012).

expression from a loxP-based nuclear Cre-reporter allele (Ai19; Fig. 1.24.3B; Madisen et al., 2010).

We hypothesized that the transcriptional interferences we observed between DO/DIO rAAVs and the somatic Cre-reporter allele were due to intermolecular, Cre-mediated recombination between compatible lox sites. Therefore we designed an alternative Cre-Off rAAV system that uses a third lox variant (loxFAS) which recombines with loxP and lox2272 at very low efficiencies (Fig. 1.24.1B; Siegel et al., 2001). In FAS rAAVs, Cre-Off expression is achieved through transgene excision by flanking, similarly oriented loxFAS sites (Fig. 1.24.2B). Intracranial injection of FAS rAAVs alone demonstrates efficient Cre-Off expression (Saunders et al., 2012). Moreover, consistent with our hypothesis, when injected with DIO rAAVs or into Cre-reporter mice, FAS rAAVs do not interfere with expression (Fig. 1.24.3A,B). These data demonstrate that differential Cre-On/Off expression can be achieved by co-injection of DIO and FAS rAAVs. They also demonstrate that caution must be used when multiple Cre-sensitive elements are

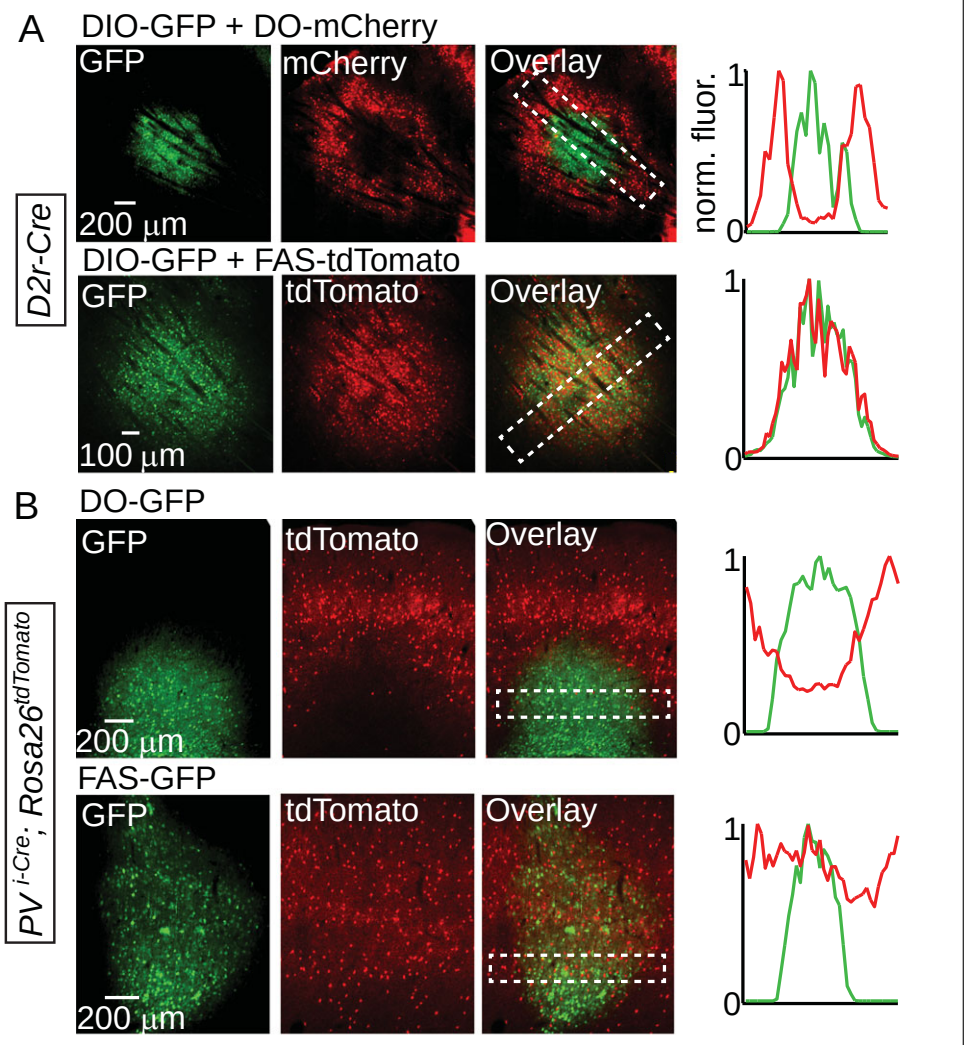

Figure 1.24.3 FAS rAAVs achieve Cre-Off expression compatible with Cre-On rAAVs or Cre-activated genomic alleles. Comparison of DO and FAS Cre-Off rAAV expression in conjunction with DIO Cre-On rAAVs and Cre-reporter alleles. Left, image of the primary infection. Right, normalized mean fluorescent values. (**A**) Simultaneous injection of DO/DIO versus FAS/DIO rAAVs to test for differential Cre-Off/On transgene expression. rAAVs were mixed and injected into the striatum of *D2r-Cre* mouse, where Cre^+ iSPNs are intermingled with Cre^- dSPNs and interneurons. DIO-GFP and DO-mCherry co-injection (top) results in expression patterns which are segregated and antagonistic. DIO-GFP and FAS-tdTomato co-injection (bottom) results in expression patterns which are spatially congruent. (**B**) Comparison of DO versus FAS Cre-Off rAAV expression in a Cre-reporter mouse. DO-GFP and FAS-GFP rAAVs were independently injected into the cortex of *PV $^{i\text{-}Cre}$;Rosa26^{tdTomato} (Ai19)* mice. Cre expression in PV^+ interneurons activates the tdTomato reporter through excision of loxP flanked stop cassette. In the area containing the DO-GFP infection, tdTomato reporter expression is greatly diminished. This inhibition is specific to DO rAAVs, as FAS-GFP infection does not affect reporter fluorescence. Figure adapted from Saunders et al. (2012).

combined in the same organism, as cryptic recombination may occur between viral and nuclear DNA containing compatible lox sites.

We also engineered a single rAAV system to achieve differential Cre-On/Off expression of distinct fluorophores (Cre-Switch; Saunders et al., 2012). The Cre-Switch rAAV carries a transgene with two open reading frames for GFP and tdTomato, inverted and

separated by a stop codon (Fig. 1.24.2A). In the starting orientation, functional tdTomato mRNA is transcribed; after Cre mediated inversion, the functional mRNA encodes GFP. Thus the Cre-Switch rAAV is equivalent to combined DIO-GFP/DO-tdTomato rAAVs. There are advantages and disadvantages to using the single rAAV Cre-Switch versus co-injection of mixed DIO and FAS rAAVs. First, the Cre-Switch system ensures that differential Cre-On/Off labeling observed is not skewed by differences in the number of DIO versus FAS rAAV particles transduced in each cell. If the goal of the experiment is to achieve equivalent differential labeling throughout the injected area, this could be an advantage. Cre-Switch rAAVs have two distinct disadvantages. First, the large size of the double transgenes runs up against the intrinsic packaging size limits of rAAVs (maximum ~4.5 kbp ITR to ITR) and thus may not be suited for larger transgenes.

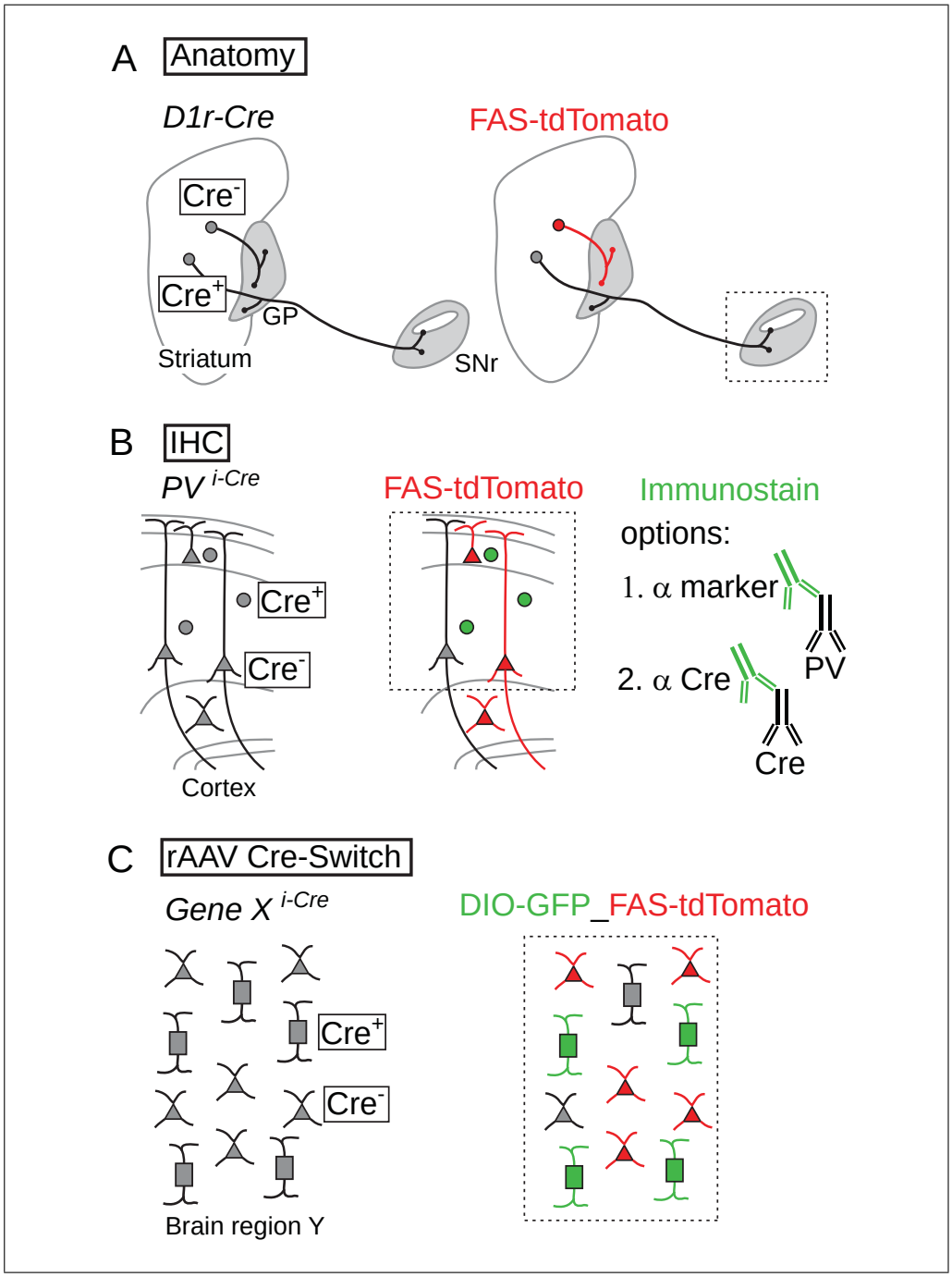

Figure 1.24.4 *(legend appears on next page)*

Second, whereas individual DIO and FAS rAAVs can be mixed and co-injected in many combinations, Cre-Switch rAAVs have to be uniquely cloned and packaged for each pair of differentially expressed transgenes.

STRATEGIC PLANNING

Design Principles for Cre-On/Off rAAV Experiments

The Cre-On/Off rAAV systems are ideal for comparing the anatomy or function of intermingled Cre^+ and Cre^- neurons within individual animals. These versatile rAAV systems complement the use of transgenic Cre for cell type specific expression or introduction of Cre through viruses or electroporation for conditional knockout of individual genes. Designing experiments with Cre-On/Off rAAVs requires understanding the factors that are general to all rAAV expression experiments in the brain, in addition to specific caveats of Cre-On/Off rAAVs. We refer the reader to several excellent recent reviews covering the role of rAAV serotype, promoter, and titer (Asokan et al., 2012; Murlidharan et al., 2014). Here we focus on Cre-On/Off experimental design.

Cre Concentration and Expression Behavior of Cre-On and Cre-Off rAAVs

Let us consider the caveats of using Cre-On/Off rAAVs to study Cre^+ and Cre^- neuron populations. Cre-On DIO-rAAVs produce functional transcripts only after Cre-mediated recombination. Thus, DIO expression is functionally "off" until it is "on." Experimentally, this translates to an absence of false positives, as Cre-On expression only occurs in cells with Cre activity. False negative expression—where nuclear Cre concentration is too low to recombine the DIO rAAVs—will be indistinguishable from the nontransduced cells.

Figure 1.24.4 *(image appears on previous page)* Strategies for validating Cre-Off rAAVs. Cre-Off rAAVs are transcriptionally active until Cre mediated recombination inactivates transgene expression. Since Cre-Off efficiency is dependent on Cre concentrations in the nucleus, Cre-Off rAAVs should be validated for each driver line, brain region and developmental period for proper experimental interpretation. We suggest three methods of validation: anatomical, immunohistochemical (IHC), and rAAV Cre-Switch. We summarize these methods with cartoons in which each depicts fully efficient Cre-Off inactivation. The dotted boxes highlight the regions for image analysis. (**A**) Anatomical. When the Cre^+ neuron population has well-described axonal or dendritic anatomy, and the full penetrance of Cre expression in that cell type has been confirmed, the experimenter can test Cre-Off expression through anatomical exclusion. For example, dopamine 1 receptor (D1r) expressing direct pathway spiny projection neurons (dSPNs) contribute the entire axonal projection from the striatum to the Substantia Nigra reticulata (SNr). To determine whether Cre targeted to dSPNs in *D1r-Cre* transgenic mice is sufficient to inactivate rAAV FAS-tdTomato expression, one could assess the density of $tdTomato^+$ axons in the SNr (dotted box) in comparison to a similar injection in a wild-type (WT) control mouse. Efficient inactivation would lead to an absence of $tdTomato^+$ axons in the SNr, but should not affect the Cre^- indirect pathway spiny projection neuron (iSPN) projection to the globus pallidus externus (GP). (**B**) IHC. Many Cre driver lines reproduce endogenous patterns of the driver gene. If antibodies against the driver gene products are available, IHC may be used to quantify exclusion of Cre-Off rAAV expression from IHC immunopositive $marker^+$ cells. Alternatively, IHC can be performed against Cre itself. For example, to determine if FAS-tdTomato expression is inactivated from $PV^{i\text{-}Cre}$ interneurons of the cortex following infection, IHC can be performed against parvalbumin or Cre and degree of co-localization measured with confocal microscopy. Similar experiments in WT controls inform to what degree the lack of co-localization is due to transduction inefficiency rather than inactivation. Importantly, transient Cre expression could inactivate rAAV expression but become undetectable at the time of IHC. (**C**) rAAV Cre-Switch. Even with no anatomical knowledge or marker antibodies for a Cre^+ population, Cre-Switch rAAVs provide a well-controlled system for assaying recombination efficiency. For example, if interested in the cell population expressing *Gene X* in Brain Region Y, one could simply inject the Cre-Switch rAAV DIO-GFP_FAS-tdTomato and quantify the degree of co-localized GFP and tdTomato expression. Since $transgene^+$ cells are typically transduced by more than one rAAV particle, low levels of Cre will recombine some but not all of the Cre-Switch genomes, leading to cells double positive for GFP and tdTomato. High levels of Cre will efficiently recombine all Cre-Switch genomes, leading to non-overlapping GFP^+ and $tdTomato^+$ cells.

In contrast, Cre-Off DO and FAS rAAVs are "on" until they are "off." Thus DO and FAS expression can occur initially in all transduced neurons, and only those cells with sufficient levels of Cre will inactivate rAAV transcription. If Cre expression is low, the chance for false positive expression is high. Moreover, true positives—where Cre effectively inactivates transcription—are indistinguishable from nontransduced cells. In practice, effective Cre-Off expression requires waiting long enough after injection for Cre to recombine "off" all rAAV genomes present in the nucleus and for any protein expressed before recombination to be degraded. This wait period is thus dependent on concentrations of Cre in the nucleus and the half-life of the recombinant protein. For typical experiments involving Cre-Off rAAVs, we typically wait >2.5 weeks.

Since nuclear Cre concentration determines the efficacy of Cre-Off expression, it is worth commenting on common experimental scenarios that affect levels of Cre expression. Inactivation of Cre-Off rAAVs is efficient when Cre expression is high and stable, such as when Cre vectors with strong promoters are introduced through electroporation or viral transduction. Cre expression can be more variable due to bacterial artificial chromosome (BAC) or knock-in alleles, which engage a subset or full complement of genomic regulation associated with the targeted gene. For example, inactivation of Cre-Off rAAVs may be less efficient in animals where Cre is driven off of loci that are regulated by development or neural activity.

Validation of Cre-Off rAAV Systems

To properly interpret Cre-On/Off experiments, Cre-Off rAAV inactivation must be validated in each transgenic line and brain region. Here we describe three imaging-based validation strategies (Fig. 1.24.4) and provide the corresponding protocols below. When possible, we suggest validating with multiple strategies. Strategy choice depends on the anatomical and molecular knowledge of your Cre^+ cell type and reagent availability. It is possible, however, to validate Cre-Off rAAVs knowing nothing about the Cre^+ cell type.

1. *Anatomical validation.* In some brain regions, a particular cell type may be known to completely account for a unique axonal projection. If a fully penetrant Cre driver line exists to target Cre to this cell type, the efficiency of Cre-inactivation following transcranial injection of DO or FAS rAAVs into this region can be judged by the presence (inefficient inactivation) or absence (efficient inactivation) of transgene expression in this axonal projection. For example, dopamine 1 receptor-expressing (D1r) direct pathway spiny projection neurons (dSPNs) are the only striatal cell type to innervate the Substantia Nigra reticulata (SNr). Thus efficient inactivation of Cre-Off rAAVs injected into the striatum of D1r-Cre BAC transgenic mice should lead to no axonal expression in the SNr (Fig. 1.24.4A).
2. *Imunohistochemical (IHC) validation.* Many Cre driver lines target cell types with known IHC markers. Following transcranial injection of DO or FAS rAAVs, post-hoc immunostaining for these molecular markers can be used to validate that transgene expression is excluded from the Cre-expressing cell type. This strategy assumes the driver line has been validated to ensure Cre is expressed selectively in the appropriate cell type. IHC can also be used to visualize Cre itself. In our experience, cells that stain positive for nuclear Cre efficiently inactivate DO or FAS rAAVs. For example, injection of DO or FAS rAAVs into the cortex of a mouse where Cre is expressed from the endogenous *Parvalbumin* locus ($PV^{i\text{-}Cre}$) should lead to expression excluded from PV or Cre immunopositive interneurons (Fig. 1.24.4B).
3. *Cre-Switch rAAV validation.* Cre-Off inactivation can also be assessed in the presence of Cre-On rAAV expression. Highly efficient Cre-mediated inactivation should lead to an absence of Cre-Off and Cre-On expression from occurring in the same cells. While this overlap could be assessed by DIO/FAS rAAV co-injection, each

transduced nucleus could harbor a different number of DIO and FAS genomes leading to recombination and expression bias. To eliminate the bias caused by transduction of multiple viruses, we recommend using the single rAAV Cre-Switch system for differential labeling. In this case, a single type of rAAV genome reports the efficiency of Cre-mediated recombination (Fig. 1.24.4C).

PREPARATION OF Cre-ON/OFF rAAVs FOR INTRACRANIAL INJECTION

BASIC PROTOCOL 1

Typically each rAAV is packaged separately and supplied frozen at high concentration (10^{11} to 10^{13} particles/ml) in 1× PBS with 350 mM NaCl and 5% (w/v) sorbitol. Concentrations are determined by dot blot (viral particles/ml). To perform stereotactic injection with multiple rAAVs, simply mix the rAAVs at the relative concentrations for the desired experiment.

Materials

Optional: 1× PBS (*APPENDIX 2A*) with 350 mM NaCl and 5% (w/v) sorbitol (for dilution)
Microcentrifuge
PCR tubes (individual or strip)
Stereotactic injection setup and pipet (*UNITS 1.20 & 4.35*)

1. Determine the ratio of Cre-On/Cre-Off rAAV particles to combine for desired differential expression.

   This ratio can be estimated from the quantified viral particles/ml or determined experimentally by trial and error, as expression will be affected by promoter, serotype, and other variables. For 1:1 Cre-On/Cre-Off labeling at high density, we suggest starting by combining equal volumes of undiluted Cre-On/Cre-Off rAAVs.

2. Thaw desired volume of Cre-On and Cre-Off rAAVs on ice. Spin down tubes in microcentrifuge.

3. On ice, combine desired volumes of Cre-On and Cre-Off rAAVs. Use either individual or strip PCR tubes, or for larger volumes, microcentrifuge tubes (0.5-ml to 1.5-ml). Pipet up and down to mix.

   Use PBS/NaCl/sorbitol solution to further dilute Cre-On/Cre-Off rAAVs if necessary.

4. Aliquot the mixed rAAVs (typically 2 to 6 μl) into PCR tubes. Spin down in microcentrifuge.

   Store at −80°C until use, avoiding freeze thaw cycles.

5. Before stereotactic injection, thaw a single aliquot of mixed rAAVs and load into injection pipet as described in *UNITS 1.20 & 4.35*.

FIXED TISSUE VALIDATION TECHNIQUES FOR Cre-OFF rAAV EXPRESSION

BASIC PROTOCOL 2

Cre-Off rAAVs express transgenes until recombination inactivates functional transcription. Therefore, Cre-Off behavior must be validated in each preparation for proper experimental interpretation. Here we describe a general protocol for generating slices of fixed brain tissue to analyze Cre-Off expression using: (1) neuronal anatomy, (2) immunohistochemistry (IHC), or (3) Cre-Switch expression.

For details on injection protocols and tissue processing, see *UNITS 1.20 & 4.35*.

Materials

4% paraformaldehyde (PFA; ~10 ml/mouse; see recipe)
1× PBS (*APPENDIX 2A*)
Mounting media (for mounting and imaging tissue sections; e.g., Life Technologies, cat. no. P36935)

Fixative-only dissection tools (for brain removal)
20-ml glass scintillation vials (for brain storage)
24-well plate
Supplies for mounting and imaging tissue sections

Small paint brush
Glass slides
Cover slips
Fluorescent microscope

Additional reagents and equipment for intracardiac perfusion fixation and fixed tissue sectioning (*UNIT 1.1*) and for stereotactic injections (*UNITS 1.20 & 4.35*)

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be followed accordingly.

Experimental design and stereotactic injection of rAAVs

1. Choose an experimental design for validating Cre-Off rAAV transcription in your system, consulting the Introduction and Figure 1.24.4.
2. Perform stereotactic injections using Cre-Off or Cre-Switch rAAVs into your transgenic mouse and area of interest as described in *UNITS 1.20 & 4.35*.

Preparation of fixed tissue brain slices

3. After a post-injection wait time of 2.5 to 14 weeks, perform intracardiac perfusion with 4% PFA according to standard protocols (*UNIT 1.1*). Remove brain and place into scintillation vial containing 4% PFA.
4. Allow brain to post-fix in 4% PFA for 24 to 48 hr at 4°C.
5. Rinse brain three times with PBS
6. Section tissue (40 to 50 μm) using standard protocols. Store sections in a 24-well plate in PBS at 4°C.

Analysis of Cre-Off transcription

7. For purely anatomical or Cre-Switch based validation, mount slices containing region of interest on slides and perform fluorescent imaging.

   For Cre-Switch imaging of GFP/tdTomato co-localization, confocal imaging is recommended.

8. For IHC validation, transfer slices containing the injected region from storage plate to a new 24-well plate for IHC. Place one to seven slices into each well filled with PBS.
9. Perform IHC using standard protocols.

A basic IHC protocol effective for immunostaining Cre recombinase and other widely used antibodies for defining brain cell types is provided in Basic Protocol 3.

10. After IHC, mount slices on slides and perform confocal imaging of rAAV expressed fluorophores and/or immunostained proteins.

11. Determine the degree of co-localization using standard image analysis techniques.

IMMUNOHISTOCHEMISTRY ON FREE-FLOATING BRAIN SLICES

BASIC PROTOCOL 3

Immunohistochemistry (IHC) on free-floating sections is a widely used technique for immunostaining brain tissue. Here we provide a stripped-down protocol which we routinely use for immunodetection of Cre recombinase and commonly used marker proteins for other cell types (e.g., choline acetyltransferase, parvalbumin, and tyrosine hydroxylase). Other IHC protocols may provide equal or better results.

Materials

1× PBS (*APPENDIX 2A*)
Blocking/dilution buffer (see recipe)
Primary antibody (e.g., mouse monoclonal Cre recombinase; Millipore, cat. no. MAB 3120)
Secondary antibody (e.g., donkey anti-mouse Alexa Fluor 647; Life Technologies, cat. no. A-31571)
Mounting media (for mounting and imaging tissue sections; e.g., Life Technologies, cat. no. P36935)
Optional: Nuclear counterstain (for mounting and imaging tissue sections)

24-well plate
Paint brush
Benchtop shaker
Aluminum foil
Supplies for mounting and imaging tissue sections
    Glass slides
    Cover slips
    Fluorescent microscope

1. Select brain sections for immunostaining. Gently transfer the sections to a fresh 24-well plate filled with PBS using a paint brush, including one to seven sections/well.

   All steps are at room temperature unless otherwise noted.

2. To remove fixative, wash slices three times for 5 min with PBS.

   Place plate on benchtop shaker such that slices are gently agitated (60 to 90 rpm).

   Fill the each well three-quarters of the way full with PBS. When removing PBS, tilt plate at an angle and remove solution with a P1000 pipettor or plastic Pasteur pipet, taking care not to accidentally remove any slices. After fresh PBS is added, gently shake the plate to ensure the slices are floating freely and are not stuck to each other before each wash.

3. During the wash steps, prepare the blocking/dilution buffer which will be used for steps 4, 6, and 9. You will need 1.5 ml × wells (500 µl for blocking, 500 µl for primary antibody reaction, and 500 µl for secondary antibody reaction).

   Kept at 4°C, we use the blocking/dilution for up to 2 weeks.

4. Blocking: After final PBS wash, remove all excess PBS, and replace with 500 µl blocking/dilution buffer. Gently shake to ensure slices are freely floating, and place on the benchtop shaker (60 to 90 rpm) for 1 hr.

5. During the blocking step, add the primary antibody to an aliquot of the blocking/dilution buffer.

   The concentration of antibody dilution will depend on the antibody itself. For Cre-recombinase mouse monoclonal antibody (MAB 3120), we use 1:250. The total volume of the blocking/dilution buffer with primary antibody should be 500 µl × number of wells.

6. Primary antibody reaction: Remove the antibody-free blocking/dilution buffer from each of well. Replace with 500 µl of the blocking/dilution buffer containing the primary antibody. Transfer to a benchtop shaker (60 to 90 rpm) at 4°C. Allow reaction to proceed for 24 to 48 hr.

7. Remove the blocking/dilution buffer with primary antibody, and perform three washes for 5 min with PBS.

8. During the PBS washes, add the secondary antibody to an aliquot of the blocking/dilution buffer.

   We typically use a dilution of 1:500 for secondary antibodies, although this concentration can be varied to improve signal and reduce background. The total volume of the blocking/dilution buffer with secondary antibody should be 500 µl × number of wells.

9. Secondary antibody reaction: Following the final wash, remove the PBS. Add 500 µl of blocking/dilution buffer with secondary antibody. Shake to ensure slices are free floating and not stuck to each other. Cover with foil to prevent light exposure, and place on lab shaker (60 to 90 rpm) for 1 to 2 hr depending on the desired intensity of secondary immunolabeling.

10. Remove the blocking/dilution buffer with secondary antibody, and perform three washes for 5 min with PBS.

11. Mount sections on slides using standard protocols, including any necessary counterstaining for nuclei visualization.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A.

Blocking/dilution buffer

To prepare 25 ml, combine 2.5 ml of 10× PBS (pH 8.0), 1.25 ml normal serum from a different species as the secondary antibody (e.g., horse serum when the secondary antibody is raised in goat), 75 µl Triton X-100, and 21.25 ml water.

Other protocols suggest using the serum from the same species as the secondary; in our hands, this strategy has resulted in a loss of immunosignal in a number of cases.

Paraformaldehyde (PFA), 4%

To prepare 50 ml, add 5 ml of 10× PBS (pH 8.0) and 10 ml of 20% paraformaldehyde to a 50-ml conical centrifuge tube. Add remaining 35 ml water.

Store at 4°C for up to 3 days.

COMMENTARY

Background Information

Visualizing the morphology of neurons is at the heart of modern neuroscience, beginning with Golgi and Cajal at the turn of the 20th century. Over the course of the next 100 years, many chemical and protein-based techniques have been used for unbiased tracing of dendrites and axonal projections. The advent of transgenic mice expressing Cre recombinase has allowed for an unprecedented opportunity to explore the anatomy and function of genetically-defined cell types in vivo. In 2008, the DNA-based genome of rAAVs was engineered to activate transcription in the presence of Cre (Atasoy et al., 2008), allowing cell types of particular brain regions and developmental times to be visualized or manipulated following stereotactic injections. While Cre-Off rAAVs were considered along with the initial development of Cre-On rAAVs, only in 2012 were alternative Cre-Off rAAVs engineered to function alongside Cre-On rAAVs (Saunders et al., 2012), and a collection of Cre-Off rAAVs carrying common fluorophore and optogenetic constructs was deposited to Addgene.

Critical Parameters and Troubleshooting

The concentration of nuclear Cre is the single most important parameter to achieve efficient Cre-Off rAAV mediated transcription. In practice, Cre expression can vary widely across transgenic mice, depending on the transgenesis approach (BAC versus knock-in) and locus of expression. In brains, gene expression is highly regulated by development, behavioral state, and neural activity. Therefore, care must be taken to characterize Cre-Off rAAV behavior in light of these factors. Undoubtedly, complete Cre-Off recombination will not be feasible in all situations. Since Cre-Off rAAVs can be used in wild-type animals, it is often helpful to include wild-type controls to compare the efficacy of Cre-off behavior.

Achieving "balanced" Cre-On/Off labeling depends on a number of factors. We consider balanced labeling to mean equal transduction/expression efficiency per Cre^+ or Cre^- neuron. In some brain regions or experiments, Cre^+ cells could make up the vast majority or minority of cells present. This relative density will affect how balanced labeling appears. For example, if the goal is to label with high efficiency a very minor Cre^+ population, it makes sense to increase the relative concentration of Cre-On particles. Of course, the intrinsic molecular differences between Cre^+ and Cre^- cells could also affect labeling through rAAV transduction or expression efficiency, which depend on the rAAV serotype and promoter respectively.

Anticipated Results

We have observed robust Cre-Off rAAV expression when Cre is introduced with another rAAV, through in utero electroporation, or in transgenic animals with both BAC and knock-in alleles. When Cre levels are high enough to be visualized by immunostaining, Cre-Off recombination appears to be highly efficient. In a number of transgenic lines where Cre is driven off of loci with known activity dependence or low expression, Cre-Off recombination has been characterized as incomplete. Users should anticipate that Cre-Off rAAVs should follow these general rules.

Time Considerations

Stereotactic injections can be performed in ~1 hr for each mouse, depending on complexity of the brain region targeted and the number of injections made per animal. Approximately 0.5 hr should be factored in for both preparation and clean up. While rAAV transgene expression can be observed starting around a week after injection, we advise waiting longer (>2.5 weeks) to allow ample time for Cre recombination to inactivate rAAV expression and for residual transgenic protein to be degraded. Animal perfusion takes ~20 min per animal. Brains should be post-fixed for 24 to 48 hr before sectioning. Preparing sections takes 0.5 to 1 hr per hemisphere, depending on the number of sections required and the sectioning method. IHC setup takes approximately 1 hr and includes two reaction steps; the primary antibody reaction usually runs overnight, whereas the secondary antibody reaction lasts 1 to 2 hr. Slide mounting and drying takes 1 to 3 hr. The time frame for imaging depends on the number of cells necessary for assaying Cre-Off transcription. We recommend analyzing co-localization in >200 cells across multiple animals.

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Internet Resources

http://www.addgene.org/Bernardo_Sabatini/

DO, FAS, and Cre-Switch rAAVs available from Addgene.