

## **INTRSECT: targeting specific cell types in the nervous System:**

### **A guide to creating and testing new INTRSECT constructs**

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### **Abstract**

As the power of genetically encoded interventional and observational tools for neuroscience expands, the boundary of experimental design is increasingly defined by limits in selectively expressing these tools in relevant cell types. Single recombinase-dependent expression systems have been widely used as a means to restrict gene expression based on single features by combining recombinase-dependent viruses with recombinase-expressing transgenic animals. This protocol details how to create INTRSECT constructs and use multiple recombinases to achieve targeting of a desired gene to subsets of neurons that are defined by multiple genetic and/or topological features. This method includes the design and utilization of both viruses and transgenic animals: these tools are inherently flexible and modular and may be used in different combinations to achieve the desired gene expression pattern.

### **Keywords**

Optogenetics; neuroscience; virus; INTRSECT; synthetic biology

## **INTRODUCTION**

Understanding the contribution of neuronal subtypes to neural circuit function is a core goal of neuroscience (Crick, 1979). The expansion of genetically-encoded tools for assaying both the necessity and sufficiency of defined cell types with temporal precision through optical modulation (optogenetics (Boyden et al., 2005; Fenno et al., 2011; Yizhar et al., 2011)) has enabled the mapping of neuronal subtype activity onto circuit dynamics and behavior. As the neuroscience toolbox continues to expand in its breadth and power, scientific questions are increasingly bound by limits in selectively modulating defined populations of neurons.

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The use of phage P1-derived Cre (Sternberg et al., 1981) recombinase and the Double Inverted Open Reading Frame (DIO) Cre-dependent expression system (Atasoy et al., 2008; Tsai et al., 2009) has proven to be a powerful and diverse strategy for restricting gene expression to defined cell types within the mammalian brain. Genetic cell-type (Luo et al., 2008) specificity may be achieved with high acuity through genome engineering to create transgenic animals expressing Cre recombinase within a defined cellular population and viruses encoding Cre-inducible genetic payloads (Atasoy et al., 2008; Saunders et al., 2012; Tsai et al., 2009). Notably, this approach dissociates the targeting and delivery modalities, capitalizing on both the genetic specificity achievable via transgenic animals and the flexibility of genetic payload achievable using viruses. However, this approach is limited in that cells may only be targeted based on a single genetic parameter, whereas in many cases a meaningful definition of cell type would include multiple genetic parameters, or would include both genetic and topologic features.

To overcome these limitations, we have created a system termed INTRSECT (Fenno et al., 2014), for ‘intronic recombinase sites enabling combinatorial targeting’. INTRSECT includes single vectors containing engineered introns, enabling expression that is conditional upon multiple recombinases (e.g. both Cre recombinase and the yeast-derived FLP (McLeod et al., 1986) recombinase), and which therefore may be conditional upon multiple cell-type features (genetic and/or topologic). Furthermore, INTRSECT enables expression based on Boolean logic as determined by the presence or absence of multiple recombinases.

The Basic Protocol provides a roadmap for designing and implementing INTRSECT targeting to express a gene of interest in a cell population positively defined by two genetic features (genetic marker X AND Y). The Alternate Protocols describe targeting a cell population positively defined by genetic and topologic features (genetic marker AND projection), targeting cell populations using different Boolean operations (e.g. AND NOT), and creating INTRSECT constructs for genes with a single open reading frame (ORF). The Support Protocols further detail testing INTRSECT reagents by transfection in cultured cells, directly examining intron splicing by reverse transcription PCR (RT-PCR), analyzing specificity and activity of constructs by flow cytometry, and assaying the identity of virus-expressing cells by immunohistochemistry of sectioned brain samples.

## **BASIC PROTOCOL 1**

### **INTRSECT targeting of genetic marker X AND genetic marker Y with a fusion gene**

The selective expression of genetic material in pre-specified cell types using recombinase-based targeting has become a backbone of interventional biological studies, especially in the mammalian central nervous system. A key advantage of recombinase-based strategies is the separation of the targeting modality and the gene to be expressed: a single genetically modified animal expressing a recombinase in a given cell type presents theoretically limitless experimental opportunities, as any genetic material may be introduced into the targeted cell type.

Where multiple recombinase-expressing animal lines are combined with viral gene delivery, the production of targeting vectors requires careful, but routine, engineering. Here we

outline the steps involved in producing constructs for use with the INTRSECT targeting system. Before beginning this protocol, a researcher must determine which genetically encoded tool or material is to be expressed, the number of separate reading frames, and the logic that they wish to use for expression (Cre AND Flp, Cre NOT Flp, Flp NOT Cre; referred to throughout as  $C_{on}/F_{on}$ ,  $C_{on}/F_{off}$ , and  $C_{off}/F_{on}$ ). This Basic Protocol details the production of a double reading frame (e.g. ChR2-YFP) INTRSECT construct governed by AND logic (requiring both Cre AND Flp for expression). Alternative methods for producing double reading frame constructs for AND NOT logic as well as the production of INTRSECT constructs for single reading frame genes are detailed in alternative protocols. Note that a growing number of INTRSECT-compatible constructs are freely available for many optogenetic tools (<http://www.optogenetics.org>).

#### **Materials**—Molecular cloning software

- a. Options include commercial (VectorNTI, SnapGene), free (ApE), and cloud-based ([www.everyvector.com](http://www.everyvector.com)) software

#### INTRSECT backbone vector and sequence

- a. Available from [www.optogenetics.org](http://www.optogenetics.org)

#### Full sequence of gene of interest

#### Recombinase expression constructs

#### Standard molecular cloning reagents.

#### E-Coli suitable for cloning constructs with repetitive sequence (Stb13)

- a. The AAV backbone used for cloning INTRSECT constructs and making AAV has long, repetitive sequences that may impair construct stability in bacteria able to homologously recombine DNA.

#### Endotoxin-free large scale DNA preparation kit

- a. Endotoxin-free DNA is important for primary neuron transfection and virus production

#### Cultured mammalian cell line of interest (HEK-293, primary neuronal cultures, etc)

#### Transfection reagents (Lipofectamine, CaPO<sub>4</sub>, etc).

#### Cre;Flp double transgenic driver mice

- a. Mice may be acquired from many sources, including the Jackson Labs ([www.jax.org](http://www.jax.org)), the GENSAT project ([www.gensat.org](http://www.gensat.org)), and the Allen Institute ([www.brain-map.org](http://www.brain-map.org))

#### Cre-conditional (cDIO) and Flp-conditional (fDIO) adeno-associated viruses

- a. Many options are available at <http://www.optogenetics.org>

**Protocol**—The cloning protocol is divided into construct design *in silico*, molecular cloning, *in vitro* testing, and *in vivo* testing. The *in silico* design work differs between single and double ORF genes, but the remaining portions are identical for both approaches.

**Construct design in silico**—The first step is picking optimal sites within the ORF for intron insertion and creating a map of the construct that will guide primer design during molecular cloning. Good intron sites minimize alternative splicing and separate the construct into three exons, none of which are able to express functional protein on their own, which ensures that the gene payload will only be expressed in the presence of both Cre AND Flp.

1. Import all gene sequences into your molecular biology cloning software of choice.
2. Ensure that your construct has two separate open reading frames (ORFs). For instance, fusion genes that link fluorescent proteins onto other genes (e.g. Chr2-YFP) are double ORF genes. A single fluorophore (Yellow Fluorescent Protein alone) is a single ORF gene.
  - a. See alternate protocol 2 for design and production of double ORF AND NOT constructs and alternate protocol 3 for single ORF INTRSECT construct design and production.
3. Open the sequence file of your gene of interest and isolate the ORF (Figure 1a; including START and STOP codons). The sequence will be divided into three fragments that will be fused with portions of the intron sequence and will be ligated into the INTRSECT backbone using these restriction sites:

	Cre AND Flp	Restriction Sites
Site 1	Exon 3	Ascl; Sall
Site 2	Exon 2	SpeI; KpnI
Site 3	Exon 1	NheI; XbaI/SacI

- a. Note that Exon 1 and Exon 3 should contain only sequence derived from the first and second ORF, respectively, while Exon 2 should contain material from both the first and second ORF, including the gene fusion site (Figure 1b). This strategy helps to ensure that no single exon will contain a functional ORF.
- b. Consider whether fragments of either ORF will function in isolation and avoid including these in their entirety within any single exon.
- c. It is prudent to ensure that exon fragments do not contain the restriction sites that will be used for ligation of that fragment.
- d. Constructs with the EF1a or nEF promoter use XbaI, constructs with the hSyn promoter use SacI.

- e. The INTRSECT cloning cassette is designed for modular cloning (Figure 2a):

BamHI | **Flp Cassette** | AscI | **Exon 1** | SalI | **Cre Cassette** | SpeI | **Exon 2** | KpnI  
| **Cre Cassette** | NheI | **Exon 3** | XbaI/SacI | **Flp Cassette** | EcoRI

4. Intron placement is dependent on a number of parameters, including natural restriction site locations within the sequence, the functionality of protein fragments, and the influence of coding sequence on splicing fidelity.
- a. The consensus sequences for splicing (Gao et al., 2008; Mount, 1982; Zhang, 1998) are:

Exon	Intron	Exon
(A/C)AG	GT(A/G)AGT.....(C/T)11AG	G

- b. For the exon splice site, either A or C will work, but C is generally scored higher in splice prediction programs.
5. There are two intron insertions: one into the first reading frame and one into the second reading frame (Figure 1c). For each ORF of the fusion protein, take into consideration your criteria from Step 3 (restriction sites and fragment functionality), and pick three of these 4bp sites for each intron (candidate sites).
6. Copy and paste the intron sequence into each of the candidate sites.
- a. The intron for the first gene is derived from the CMV IE gene (Chapman et al., 1991) and is the following sequence:
- b. 5' -  
gta**A**gtgtcggggttgtgccccctttttataaaattgtattaatgttatatacatatctcctgatgtg  
acctatgtcttatgactctatttctcatgtttag - 3'
- c. The highlighted **A** is a change that was made from the native sequence during intron optimization that was found to significantly enhance functional expression
- d. This intron is inserted into the first gene between the two G's: (A/C)AG | **insert intron here** | G
7. Once you have pasted this sequence into the gene, copy the entire reading frame (START to STOP), including the inserted intron, and paste it into <http://www.cbs.dtu.dk/services/NetGene2/> (Brunak et al., 1991). You will get an output that looks like this (Figure 1d):
8. The donor site will be the exon sequence on the upstream, 5' side of the inserted intron and should end with (a/c)AG. The acceptor site will be the exon sequence on the 3' side of the inserted intron and should begin with G. In evaluating the output of the analysis, you want to take into consideration two things:

- a. That the site you have inserted the intron into gives the **highest confidence** value of any options (this is an analysis of ChR2-YFP with two introns: the donor confidence values are 0.95 and 1.00, while the acceptor confidence values are 0.93 and 0.83).
  - b. That your insertion site **minimizes alternative splice sites**, as indicated by the number of alternative options and their confidence values.
9. After running the analysis for the first insertion site, note the confidence values of the acceptor and donor for the correct splicing option. Repeat steps 6–8 with the other candidate sites. Choose the option with the highest confidence value; if more than one choice exists, choose the one that generates the fewest number of high-value alternatively spliced products.
  10. Next, repeat the process with the second intron (Ishida et al., 1982) (into the second ORF):
    - a. 5' -  
 gtgagtacaggaggtggagagtggccagcccttctcatgttcagagaacatggtaactggtaagtc  
 atgctgtcccacag - 3'
  11. Once you have identified the most appropriate sites for the two introns, create a vector map that has both of these inserted into the ORF. This will be used in the next section to aid in primer design for cloning the INTRSECT construct.

**Molecular cloning**—Having now created a map of your construct with artificially inserted intron sequences, the next step is to attach portions of the introns that must be directly fused to the exon sequence and attach restriction sites that will facilitate cloning into the INTRSECT backbone. This is usually accomplished using overlapping PCR, but you may also build the entire construct *in silico* and have it synthesized. As the price of *de novo* synthesis has decreased, having the construct synthesized from scratch may be more economical for labs that are interested in making a single INTRSECT construct or that have less experience with molecular cloning. Many labs take a combined approach, where the fragments are synthesized *de novo*, but then cloned into the standard restriction sites in order to make constructs with different logical gates, but containing the same payload. Some DNA production companies may have difficulty synthesizing portions of the INTRSECT backbone that contain stretches of repeated sequence. Note that the final construct will have the exons rearranged and with some in the anti-sense (reverse complement) orientation (Figure 2a):

	$C_{on}/F_{on}$	Restriction Sites
<b>Site 1</b>	Exon 3 (anti-sense)	5' – Ascl; 3' – Sall
<b>Site 2</b>	Exon 2 (sense)	5' – Spel; 3' – KpnI
<b>Site 3</b>	Exon 1 (anti-sense)	5' – NheI; 3' – XbaI/SacI

12. The following fragments will be created (all sequences in sense orientation; Figure 2b):

- a. 5' – XbaI/SacI | Exon 1 | Intron 1 donor | NheI – 3'
- b. 5' – SpeI | Intron 1 acceptor | Exon 2 | Intron 2 donor | Kpn I – 3'
- c. 5' – SalI | Intron 2 acceptor | Exon 3 | AscI – 3'
- d. The intron fragments **must be fused directly** to your exon sequence (Figure 2c). There can be **no intervening sequence** between the exon and intron sequences.
- e. Note that Exon 1 must contain both the Kozak sequence and Start codon (commonly, GCCACC and ATG, respectively; Figure 2c)
13. The exon sequence **must** be split from the complete gene sequence at the following sites previously identified as splice donor/acceptor sites:
- a. 5' - Exon 1 (A/C)**AG** | intron 1 donor – 3'
- b. 5' - Intron 1 acceptor | G Exon 2 (A/C)**AG** | intron 2 donor – 3'
- c. 5' - Intron 2 acceptor | G Exon 3 – 3'
- d. Note that the G of Exon 2 corresponds to the last nucleotide of the four base sequence identified between Exons 1 and 2 (and the same is true for Exons 2 and 3).
14. Amplify individual exons by PCR using primers that are suitable for the overlapping addition of the intron sequences (Figure 2c; restriction site sequences underlined):
- a. **Intron 1 donor (15 bp):** 5' - gtaAgtgtcggggttGCTAGC – 3'
- b. **Intron 1 acceptor (97 bp):** 5' - ACTAGTtgtgcccccccttttttataaaattgtattaatgtatatacatatctcctgtatgtgacccatgtgcttatgactctatttctcatgtgttag – 3'
- c. **Intron 2 donor (14 bp):** 5' - gtgagtacaggaggGGTACC – 3'
- d. **Intron 2 acceptor (68 bp):** 5' - GTCGACtggagagtggccagcccttctcatgttcagagaacatggttaactggttaagtcatgtctcccacag – 3'
- e. The addition of the intron segments to your exons will form the basic units for any logical targeting construct; however, INTRSECT constructs with other logic gates will require the addition of different restriction sites (see Additional Protocol 2).
15. Ligate these fragments into the INTRSECT backbone using standard restriction enzyme digestion and ligation (Figure 2d).
- a. See <https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finderfordoubledigestguidelines>.
- b. Transform ligation reactions into Stbl3 or other suitable homologous recombination-deficient E-coli

16. Sequence entirety of completed construct, paying special attention to exon/intron fusion sites, presence of recombinase recognition cassettes, and modified directionality of exons 1 and 3.
17. Prepare endotoxin-free large scale purified stock of completed INTRSECT construct.

### In vitro

18. To directly test your construct in culture (Figure 3), prepare four wells of a mammalian cell line (we use both HEK293 and primary neuronal cultures) and transfect with the following combinations of recombinases and your construct. Following transfection reagent manufacturer guidelines, determine the correct amount of DNA for the triple (INTRSECT construct, Cre, and Flp) transfection condition for equimolar concentrations of each. Use the **same amount** of DNA in the other conditions (e.g. if you use 100ng INTRSECT, 150ng Cre, and 200ng Flp for the “+Cre AND Flp” transfection, also use 100ng INTRSECT for the “alone” condition).
  - a. INTRSECT construct without recombinase (“alone”)
  - b. INTRSECT construct + Cre recombinase (“+Cre”)
  - c. INTRSECT construct + Flp recombinase (“+Flp”)
  - d. INTRSECT construct + Cre recombinase + Flp recombinase (“+Cre AND Flp”)
  - a. We use Lipofectamine 2000 for HEK293 cells and CaPO4 for primary neuronal cultures
  - b. Fluorophore- or epitope-tagged recombinases are useful for post-hoc analysis of recombinase efficiency and co-localization.
19. With fusion opsin-fluorophore constructs we generally see expression within 6–8 hours after transfection using a standard epifluorescence microscope.
20. Expression should only be apparent in triple transfected (construct + Cre + Flp; **Figure 3b,c**) condition.
21. Splicing efficiency may be directly evaluated by RT-PCR (Figure 3a; see Support Protocol)
22. Recombination efficiency and specificity may be assayed by flow cytometry analysis (Figure 3b; see Support Protocol)
23. It is recommended that gene-appropriate *in vitro* testing be carried out prior to *in vivo* testing (Figure 3d).

### In vivo testing

24. Produce Adeno-Associated Virus (AAV) using your INTRSECT construct

- a. See Critical Parameters for additional information about promoters, viral serotypes, and viral production.

### **Perform positive control: targeting the genetically-defined cell populations**

25. In Cre;Flp double transgenic animals, inject one animal with a fluorophore-expressing Cre-conditional adeno-associated virus (e.g. AAV-cDIO-YFP) and a fluorophore-expressing Flp-conditional adeno-associated virus (e.g. AAV-fDIO-mCherry) using standard stereotactic neurosurgical techniques in compliance with IACUC and all applicable institutional regulations.
  - a. Parameters will vary based on viral titer and the size of the target region, but a typical injection would use ~500nL (for a viral titer on the order of  $10^{12}$ ).
  - b. It is advisable that all viruses are matched in viral titer, serotype, and promoter in order to minimize variability in expression pattern.
26. Wait for approximately 1–4 weeks for viral expression.
  - a. The optimal time to wait for expression is dependent upon multiple factors including viral titer, serotype, and promoter, and will therefore need to be empirically determined in each instance.
27. Confirm the presence of fluorophore-expressing cells in the target region and perform immunostaining for the marker of interest to confirm that the driver line is highly specific in the target region.

### **Target the gene A AND gene B-defined cell population**

28. Inject AAV with INTRSECT payload into the region of interest within double transgenic animals. Inject identical volume of virus into the same location in single transgenic Cre and Flp animals, as well as a wild-type animal as negative controls.
29. Wait for approximately 1–4 weeks for viral expression.
  - a. Expression times will differ as noted in step 24.
  - b. We recommend examining expression time with new constructs directly using a time series with multiple animals at regular intervals (weekly).
30. Confirm the presence of viral expression in the region of interest using appropriate visualization technique (e.g. identification of genetically encoded fluorophore expression, immunohistochemistry, etc; Figure 3e).
31. Perform double immunohistochemistry for expression of the genes of interest and quantify co-expression with viral expression to confirm that the expression is specific to cells with the desired genetic expression profile (see Support Protocol).

## ALTERNATE PROTOCOL 1

### INTRSECT targeting of genetic marker X AND downstream projection Y

Projection-based targeting strategies enable the expression of genetically encoded tools based on the downstream (efferent) connectivity of a neuron (Lammel et al., 2013; Luo et al., 2008; Wickersham et al., 2007). Integrating INTRSECT with this approach enables the selection of neurons based on both genetic and projection-based criteria. For example, targeting the dopaminergic cells in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc) requires an intersectional strategy as neither all dopaminergic VTA neurons (that project to numerous downstream areas) nor the VTA-NAc projection (that includes both dopaminergic and non-dopaminergic cells (Van Bockstaele and Pickel, 1995; Xia et al., 2011)) exclusively describe this population.

As detailed below, this intersectional targeting may be achieved by combining transgenic animals, retrograde viruses, and multiple recombinases. Continuing the example of targeting dopaminergic VTA cells projecting to the NAc, the transgenic animal is used to define the genetic parameter, with expression of one recombinase (Cre) restricted to cells expressing the genetic marker of interest (Tyrosine Hydroxylase::Cre, expressing recombinase in dopaminergic cells). The retrograde virus is used to define the projection-based parameter: it is injected into the downstream region of interest where it delivers the second recombinase (injecting virus expression Flp in the NAc). The final component is a conditional virus injected directly into the target region of interest (C<sub>on</sub>/F<sub>on</sub> in the VTA). Notably, this same expression pattern may alternatively be achieved using a different strategy (Figure 4a) (Fenno et al., 2014; Stamatakis et al., 2013), in which the recombinase delivered via the retrograde virus is configured such that it is activated only in the presence of the genetically-expressed recombinase (e.g. cDIO-Flp) and the conditional virus is then activated by the second recombinase alone (e.g. fDIO). In theory, these two approaches should result in the same ultimate expression pattern, and experimenters may choose whichever is more convenient in practice.

The protocol below assumes that the transgenic animal expresses Cre recombinase and that the retrograde virus expresses Flp recombinase. In practice, any combination of compatible recombinases may be used per the experimenter's preference.

#### Materials—Cre transgenic driver mice

- a. Mice may be acquired from many sources, including the Jackson Labs ([www.jax.org](http://www.jax.org)), the GENSAT project ([www.gensat.org](http://www.gensat.org)), the Allen Institute ([www.brain-map.org](http://www.brain-map.org))

#### Flp-expressing retrograde virus

- a. Obtain a retrograde virus (e.g. LT-HSV (Kim et al., 2013)) encoding Flp recombinase along with a fluorophore to enable easy identification of expressing cells.
- b. As an alternative to creating a fusion protein (e.g. Flp-mCherry), it may be preferable to separate the fluorophore from the recombinase using an IRES

sequence (e.g. mCherry-IRES-Flp or mCherry-p2a-Flp), resulting in separate translation of the two proteins and possibly leading to higher activity levels of the recombinase.

Cre- and Flp-conditional ( $C_{on}/F_{on}$ ) adeno-associated virus (AAV) encoding gene of interest

- a. See Basic Protocol 1

Cre-conditional (cDIO) adeno-associated viruses

- a. Many options are available at <http://www.optogenetics.org>

### **Perform positive control #1: targeting the genetically-defined cell population**

1. Follow steps 24–26 in Basic Protocol 1 using the Cre-driver mouse and the cDIO virus
2. Confirm the presence of fluorophore-expressing fibers in the downstream region of interest (Figure 4b).
  - a. If no such fibers are seen, then the genetically-targeted cells may not include those that project to the region of interest. This may be due to deficiency in the transgenic animal (which may not express Cre in the desired cells), the viral serotype (which may have a tropism that excludes the desired cells), the viral promoter (if a non-ubiquitous promoter is used), or the specific targeting coordinates.

### **Perform positive control #2: targeting the topologically-defined cell population**

3. In wild-type animals, inject a fluorophore-expressing Flp-conditional adeno-associated virus (e.g. AAV-fDIO-YFP) in the target region (where the cell bodies are located).
4. In the same animals, inject a Flp-expressing retrograde virus (e.g. LT-HSV-mCherry-IRES-Flp) in the downstream region (Figure 4c).
5. Wait for approximately 1–4 weeks for viral expression.
  - a. The optimal time to wait for expression is dependent upon the retrograde virus type, the distance between the target and downstream regions, as well as the multiple additional variables detailed above. It will again need to be empirically determined in each instance.
6. Confirm the presence of mCherry- and YFP-expressing cell bodies in the target region, as well as YFP-expressing fibers in the downstream region.
  - a. If no YFP-expressing cells are seen in the target region, the presence or absence of mCherry-expression can guide the troubleshooting process. The absence of mCherry expression would imply a failure to retrogradely infect cells in the target region, a failure to wait long enough for expression of the retrograde virus, or possible toxicity of the retrograde virus. The presence of mCherry would imply possible

mistargeting of the AAV, or a failure to wait long enough for expression of the AAV.

7. Perform immunostaining for the genetic marker of interest, and quantify co-expression with YFP to confirm that the projecting cells include those with the desired genetic marker.

#### **Perform negative control #1: no recombinase in the genetically-defined cell population**

2. In wild-type animals, inject a Cre- and Flp-conditional ( $C_{on}/F_{on}$ ) INTRSECT adeno-associated virus (AAV) in the target region (where the cell bodies are located).
3. In the same animals, inject a Flp-expressing retrograde virus (e.g. LT-HSV-mCherry-IRES-Flp) in the downstream region.
4. Wait for approximately 1–4 weeks for viral expression (as per above).
5. Confirm the absence of YFP expression.

#### **Perform negative control #2: no recombinase in the topologically-defined cell population**

8. In Cre-driver animals, inject a Cre- AND Flp-conditional ( $C_{on}/F_{on}$ ) adeno-associated virus (AAV) in the target region (where the cell bodies are located).
9. Wait for approximately 1–4 weeks for viral expression (as per above).
10. Confirm the absence of YFP expression.

#### **Target the genetically AND topologically-defined cell population**

11. In Cre-driver animals, inject a Cre- AND Flp-conditional ( $C_{on}/F_{on}$ ) adeno-associated virus (AAV) in the target region (where the cell bodies are located).
12. In the same animals, inject a Flp-expressing retrograde virus (e.g. LT-HSV-mCherry-IRES-Flp) in the downstream region (Figure 4d).
13. Wait for approximately 1–4 weeks for viral expression (as per above).
14. Confirm the presence of mCherry- and YFP-expressing cell bodies in the target region.
15. Confirm the presence of YFP-expressing fibers in the downstream region.
  - a. An alternative approach to confirm that the expressing cells are projecting to the downstream region of interest would be to employ CLARITY (Chung et al., 2013) in order to perform whole-brain imaging and projection tracing.
16. Perform immunostaining for the marker of interest, and quantify co-expression with YFP to confirm that the expression is specific to cells with the desired genetic marker (Figure 4a).

## ALTERNATE PROTOCOL 2

### INTRSECT targeting of genetic marker X AND NOT genetic marker Y with a fusion gene

As an extension of Basic Protocol 1, we here outline the steps involved in producing double open reading frame constructs for use with the INTRSECT targeting system to be selectively expressed in cells that express one recombinase (but not the other recombinase nor both recombinases; Figure 5).

#### Materials

- a. See Basic Protocol 1

#### Molecular Cloning Protocol

1. Follow Basic Protocol 1 Steps 1 and 2
2. Open the sequence file of your gene of interest and isolate the ORF. The sequence will be divided into three fragments that will be fused with the intronic sequence and recombinase recognition sites as follows, using the indicated restriction sites:

	$C_{on}/F_{off}$	$C_{off}/F_{on}$	Restriction Sites
<b>Site 1</b>	Exon 1 (sense)	Exon 3 (anti-sense)	5' – AscI; 3' – SalI
<b>Site 2</b>	Exon 2 (anti-sense)	Exon 2 (anti-sense)	5' – SpeI; 3' – KpnI
<b>Site 3</b>	Exon 3 (sense)	Exon 1 (anti-sense)	5' – NheI; 3' – XbaI/SacI

- a. Observe suggestions from Step 2 of Basic Protocol 1
3. Follow Basic Protocol 1 Steps 3 through 11
4. Generate three fragments, which will differ depending on the logic of expression:
  - a. For Cre AND NOT Flp constructs
    - i. 5' – AscI | Exon 1 | Intron 1 donor | SalI – 3'
    - ii. 5' – KpnI | Intron 1 acceptor | Exon 2 | Intron 2 donor | SpeI – 3'
    - iii. 5' – NheI | Intron 2 acceptor | Exon 3 | XbaI/SacI – 3'
  - b. For Flp AND NOT Cre constructs
    - i. 5' – XbaI/SacI | Exon 1 | Intron 1 donor | NheI – 3'
    - ii. 5' – KpnI | Intron 1 acceptor | Exon 2 | Intron 2 donor | SpeI – 3'
    - iii. 5' – SalI | Intron 2 acceptor | Exon 3 | AscI – 3'

- c. The following intron pieces **must be fused directly** to your gene. There can be **no intervening sequence** between the exon and intron sequences
  - d. See Basic Protocol 1 Step 14 for intron sequences. **Note that restriction site sequence (underlined) will differ as indicated above.**
5. Follow Basic Protocol 1 Steps 15–30

## ALTERNATE PROTOCOL 3

### INTRSECT targeting of genetic markers with a non-fusion gene

To compliment Basic Protocol 1, we here outline the steps involved in producing single open reading frame constructs for use with the INTRSECT targeting system to be selectively expressed in cells based on recombinase expression patterns. The major difference between the design of constructs with a single reading frame and constructs with multiple reading frames is that only a single intron is inserted into single ORF constructs. In this case, the direction of gene fragments 5' and 3' of the intron are independently manipulated by separate recombinases that do not have nested recombinase recognition cassettes.

#### Materials

- a. See Basic Protocol 1

#### Molecular Cloning Protocol

1. Follow Basic Protocol 1 Step 1
2. Ensure that your construct has one single ORF. For instance, adapting a genetically encoded fluorophore (such as Yellow Fluorescent Protein) to INTRSECT-based expression. For constructs containing two independent reading frames (e.g. Chr2-EYFP), see Basic Protocol 1 and Alternate Protocol 2.
3. Open the sequence file of your gene of interest and isolate the ORF. The sequence will be divided into two fragments that will be fused with the intronic sequence and recombinase recognition sites as follows, using the indicated restriction sites:

	C <sub>on</sub> /F <sub>on</sub>	C <sub>on</sub> /F <sub>off</sub>	C <sub>off</sub> /F <sub>on</sub>	Restriction Sites
Site 1	Exon 1	Exon 1	Exon 1	5' – SacI; 3' – NheI
Site 2	Exon 2	Exon 2	Exon 2	5' – AclI; 3' – XbaI

- a. Note that the two exons should be chosen to ensure that neither exon in isolation will contain a functional protein fragment.
- b. It is prudent to ensure that exon fragments do not contain the restriction sites that will be used for ligation later on.
- c. The INTRSECT cloning cassette is designed for modular cloning:

BamHI | **Cre Cassette** | Sall | **Site 1** | NheI | **Cre Cassette** | KpnI | **Flp Cassette** | AscI | **Site 2** | XbaI | **Flp Cassette** | EcoRI

4. Locate a suitable insertion site for the single intron necessary for this approach by following Basic Protocol 1 Steps 4–11. Use the intron described in Basic Protocol 1 Step 6.

### Molecular Cloning

5. See introduction to *Molecular Cloning* in Basic Protocol 1
6. The following fragments will be created, with restriction sites as noted:
  - a. 5' Restriction Site | Exon 1 | Intron 1 donor | 3' Restriction Site
  - b. 5' Restriction Site | Intron 1 acceptor | Exon 2 | Intron 2 donor | 3' Restriction Site
  - c. The following intron pieces **must be fused directly** to your gene. There can be **no intervening sequence** between the exon and intron sequences

	C <sub>on</sub> /F <sub>on</sub>	C <sub>on</sub> /F <sub>off</sub>	C <sub>off</sub> /F <sub>on</sub>
<b>Site 1</b>	Exon 1 (anti-sense)	Exon 1 (anti-sense)	Exon 1 (sense)
<b>Restriction Sites</b>	5': NheI 3': Sall	5': NheI 3': Sall	5': Sall 3': NheI
<b>Site 2</b>	Exon 2 (anti-sense)	Exon 2 (sense)	Exon 2 (anti-sense)
<b>Restriction Sites</b>	5': XbaI 3': AscI	5': AscI 3': XbaI	5': XbaI 3': AscI

7. The exonic sequence **must** be split at the following sequences that you previously identified as splice donor/acceptor sites:
  - d. 5' – restriction site 1 | Exon 1 (A/C)**AG** | intron 1 donor | restriction site 2 – 3'
  - e. 5' – restriction site 1 | Intron 1 acceptor | G Exon 2 | restriction site 2 – 3'
  - f. Note that the G of Exon 2 corresponds to the last nucleotide of the four base sequence identified between Exons 1 and 2.
8. Amplify
  - a. **Intron 1 donor (15 bp):** 5' - gtaAgtgtcggggtt**GCTAGC** – 3'
  - b. **Intron 1 acceptor (97 bp):** 5' - ACTAGTtgtgccccccctttttataaaattgtattaatgtatatacatatctcctgtatgtgacccatgtgcttatgactctattctcatgtgttag – 3'
  - c. The addition of the intron segments to your exons will form the basic units for any logical targeting construct—consider adding the intron fragments to the exons as a first round of PCR and adding the

restriction sites as a second round of PCR if you will be creating more than one expression logic with the same payload.

9. Complete INTRSECT construct cloning by following Basic Protocol 1 Steps 15–17.

### Testing and validation

10. Follow Basic Protocol 1 Steps 18–30

## SUPPORT PROTOCOLS

### IMMUNOHISTOCHEMISTRY

The procedures in this protocol can be used to immunolabel thin (~40µm) brain slices with antibody, in order to identify cells expressing a given marker. Note all percentages are given as volume:volume.

**Materials**—Normal Donkey Serum (NDS; Jackson ImmunoResearch 017–000-121)

Primary antibody

Secondary antibody (Jackson ImmunoResearch)

DAPI (optional)

PVA-DABCO (Sigma 10981)

Slides (Superfrost Plus recommended; VWR 48311–703) and coverslips (VWR 48393 059)

Triton X-100 (Sigma X100)

1x PBS

4% paraformaldehyde (PFA)

30% sucrose in 1x PBS

- a. Gentle heating and stirring are required to dissolve sucrose; do not boil
- b. Filter sterilize 0.2µm

Freezing microtome (Leica SM2000 R)

Cryoprotectant (25% glycerol, 30% ethylene glycol, 45% 1x PBS, pH 6.7)

- a. Filter sterilize 0.2µm

### Prepare thin slices

1. Following virus injection and empirically determined expression time, anesthetize and perfuse the animal transcardially with 20mL ice-cold 1x PBS followed by 20mL 4% paraformaldehyde (PFA) and carefully dissect out the brain.

2. Post-fix the brain overnight in PFA at 4°C
3. Equilibrate in sterile 30% sucrose for at least 24 hours (until the brain sinks to the bottom of the tube).
4. Section tissue at 40  $\mu\text{m}$  using a freezing microtome. Collect the slices surrounding the regions of interest and store in sterile cryoprotectant.

#### Immunostain slices

5. Wash slices 3 times for 5 min in 1x PBS.
6. Block/Permeabilize for 30 min in 1x PBS with 0.3% Triton-X and 3% NDS
7. Incubate in primary antibody diluted in 1x PBS with 3% NDS. Optimal incubation conditions differ based on the antibody, but a typical incubation is overnight at 4°C.
8. Wash slices 3 times for 10 min in 1x PBS
9. Incubate in secondary antibody diluted in 1x PBS with 3% NDS. Optimal incubation conditions will differ based on the antibody, but a typical incubation is 1–3 hours at room temperature.
  - a. Choose a fluorophore that is spectrally separable from those expressed by the injected viruses. For example, if the retrograde virus includes mCherry and the INTRSECT AAV includes YFP, a far-red secondary antibody would be appropriate.
10. Wash slices 3 times for 10 minutes in 1x PBS
  - a. You have the option of including DAPI (1:50,000; or other pan-cellular stain) in the second to last wash if nuclear labeling is desired.
11. Mount sections on superfrost+ slides and coverslip using PVA-DABCO. Dry thoroughly at room temperature, and image.
  - a. We prefer PVA-DABCO over other mounting medias due to the stability of fluorophores in this media.

#### INTRSECT construct analysis using RT-PCR

The procedures in this protocol can be used to analyze the splicing efficiency of all INTRSECT constructs.

**Materials**—Cultured cell line (we use HEK cells; ATCC CRL-1573 or CRL-3216; ThermoFisher R70007)

INTRSECT construct ([www.optogenetics.org](http://www.optogenetics.org))

Recombinase expression constructs ([www.optogenetics.org](http://www.optogenetics.org))

Transfection reagents (Lipofectamine; ThermoFisher 11668030)

mRNA isolation and RT-PCR reagents (SuperScript III One-Step; ThermoFisher 12574018)

Direction-specific primers

Ice bucket

Standard PCR reagents (Platinum Taq DNA Polymerase; ThermoFisher 10966018)

Gel purification reagents (optional; Qiagen 28704)

Sequencing primers (optional)

### **Transfect and prepare cultured cells**

1. Design direction-specific oligonucleotide primers that span completely across introns
  - a. For Cre AND Flp constructs, create forward primers that sit in exons 1 and 2 and reverse primers that sit in exons 2 and 3 to be used for three separate analyses (splicing across intron 1, splicing across intron 2, and splicing across both introns).
2. Prepare 12-well plates with cultured cell line
3. Transfect at approximately 60–75% confluency with the INTRSECT construct and appropriate recombinase(s).
4. Allow an appropriate amount of expression time to elapse in culture
  - a. With HEK cells we analyze expression at five days post-transfection
5. Prepare mRNA according to manufacturer protocol and synthesize cDNA using your preference of RT primer (we use template-specific primers).
6. Amplify cDNA using primer sets to analyze splicing of intron 1 (exon 1 F; exon 2 R), splicing across intron 2 (exon 2 F; exon 3 R), and potential splicing across both exons (exon 1 F; exon 3 R), including standard PCR negative controls. Include an additional sample consisting of INTRSECT construct template DNA with the same primers
  - a. The template DNA will include unspliced introns and serves as a negative control for analyzing splicing across intron 1 and splicing across intron 2. For constructs with two ORF, primers in exon 1 and exon 3 will amplify a band that is larger than the cDNA band, as it contains the intron sequence that is removed during mRNA processing.
7. Separate PCR products by gel electrophoresis and visualize. Amplicon sizes should be apparently different between spliced and unspliced constructs (Figure 3a).
  - a. Multiple bands may indicate the presence of unspliced product or of inappropriately spliced products. We recommend sequencing all bands to differentiate between these two options.

8. [Optional] Excise bands and purify DNA fragments from gel. Sequence and compare to source template to assess splicing efficiency and specificity (Figure 3a).

### **INTRSECT construct analysis using flow cytometry analysis**

The procedures in this protocol can be used to analyze the expression efficiency and specificity of INTRSECT constructs encoding fluorophores relative to positive and negative controls.

**Materials**—Cultured cell line (we use HEK cells; ATCC CRL-1573 or CRL-3216; ThermoFisher R70007)

INTRSECT construct ([www.optogenetics.org](http://www.optogenetics.org))

Recombinase expression constructs ([www.optogenetics.org](http://www.optogenetics.org))

Transfection reagents (Lipofectamine; TheroFisher 11668030)

Propidium Iodide (1000X stock 1mg/ml) or other spectrally appropriate vital dye

Heat block at 95 degrees C

0.25% Trypsin (Gibco 12604013)

Ice-cold 1x PBS

Flow cytometer

Flow cytometer compatible tubes

Small volume tubes (eg 1.5 mL)

Temperature-controlled bench-top centrifuge

Ice bucket

Foil

### **Transfect and prepare cultured cells**

2. Prepare 24-well plates with cultured cell line by seeding at low, single-cell density
3. Transfect at approximately 60–75% confluency in biological replicates following transfection manufacturer guidelines.
  - a. Two sets of untransfected samples (“negative” and “dead”)
  - b. One set of cells transfected without DNA (“empty”)
  - c. One set of cells with non-recombinase dependent source construct (“positive”)

- d. One set of cells with the INTRSECT construct without recombinase (“alone”)
  - e. One set of cells with the INTRSECT construct + Cre recombinase (“+Cre”)
  - f. One set of cells with the INTRSECT construct + Flp recombinase (“+Flp”)
  - g. One set of cells with the INTRSECT construct + Cre recombinase + Flp recombinase (“+Cre AND Flp”)
  - h. Following transfection reagent manufacturer guidelines, determine the correct amount of DNA for the triple (INTRSECT construct, Cre, and Flp) transfection condition for equimolar concentrations of each. Use the **same amount** of DNA in the other conditions (e.g. if you use 100ng INTRSECT, 150ng Cre, and 200ng Flp for the “+Cre AND Flp” transfection, also use 100ng INTRSECT for the “alone” condition)
4. Allow an appropriate amount of expression time to elapse in culture
    - a. With HEK cells we analyze expression at five days post-transfection
  5. Prepare both small volume centrifuge-compatible tubes and flow cytometer compatible tubes
  6. Chill PBS on ice
  7. Thaw Propidium Iodide stock at room temperature in foil
  8. Carefully aspirate media and add appropriate volume of Trypsin to each well. Allow cells to detach from plate and each other
  9. Triturate cells using cold PBS and transfer volume to individually labeled small volume centrifuge-compatible tube on ice. Place foil over tubes.
  10. Place ‘dead’ samples at 95 degrees C for 3 minutes to heat-kill.
  11. Centrifuge cells at 4 degrees C to pellet (1500 x g x 5 minutes is usually sufficient).
  12. While cells pellet, prepare PBS + PI by diluting PI stock to final concentration 1ug/ml into ice-cold PBS.
  13. After cells have pelleted, aspirate media and resuspend in 0.5–1 mL PBS+PI and transfer to flow cytometer compatible tubes on ice. Use foil to protect from light.

#### Analyze using flow cytometry

14. Using your flow cytometer of choice, prepare gates using negative, positive, and dead samples (Figure 3b).
15. Record sufficient data points from empty, alone, +Cre, +Flp, and +Cre AND Flp samples (We collect 20,000–30,000 events from each replicate)

16. Analyze expression for expression level of construct in +Cre AND Flp compared to control as well as Empty, Alone, +Cre, and +Flp compared to negative control using appropriate flow cytometry software or statistical analysis packages (eg FlowJo, R, MatLab, etc).

## COMMENTARY

### 1. Background Information

The excision and inversion properties of Cre recombinase are used together in the DIO Cre-dependent (cDIO) expression strategy (Atasoy et al., 2008; Tsai et al., 2009). Cre recombinates Lox sites, which are comprised of two characteristic palindromic sequences flanking a short, non-palindromic spacer. Given a length of DNA flanked by two recognition sites, the relative orientation of the sites determines whether the intervening sequence will be excised (same direction) or inverted (opposite direction). Sites with different spacer sequences are unable to recombine. The initial configuration of the open reading frame (ORF) is in the anti-sense orientation relative to the promoter, thereby preventing functional transcription. This inverted sequence is flanked by two sets of incompatible, inverted recognition sites; after two successive recombination events, the ORF is irreversibly locked into the forward orientation and is flanked by one of each incompatible sites.

The same principle applies for other recombinases such as Flp. Flp recombinates FRT sites, which share a common structure with Lox sites. Multiple FRT sites have been described and to date two of them (FRT and F5 (Schlake and Bode, 1994)) have been used in combination to create a Flp-dependent DIO (fDIO) (Fenno et al., 2014). INTRSECT combines these two recombinases, with heterologous introns and viruses to enable intersectional expression of genetically encoded tools.

### 2. Critical Parameters

**a. Species**—Although INTRSECT has been tested and validated only in mice, it should be easily integrated in rats, particularly as more transgenic rat lines become available (Witten et al., 2011). It may be prudent to first confirm that the recombinases are functional by performing a positive control experiment, such as a co-injection of recombinase and associated DIO viruses. As the recombinases described in this Protocol have been optimized for mammalian use (Buchholz et al., 1998; Raymond and Soriano, 2007), they would likely require modification and validation before being applied to non-mammalian systems.

**b. Transgenic lines**—For any experiment involving transgenic animals, the specificity and penetrance of the animal line is of critical importance. For positively-defined cell types, low specificity would result in “off-target” or “leaky” expression, whereas for negatively-defined cell types low penetrance would result in the same.

**c. Promoters**—The constructs in the examples above all use the ubiquitous promoter EF1 $\alpha$ , but further genetic specificity may be conferred by instead using cell-type specific promoters in any or all of the viral constructs, as desired. Furthermore, future work in viral engineering to produce custom virus/receptor pairs (Marshall et al., 2010; Wickersham et al.,

2007), pseudotyped coat proteins(Kato et al., 2011a; Kato et al., 2011b; Kim et al., 2016), and engineered trans-synaptic binding peptides(Gradinaru et al., 2010) are easily integrated with the techniques outlined here for targeting based on more highly refined criteria.

**d. Recombinases**—The protocols above involve the combined use of Cre and Flp recombinase, both members of the tyrosine recombinase family, but INTRSECT strategies may also be designed using other combinations of recombinases, including Dre(Sauer and McDermott, 2004), VCre, and SCre(Suzuki and Nakayama, 2011). DNA recognition sites for all of these enzymes share a common structure: two characteristic palindromic sequences flank a short, non-palindromic spacer. The sequence of the palindromic region determines its associated recombinase enzyme, while the sequence of the spacer region confers both directionality and identity, such that each enzyme acts only on its own associated recognition sites. However, in practice it is critical to first confirm that any recombinases that will be used in combination do, indeed, function independently. Notably, Cre and Dre have demonstrated cross-reactivity both *in vitro* and *in vivo*(Fenno et al., 2014) and therefore should not be used in combination.

**e. Choice of viruses**—Retrograde delivery of recombinase may in theory be performed by any one of the several retrograde viruses available for standard use in neuroscience, including LT-HSV(Fenno et al., 2014; Kim et al., 2013; Stamatakis et al., 2013), rabies virus(Osakada et al., 2011; Reardon et al., 2016), pseudorabies virus(Oyibo et al., 2014), and CAV-2(Bru et al., 2010). An important consideration is that INTRSECT targeting strategies requires waiting for sufficient levels of AAV-mediated expression, so it is important to select a retrograde virus that is non-toxic on that timescale. Rabies viruses have the disadvantage of a short expression window prior to the onset of cytotoxicity and neuron death(Wickersham et al., 2007). Highly expressed AAV serotypes such as AAV-DJ(Grimm et al., 2008), a synthetic serotype produced by gene shuffling, will express more rapidly, possibly enabling the use of Rabies viruses for these experiments.

AAV may be produced in an individual laboratory following previously published protocols(Grieger et al., 2006) or may be purchased from any one of several virus core facilities (e.g. <http://www.med.unc.edu/genetherapy/vectorcore> or <http://med.stanford.edu/gvvc/>).

## Troubleshooting

**1. Extra bands in RT-PCR**—We have only observed extra bands in the RT-PCR when using the double intron approach (two ORF). These represent three distinct problems. The origin of the issue may be estimated from the size of the band, but definitive diagnosis of this problem requires PCR product or band sequencing.

- a.** Small bands. Bands that are on the order of a few hundred bases are almost guaranteed to represent direct splicing of exon 1 to exon 3 (e.g. exon skipping or alternative splicing). This happens commonly and does not appear to impact functional expression as assayed by both *in vitro* and *in vivo* assays. We have attempted to correct this issue at length (unpublished results), but have so far been unsuccessful. It is not clear that this splicing pattern is replicated *in vivo*.

- b. Large bands that are slightly too small. We have had experience twice where the band is slightly small. Both times, this represented mis-splicing originating from a cryptic splice site within the coding frame of the ORF. We were able to easily correct this once by taking advantage of codon degeneracy to change the offending DNA sequence (and thereby disrupt the unwanted splice site), but preserve the translated product. A second time (unpublished data), we did not have this option, so relied on the crystal structure of this protein to change one of the amino acids (without degrading efficacy of the protein).
- c. Large bands that are slightly too large. This usually represents mis-splicing due to sub-optimal placement of the intron within the ORF. We have successfully fixed the problem by moving the intron to an alternative site in the ORF.

## 2. Mis-expression (expressing with incorrect recombinase combination) *in vitro*

Possible reasons that we have seen mis-expression *in vitro* include:

- a. Not waiting enough time for recombinase activity (especially for infection *in vivo*)
- b. Errors in cell culture, including plate labeling, re-using tips, or contamination across wells during washes
- c. Contamination of recombinase stock with other recombinases or constructs. We have experienced this specifically when using the Invitrogen BenchPro plasmid purification robot due to a deficiency in the design of the tubes, causing them to both leak from the bottom and to become contaminated with DNA product from other tubes that leaked.
- d. Improper design for expected recombinase expression logic. Compare your design against those available from the optogenetics resource website ([www.optogenetics.org](http://www.optogenetics.org)).

**3. Low or mis-expression *in vivo***—We rarely see either of these phenomenon, but have found them to mostly originate from generic viral issues, including:

- a. Poor viral production due to excessive payload size
- b. Confounding tropism
- c. Unreliable transgene expression pattern
- d. Not waiting long enough for expression
- e. Toxic viral payload

Multiple reviews cover basics of virus design, including (Yizhar et al., 2011). Second, we have seen INTRSECT-specific mis-targeting and found that this occurs when using high viral titres. The mechanism is unknown, but may involve hetero-concatamers formed by adenoassociated viruses. Decreasing the titre by an order of magnitude has successfully remedied the mis-expression.

## Understanding Results

It is anticipated that you will design, construct, validate *in vitro*, and validate *in vivo* INTRSECT constructs to be used for targeting neuron subpopulations as described in the previous sections.

When you have validated a construct, you will then be able to inject virus that harbors your construct into a brain region of interest in order to limited expression to subpopulations of neurons based on the combination of the recombinase expression pattern and the logic gate that has been engineered into the new construct. In the case of a  $C_{on}/F_{on}$  virus injected into an animal expressing Cre and Flp recombinases in separate, but overlapping, neuron subpopulations, the expression of your virus would be limited to neurons that have both recombinases.

In a first example, AAV- $C_{on}/F_{on}$ -ChR2-YFP was injected into the hippocampus of PV-Cre;SST-Flp double transgenic mice (Fenno et al., 2014). Expression of ChR2-YFP was limited to neurons with morphology consistent with cell types that express both inhibitory neuron markers within the injected area of the hippocampus. Here, expression of the virally-delivered ChR2-YFP marks (and allows the experimenter to manipulate the activity of) neurons that are PV+ AND SST+. Injection of this same virus into single transgenic PV-Cre or SST-Flp animals did not result in expression of ChR2-YFP.

A second example used dual virus expression paradigms to restrict expression of ChR2-YFP based on a combination of transgenic and virally-delivered recombinases (Tovote et al., 2016). First, to neurons that express Flp AND NOT Cre using AAV- $C_{off}/F_{on}$ -ChR2-YFP virus co-injected with a pan-neuronal AAV-Flp into Chat-Cre mice. Here, all neurons *except* those that express Cre (in this case, Chat+ neurons) expressed ChR2-YFP. In a separate experiment, the authors injected  $C_{on}/F_{on}$ -ChR2-YFP virus into Vglut2-Cre mice in a region with cells known to project to a pre-specified downstream region. In this downstream region, they injected the retrograde virus HSV-Flp. In this case, HSV-Flp is taken up at the axon terminal and trafficked back to the cell body. Expression of ChR2-YFP was limited to neurons that are Vglut2+ AND project to the region that had received the injection of HSV-Flp. A separate set of experiments similarly combined transgenic and retrograde virus delivery of recombinases to target neurons based on genetic and topological characteristics; in this case (Marcinkiewicz et al., 2016),  $C_{off}/F_{off}$ -ChR2-YFP was used, thereby *excluding* expression of ChR2-YFP from cells expressing a certain gene AND projecting to a pre-specified downstream region.

## Time Considerations

Part 1: *in silico* design: approximately 2 hours

Part 2: Molecular cloning: approximately 1–2 weeks

Part 3: *In Vitro* testing: approximately 1 week

Part 4: *In Vivo* testing: approximately 1–4 weeks

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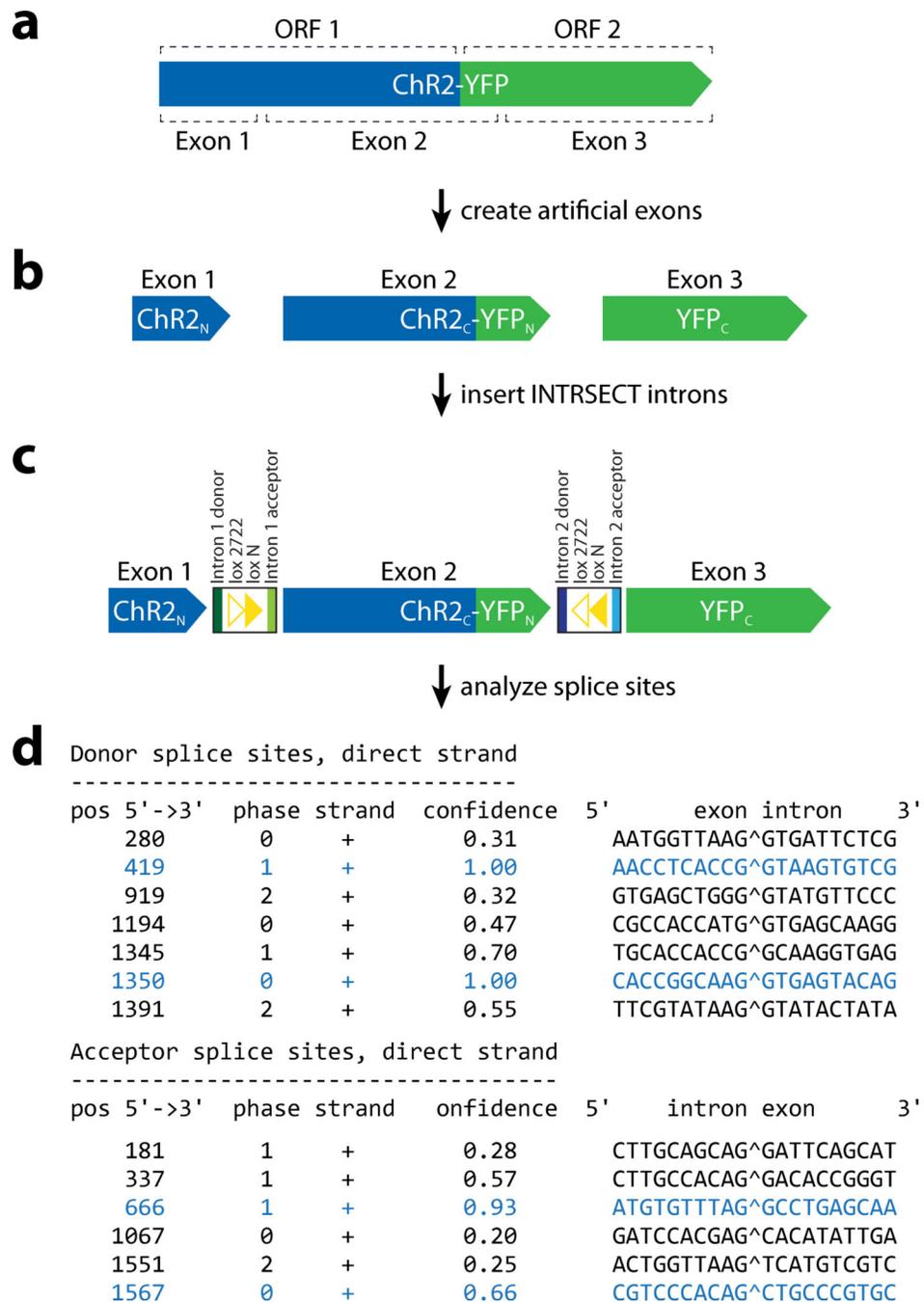
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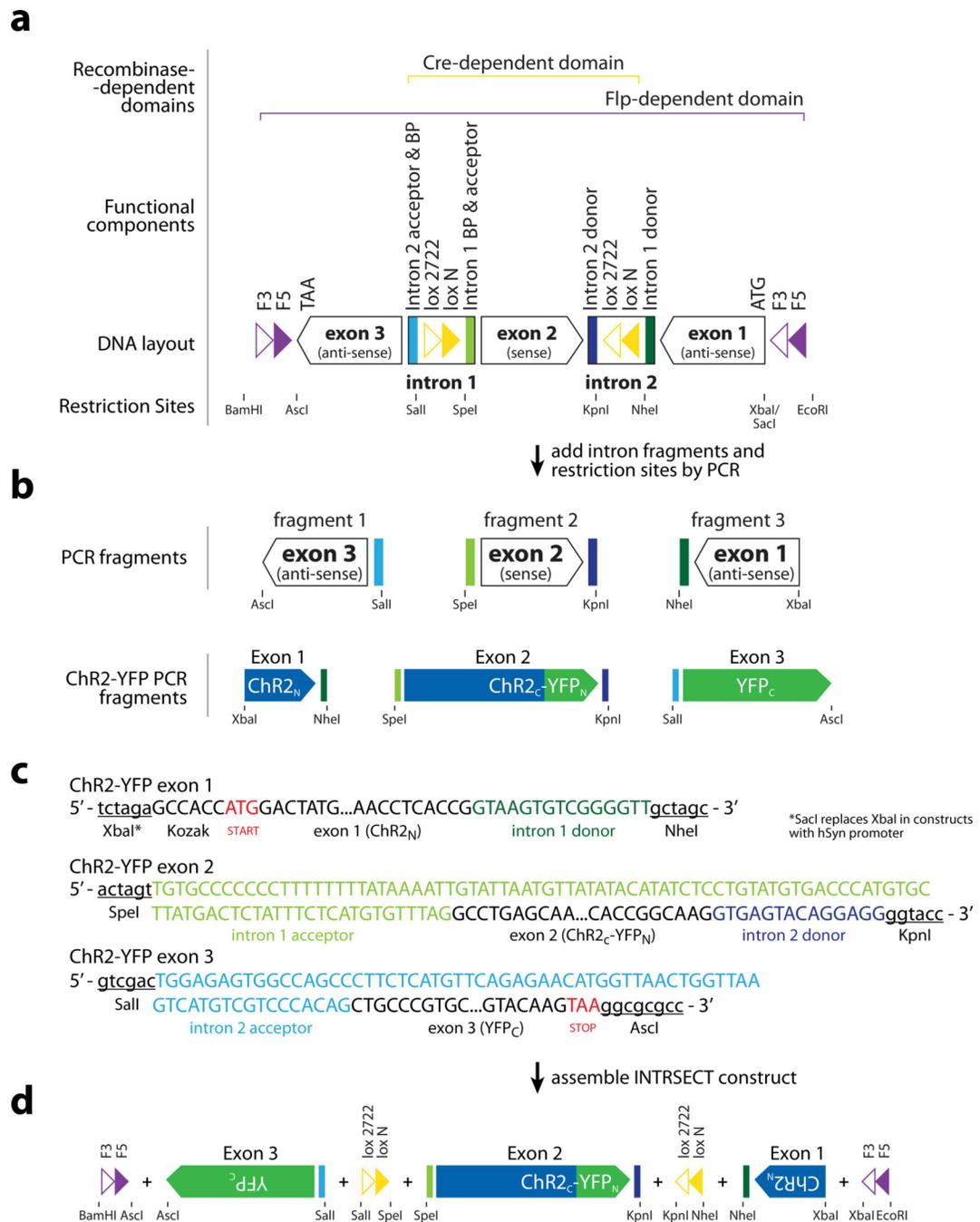
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### Significance Statement

The nervous system is composed of myriad neuron subtypes defined based on genetic signature, synaptic partners, anatomical location, or any combination of these. Many types of neurons occupy any given volume of the brain and being selective modulation of neuron subtypes, without perturbing their neighbors, is a fundamental challenge in neuroscience. We have published a method that combines off the shelf reagents to selectively express any viral payload in neurons based on either positive (AND) or negative (AND NOT) logical gates, termed INTRSECT. This expands the resolution of experiments undertaken by neuroscientists in trying to understand how neuron subtypes contribute to circuit function and behavior and health and disease. Here, we give a protocol for generating and testing INTRSECT constructs.

**Figure 1.**

Choosing and analyzing intron insertion sites for INTRSECT construct design. **a)** In genetically-encoded tools containing two open reading frames, two separate introns will be inserted, creating three exons. **b)** Search within each ORF to locate two sites that match the consensus sequences for the exon portion of intron splice sites. **c)** Insert the INTRSECT introns into the sequence at the sites you have identified using standard molecular biology plasmid design software. **d)** Analyze the splice sites using NetGene2.0 or another intron prediction tool to see that your choice of site has a high chance of being properly spliced.

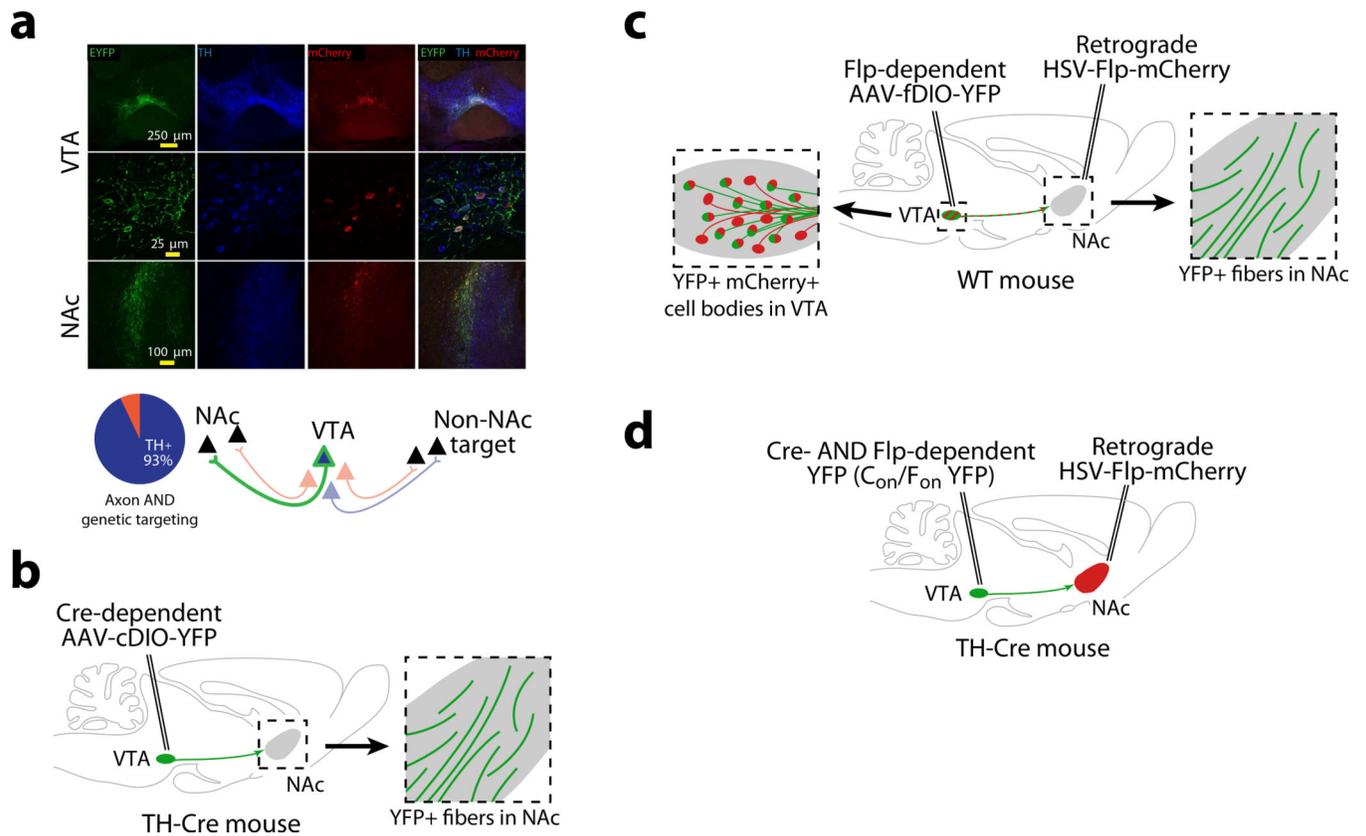
**Figure 2.**

Molecular cloning of INTRSECT constructs. **a)** The layout of a Con/Fon INTRSECT construct, with individual elements of intron one (in blue) and intron two (in green) as well as Cre-related elements (in yellow) and Flp-related elements (in purple). Exons direction (sense vs. reverse complement) indicated by exon arrow direction. Restriction sites are noted. **b)** Intron fragments and restriction sites will be added to exon fragments to create the building blocks of any INTRSECT construct. ChR2-YFP is given as an example. **c)** The donor and acceptor sequence of intron 1 (in green) and intron 2 (in blue) must be directly

fused to the exon fragments, without any intervening sequence, including restriction sites. Ensure that the Kozak sequence and START codon (in red) are included in exon 1 and the STOP codon (in red) is in exon 3. Generate these fragments by PCR, adding the restriction sites (underlined) to the oligos. **d)** Ligate exon fragments into the INTRSECT backbone using the included, unique restriction sites. ChR2-YFP is given as an example.

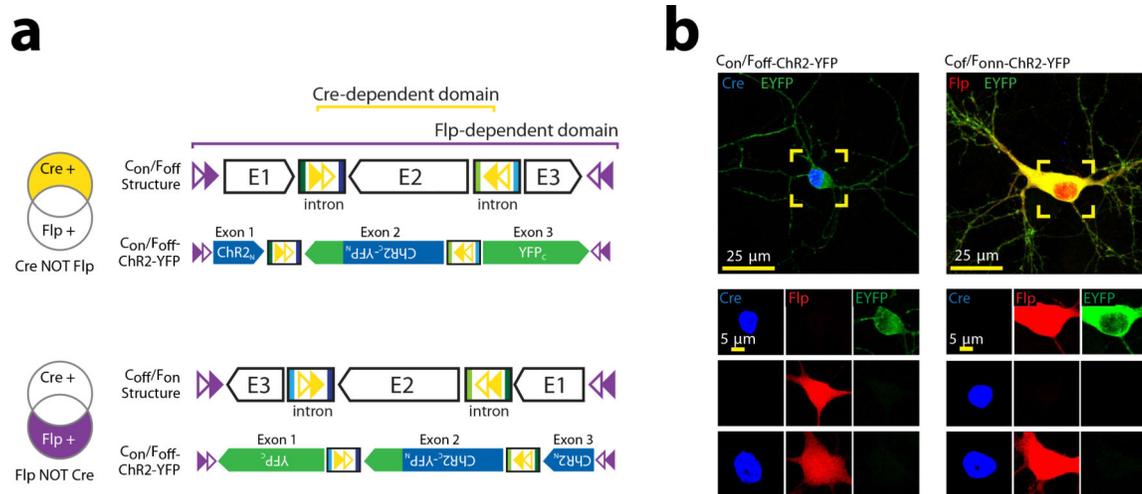


ChR2-YFP (green), as well as other non-active recombinase combinations (red, yellow, purple). **c)** Primary neurons transfected with combinations of the INTRSECT construct and recombinases shows expected ChR2-YFP trafficking as well as recombinase expression. **d)** *In vitro* functional analysis of primary neurons transfected with Con/Fon ChR2-YFP indicates that it has comparable photocurrents to WT ChR2-YFP. **e)** Injection of adeno-associated virus containing the INTRSECT construct has no expression in single Cre or Flp transgenic animals (left, middle), but robust expression in Cre;Flp double-transgenic animals (right).

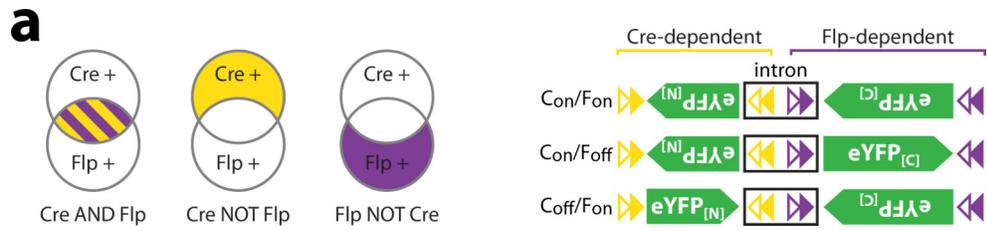


**Figure 4.**

Targeting cells based on genetic AND topological properties. **a**) Dopaminergic neurons of the ventral tegmental area projecting to the nucleus accumbens may be selectively targeted using a combination of retrograde viruses, recombinases, and recombinase-dependent viruses. **b,c**) Control experiments assaying that the transgenic population of Cre-expressing neurons projects to the desired downstream area (**b**) and that the retrograde virus is able to infect the terminals of projection neurons (**c**) are critical for each transgenic mouse line/anatomic location combination. **d**) After ensuring that the anatomy and viruses are suitable for the experimental design, a transgenic mouse (here expressing Cre in all dopaminergic neurons) is injected with a retrograde, flp-expressing virus (HSV-Flp-mCherry) in the nucleus accumbens. This is trafficked retrograde to the cell bodies of all cells projecting to the nucleus accumbens. A virus containing a Cre AND Flp dependent INTRSECT construct (Con/Fon Chr2-YFP) is injected at the target region, and will only be expressed in cells that are both dopaminergic and project to the nucleus accumbens.

**Figure 5.**

AND NOT targeting with INTRSECT. **a**) In addition to targeting neurons with both Cre AND Flp, INTRSECT may be used to target cells with Cre AND NOT Flp as well as Flp AND NOT Cre, which adds a level of resolution over using just Cre or Flp alone in targeting schemes (left). The INTRSECT backbone is designed for modular cloning, using the same exons as the Con/Fon construct, but with a modified orientation (right). Note that the intron fragments fused the exons stay with their respective exons and also change orientation (for instance, the dark green intron donor fragment fused to the 3' terminal of exon 1). **b**) Neurons transfected with Con/Foff ChR2-YFP and Coff/Fon ChR2-YFP show robust expression in the correct recombinase condition, but not with the other recombinase, nor both recombinases.



**Figure 6.** INTRSECT construct design for single ORF genes. **a)** A single intron is used for genes containing one ORF (such as YFP). Here, the gene is split into two exons and, as in the two-intron, two-ORF INTRSECT constructs, the starting orientation of the exons determines the expression logic.