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Combinatorial methods for refined neuronal gene targeting

Haojiang Luan and Benjamin H White

Methods for the selective and reproducible expression of genetically encoded tools in targeted subsets of cells are required to facilitate studies of neuronal development, connectivity, and function in living animals. In the absence of techniques for synthesizing promoters that target defined cell groups, current methods exploit the regulatory elements of endogenous genes to achieve specificity of transgene expression. However, single promoters often have expression patterns too broad to pinpoint the functional roles of specific neurons. In this review, we describe emerging combinatorial techniques that make transgene expression contingent not upon a single promoter, but upon two or more promoters. Although only a few such techniques are currently available, recent developments promise rapid growth in this area in the coming years.

Addresses
Laboratory of Molecular Biology, National Institute of Mental Health, NIH, 9000 Rockville Pike, Bethesda, MD 20892-3736, United States

Corresponding author: Luan, Haojiang (luanh@mail.nih.gov) and White, Benjamin H (benjaminwhite@mail.nih.gov)

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Introduction

Sophisticated genetic tools increasingly complement classical techniques for labeling neurons and monitoring or manipulating their function [1,2]. These tools take the form of transgenes that can be expressed in the cells of living animals, and using them to their full advantage requires equally sophisticated techniques for targeting their expression within the nervous system to specific cells in a temporally defined way. The most versatile strategies for cell type specific targeting are binary systems, which exploit the natural *cis* regulatory elements of endogenous genes to drive the expression of a primary effector, usually a transcription factor (Figure 1A) or recombinase (Figure 1B). This primary effector then activates (or in some cases, inactivates) expression of the transgene encoding a secondary effector that permits the marking, monitoring, or manipulation of the expressing cell. Binary systems have been developed for use in several genetic model organisms. The Gal4-UAS system and its variants are ubiquitously used in *Drosophila* research and increasingly in work on zebrafish [3,4]. Recombinase-based systems, based principally on the use of Cre, and the Tet-on/off systems are commonly used in mice [5,6]. While temporal control of gene expression using these systems or their derivatives has been possible for some time, restricting expression to arbitrarily small subsets of neurons has remained a challenge. Certain methods exploit stochastic processes to provide transgene expression at exquisite – even single cell – resolution [7,8], but methods that permit reproducible expression of arbitrary effector transgenes in delimited subsets of neurons have become available only recently with the development of combinatorial techniques for refined transgene targeting.

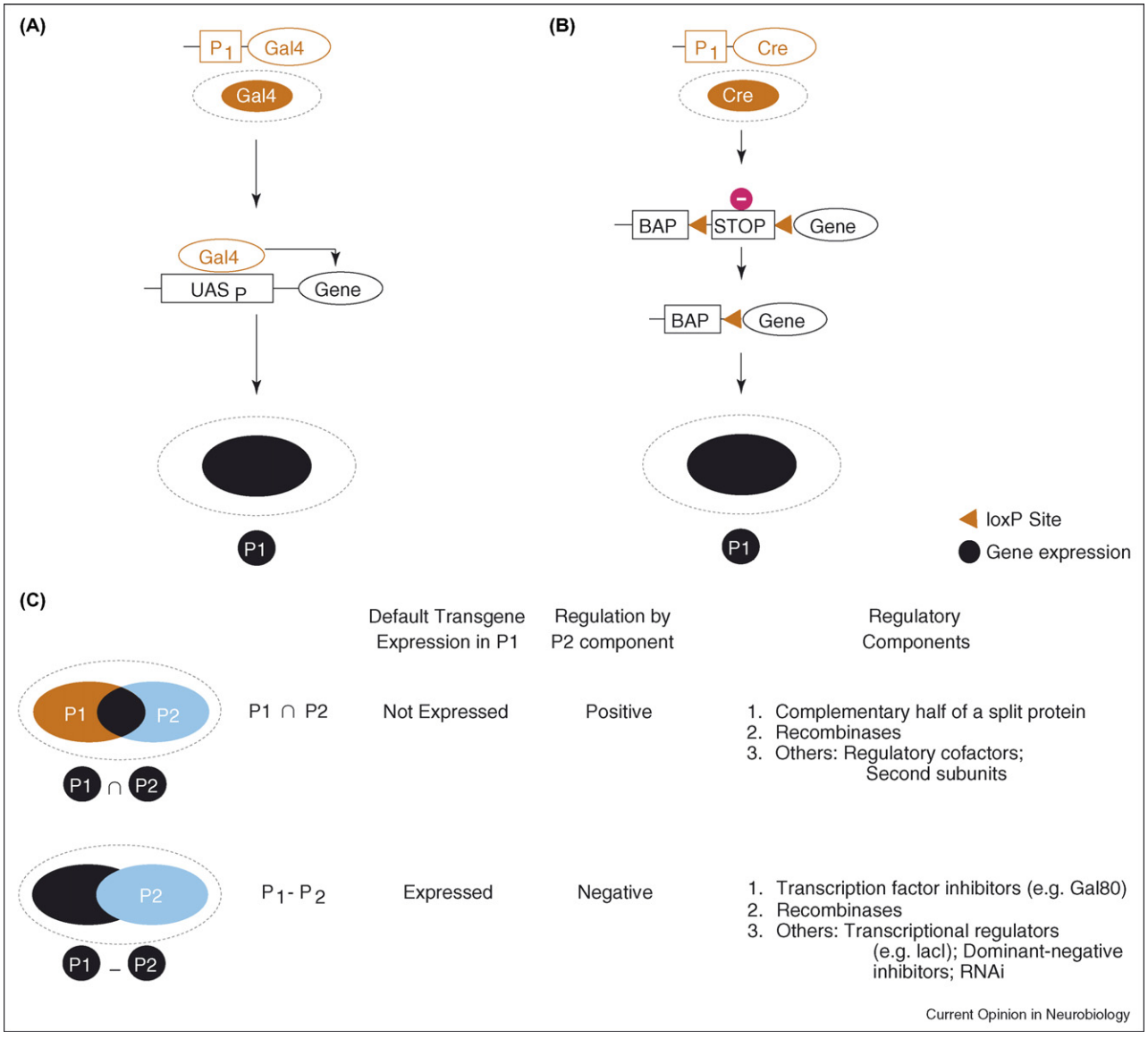
This review will summarize these recent advances, focusing on methods that permit parsing cells within a group of interest by exploiting two or more promoters, the expression patterns of which overlap. Restriction is accomplished by delimiting expression either to the intersection of these two expression patterns, that is, to cells that are included in both patterns, or to the difference between them, that is, to cells that are exclusively present in one pattern. Following Farago *et al.* [9••], we refer to these two types of restriction as 'intersectional' and 'subtractive,' respectively. The logic of these two types of restriction is outlined in Figure 1C. Each can be viewed as the implementation of a Boolean AND (∩) or NOT (−) 'expression operation' performed using the two transgene promoters. For simplicity, we will consider intersectional (∩) and subtractive (−) restrictions of one expression pattern (P1) relative to a second overlapping pattern (P2). Intersectional restriction then can be viewed as the result of positively regulating a normally inactive expression pathway in P1 (via expression of a second transgene expressed in P2), while subtractive restriction can be viewed as the result of negatively regulating a normally active expression pathway in P1. The principal types of genetic tools that have been used to implement these two types of restriction are listed in Figure 1C.

Intersectional restriction of gene expression (P_1 AND P_2)

Split protein systems for special applications

One class of strategies for intersectional restriction requires components that are inactive alone, but when combined reconstitute a desired function. Such strategies often take advantage of protein complementation, in

Figure 1



Binary gene targeting techniques and combinatorial methods of restriction. **(A)** The Gal4-UAS system of *Drosophila*, a transcription factor-based system for targeting transgene expression *in vivo*, is depicted schematically. The promoter P_1 drives expression of the transcription factor Gal4 in the pattern indicated schematically in vermilion within the dotted oval. When animals expressing Gal4 in this pattern are crossed to animals bearing a transgene of interest ('Gene') placed downstream of a universal Gal4-responsive promoter ('UAS_P'), the transgene is expressed wherever Gal4 is expressed in the progeny, indicated by the black P_1 pattern within the oval shown on the bottom. **(B)** An alternative binary gene targeting technique, commonly used in mice, that uses the site-specific recombinase Cre. In this system, Cre is expressed in the pattern dictated by the promoter P_1, again indicated in vermillion, and the transgene of interest ('Gene') is placed upstream of a broadly active promoter (BAP). The transgene is separated from the BAP by a transcription stop cassette (STOP), which is flanked by Cre target (i.e. loxP) sites (arrowheads). The stop cassette interrupts transcription from the BAP, indicated by the stop symbol (red with white dash), unless it is excised by Cre, in which case transgene expression is activated. Animals bearing the transgene construct and expressing Cre in the P_1 pattern express the transgene only in P_1, again indicated by the black oval at the bottom. **(C)** Transgene expression within the P_1 pattern (vermillion) can be restricted by either positively or negatively regulating it in subsets of cells common to the expression pattern of a second promoter, P_2 (blue). Positive regulation implies lack of transgene expression in P_1 under default conditions (i.e. in the absence of the component expressed in P_2) and results in expression at the intersection of P_1 AND P_2 (indicated by the symbol '∩' and the region shown in black). By contrast, negative regulation implies default transgene expression in P_1 that is blocked in cells that are also within P_2. Such restriction is therefore subtractive (indicated by the symbol '–') and results in expression in cells in P_1, but NOT P_2, again shown in black. The types of regulatory components that can be expressed in the P_2 pattern to effect positive and negative regulation of transgene expression are shown in the right-hand column.

which two inactive fragments of a protein associate to reconstitute function, or alternatively, of the modularity displayed by many proteins, which may have multiple subunits or multiple (and separable) domains within a single subunit that contribute independently to the final functional unit. By independently targeting the expression of the two inert halves of a split protein, reconstitution of function can be achieved at the intersection, and only at the intersection, of the expression patterns of the two promoters used to target them. Split protein effectors have been used to achieve restricted cell labeling (using a split GFP molecule [10]) or cell ablation (using a split caspase [11•]) in *C. elegans*. A similar combinatorial approach to achieve restricted neuronal silencing in mammalian neurons has been proposed, but not yet validated, using an ivermectin-sensitive chloride channel composed of two subunits [12•]. Similarly, a split luciferase molecule has recently been introduced in *Drosophila* but has yet to be used to restrict light production only to subsets of neurons of interest [13•]. This latter tool is noteworthy as an example of a promising new method of splitting proteins that employs what are called split inteins and that can be used to functionally divide (and later rejoin) even proteins that have no obvious modularity.

Although split reporter or effector molecules are useful, a more general system for performing restricted neuronal manipulations that takes advantage of existing effector constructs and does not require the generation of unique split effectors for each application is desirable. One approach to making such a system that has recently proved successful involves splitting the primary effector of a binary expression system [14••].

Split Gal4: a general, transcription factor-based method for intersectional restriction of transgene expression

Gal4, which activates transcription of downstream genes in yeast by binding to a DNA motif known as the 'Gal4 upstream activating sequence,' or UAS, is modular with separable DNA binding and transcription activation domains. This modularity previously has been exploited to make hybrid transcription factors that retain the Gal4 DNA-binding domain (BD), and therefore drive expression of UAS-transgenes, but that are coupled to alternative transcription activation domains (ADs) that render the hybrid transcription factor inducible (as in the GeneSwitch molecule [15,16]) or more active (as when the potent AD of the viral transcription factor VP16 is used [17,18]). Luan *et al.* [14••] also have exploited the modularity of Gal4 to develop the ternary, 'Split Gal4' expression system illustrated in Figure 2A, which can use either the Gal4 AD or the VP16 AD. In each case, the AD is coupled to a synthetic leucine zipper [19] that heterodimerizes with a complementary zipper fused to the Gal4 BD. In this manner, when either AD is co-expressed with the Gal4 BD, transcriptional activity is reconstituted and UAS-transgenes are expressed.

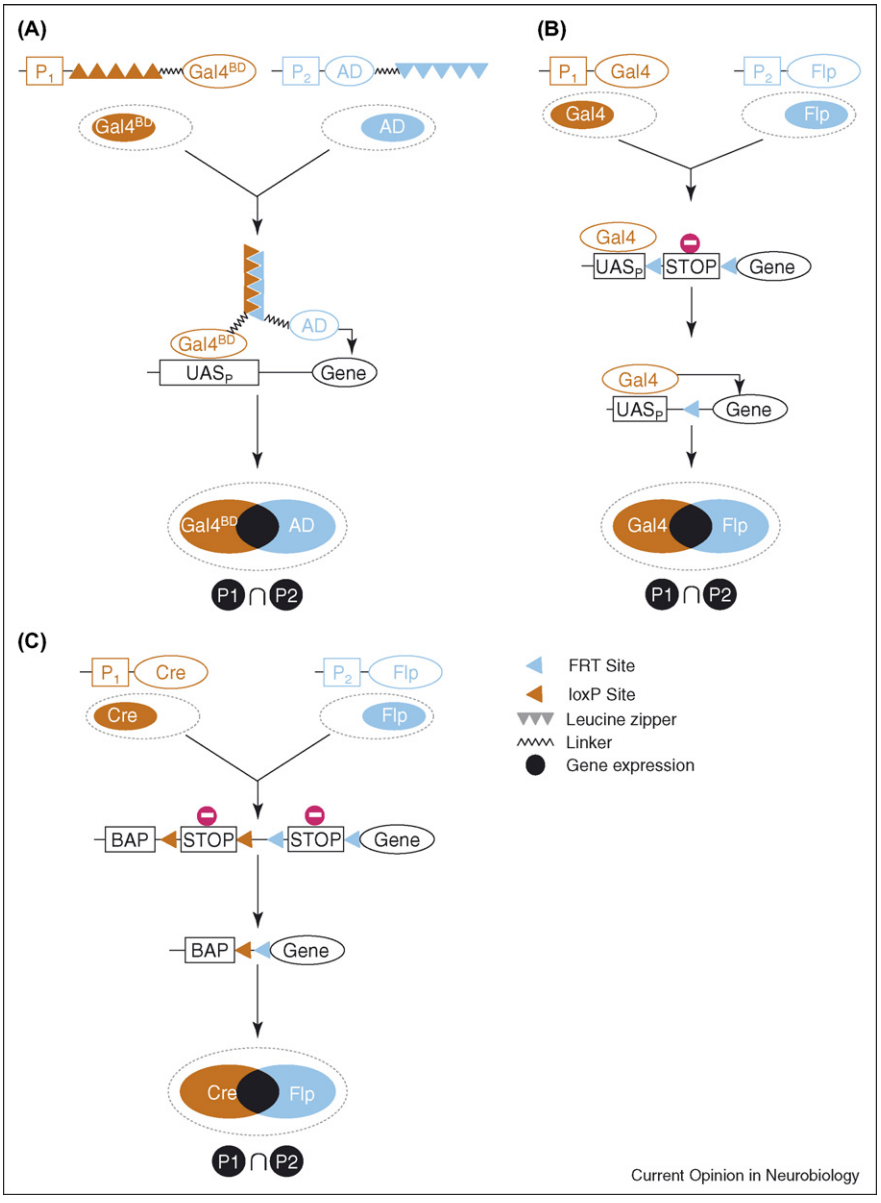
Luan *et al.* [14••] applied the Split Gal4 system to ablate targeted subsets of neurons in a network using the proapoptotic UAS-transgene, *reaper*, and the range of problems that can be addressed by this technique should be quite broad since it is compatible with many other existing UAS-effector lines available in *Drosophila*. The two implementations of the technique have different strengths, with the VP16 AD providing higher transgene expression than the Gal4 AD, and the Gal4 AD subject to a further level of (negative) regulation by the Gal4 inhibitor Gal80 (discussed below). While the original version of the VP16 AD was reported to cause ectopic expression, which constrained its range of use, this limitation has since been overcome by drosophilizing the VP16 AD sequence and ablating a potential enhancer site within it (H Luan and BH White, unpublished data).

Like all intersectional techniques employing split proteins, reconstitution of function in the Split Gal4 system requires temporal, as well as spatial, overlap of the two components. This requirement is met if the promoters used to direct expression of the BD and the AD are active at the same time in a given cell. It will also be met if the promoters are active at different times, but if the earlier expressed component is sufficiently stable to remain in the cell until the other component is expressed. It is not known how often this latter condition will obtain as the stability of the individual Split Gal4 components has not been examined systematically. It is worth noting, however, that while the requirement of temporal coincidence of expression may be a disadvantage in some cases, it can be an advantage if it adventitiously provides temporal as well as spatial restriction of transgene expression. In addition, it should be noted that the Split Gal4 technique, in principle, can be used in conjunction with UAS-Gal4 constructs [20] to persistently activate UAS-transgene expression in cells in which the Split Gal4 components are co-expressed only transiently.

Recombinase-based systems for intersectional restriction of gene expression

A second class of intersectional techniques has been developed that does not rely on split proteins, but instead makes gene expression dependent upon the action of one or more recombinases. Site-specific recombinases, such as Cre and Flp, excise DNA sequences placed between their target recognition sites. Cre specifically recognizes a 34-bp motif known as a 'locus of crossover,' or LoxP site, while Flp recognizes a sequence of similar length known as the 'Flp Recombination Target,' or FRT, site. A straightforward method of activating gene expression, first demonstrated *in vivo* in mice using Cre [21], and subsequently in *Drosophila* using Flp [22], involves excising a transcriptional 'stop cassette' placed between a promoter and the transgene of interest. The stop cassette is flanked by directly repeated loxP or FRT sites, and in cells lacking recombinase activity this cassette efficiently

Figure 2



Techniques for intersectional restriction. **(A)** The Split Gal4 system takes advantage of the modularity of the Gal4 molecule separating it into its two independent functional domains: the DNA-binding domain (BD), which recognizes the UAS_P promoter site, and the transcription activation domain (AD), which initiates transcription. Each domain is fused to a heterodimerizing leucine zipper (filled sawtooth lines) separated by a short intervening linker (black sawtooth). This insures that the two domains associate when expressed in the same cell and reconstitute transcriptional activity. Each domain is independently targeted using promoters P_1 (vermilion) and P_2 (blue). When these constructs ('hemidrivers') are brought together in crosses to flies bearing a UAS-transgene, the transgene is expressed in progeny only at the intersection of the expression patterns of P_1 and P_2 (indicated in black). **(B)** Intersectional restriction achieved by coupling a transcription factor-based system with a recombinase-based system. Gal4 and Flp are independently targeted using promoters P_1 and P_2, respectively. The transgene of interest ('Gene') is separated from the universal Gal4 promoter (UAS_P) by a transcription stop cassette (STOP) flanked by Flp recombination target sites (blue arrowheads). The transgene is therefore only expressed at the intersection of P_1 and P_2 (in black) where Flp has excised the stop cassette AND Gal4 is present to drive transcription. **(C)** The 'dual recombinase' intersectional gene targeting technique. P_1 and P_2 are used to independently target distinct recombinases (Cre and Flp), each capable of excising one of the two transcription stop cassettes placed between the transgene ('Gene') and a broadly active promoter (BAP). Transgene expression is contingent upon excision of both the Cre and Flp stop cassettes, and therefore is activated only at the intersection of the Cre (i.e. P_1) and Flp (i.e. P_2) expression patterns.

blocks transcription of the transgene. In the so-called 'Flp-out' system developed by Struhl and Basler [22], the promoter placed upstream of the stop cassette was chosen to be the ubiquitous and constitutively active actin promoter, and many subsequent implementations have similarly aimed to use broadly active promoters to insure transgene expression in as many cell types as possible at all developmental stages. Several variations of the Cre or Flp binary systems have been used [23,24], some of which couple to transcription factors to enable inducible, restricted, or enhanced expression [25–27]. The most versatile of these, from the standpoint of restriction [28••], combines the Flp-out and Gal4-UAS systems by placing a FRT-flanked stop cassette (or 'STOP') between the UAS and the transgene of interest as depicted in Figure 2B. In this intersectional system, Gal4 and Flp are independently targeted to different cell groups using promoters with distinct, but overlapping, expression patterns, and expression of UAS-STOP-transgenes occurs only within those cells that express both Gal4 and Flp.

A second type of intersectional technique, introduced by Awatramani *et al.* [29] for fate-mapping studies in mice, achieves combinatorial restriction of transgene expression by independently targeting the recombinases Flp and Cre using distinct promoters (Figure 2C). In this 'dual recombinase' system, expression of the transgene of interest is made contingent upon the excision not of one but of two stop cassettes placed between the transgene and a broadly active promoter. Each stop cassette is flanked by target sites for only one of the two recombinases so that only in cells expressing both Cre and Flp will both cassettes be removed, allowing transcription of the transgene of interest. Thus far, the dual-recombinase system has been used only to express reporter transgenes, but its extension to the expression of other transgenes of interest should be possible (for examples, see reference [5]).

Unlike the Split Gal4 system, the dual-recombinase approach does not require simultaneous expression of both independently targeted components. Excision of each stop cassette is permanent, and even if the promoters used to drive Flp and Cre expression are active at different times in a common group of cells, transgene transcription will commence in these cells as soon as both stop cassettes are excised. Both Awatramani *et al.* [29] and Farago *et al.* [9••] have reported highly efficient activation of transgene expression in separate implementations of the dual-recombinase system, though in general the extent of transgene activation will be a function of recombinase expression levels and efficiencies, which may vary with promoter and with the site of integration of the transgene-bearing construct. The ROSA26 locus in mice [30] has been favored as an insertion site for stop cassette constructs because it supports efficient recombination and broad expression, though exceptions exist [31]. Similarly, the potent hybrid 'CAG' promoter introduced by Niwa *et al.* [32] has been used in transgenic expression constructs inserted at other sites because of its strong, constitutive activity in many cell types.

Subtractive restriction of gene targeting (P_1 NOT P_2)

In contrast to intersectional restriction of transgene expression, in which transcriptional activity is induced in cells common to two overlapping expression patterns, subtractive restriction involves suppressing expression in the common subset of cells (Figure 1C). Suppression can be achieved in several ways. For binary systems, inhibition of either the primary or secondary effector represents an obvious possibility. The first approach has been successful in *Drosophila*, where Gal80, the natural inhibitor of Gal4, has been used to block Gal4 function. The second approach has proved more promising in recombinase-based systems, as Cre and Flp lack natural protein regulators.

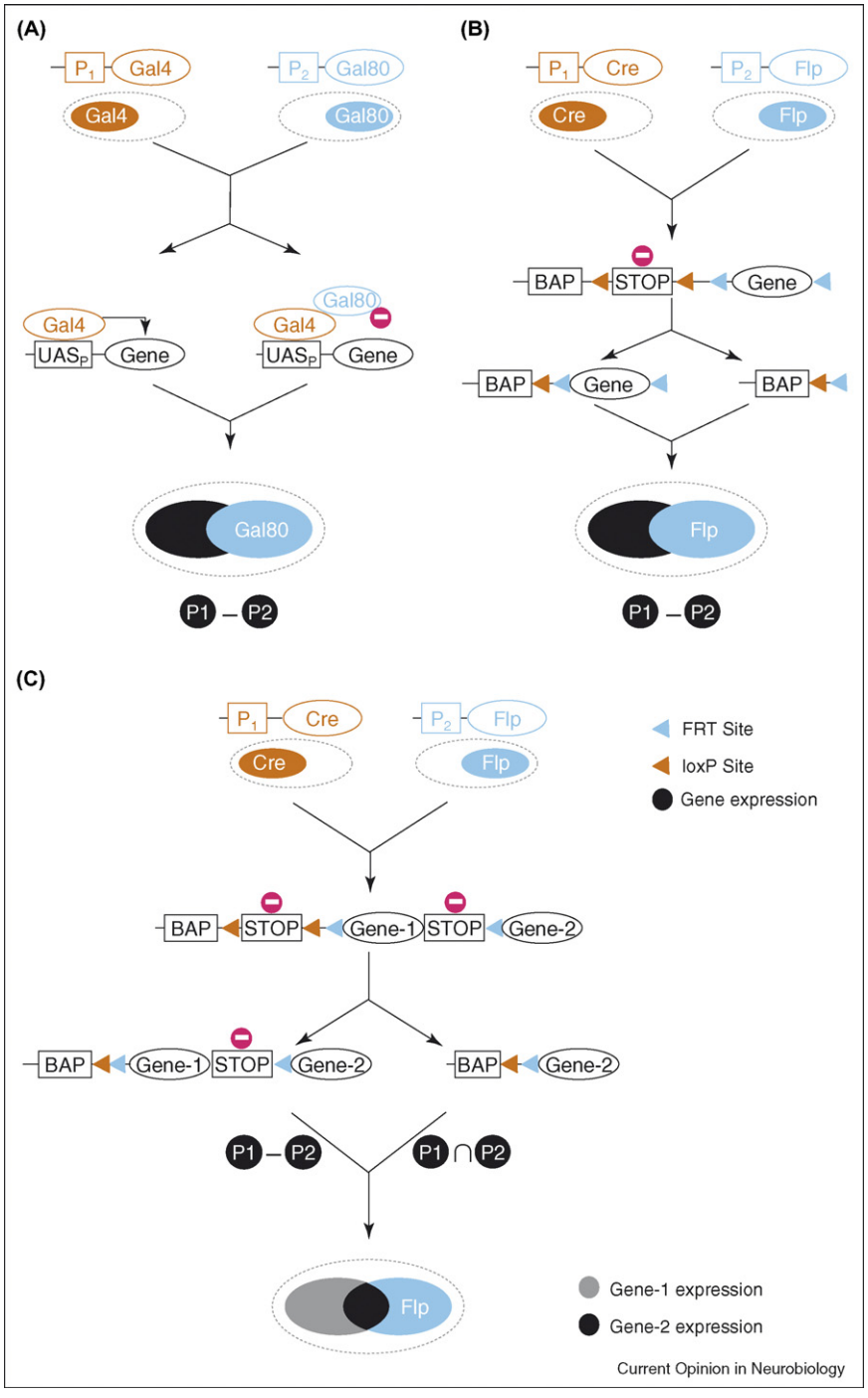
Subtractive restriction using the Gal4 inhibitor, Gal80

Subtractive gene targeting was pioneered in *Drosophila* using Gal80, which was first introduced as an essential component of the MARCM system for mosaic analysis [33]. This protein binds to the C-terminus of Gal4 and blocks transcription by interfering with the recruitment of other components of the transcriptional machinery. In addition to wild-type Gal80, a temperature-sensitive mutant (Gal80ts) that efficiently blocks transcription at low (18 °C), but not high, temperatures (30 °C) also has found widespread use in providing temporal control in the Gal4-UAS system [34]. A synthetic, conditional Gal80 molecule, which was made by inserting a temperature-sensitive intein into the Gal80 sequence, and which is active at high, but not low temperatures, has been less widely used [35].

Subtractive restriction of transgene expression using Gal80 is accomplished as shown in Figure 3A. Gal4 and Gal80 are independently targeted to overlapping subsets of neurons using different promoters, and transgene expression is restricted to those neurons that express Gal4, but not Gal80. Stoleru *et al.* [36] first used this paradigm to selectively block apoptosis in neurons expressing certain clock genes. To obtain complete suppression of Gal4 activity, a prerequisite for successful use of the system, the authors used multiple copies of the Gal80 transgenes. Directed expression of Gal80 using other promoters subsequently has been used to restrict expression of several UAS-transgenes to study such issues as learning and memory [37•] and developmentally programmed behaviors [38•].

Gal80 also has been used under conditions where its expression pattern is not directed by a specific promoter, but instead depends on enhancer elements located near

Figure 3



Techniques for subtractive restriction. **(A)** Use of the Gal4 inhibitor Gal80, which binds to the Gal4 AD, to selectively block transcription in the subset of neurons common to two promoters. Gal4 and Gal80 are independently targeted using promoters P_1 (vermillion) and P_2 (blue). Gal4 drives expression of the UAS-transgene of interest ('Gene') in all cells except those where Gal80 is also expressed (region indicated in black). These two outcomes are separately represented in the schematics on the left and right, respectively, with Gal4 block by Gal80 denoted with the stop symbol. **(B)** Use of a dual recombinase system to perform subtractive restriction of transgene expression. The logic of this system is identical to that of the intersectional method shown in Figure 2C, except that the second stop cassette now incorporates the transgene of interest ('Gene'). The transgene is therefore expressed in all Cre-expressing cells in the P_1 pattern, except those that are also within P_2 (i.e. P_1–P_2, region indicated in black). In the latter cells, Flp excises the transgene-containing cassette, abolishing transgene expression. **(C)** Simultaneous intersectional and subtractive transgene expression using the dual recombinase system. The schematic shows the implementation of subtractive restriction employed by Farago *et al.* [9••], which permitted simultaneous subtractive and intersectional restriction of expression of two transgenes ('Gene-1' and 'Gene-2'). The system differs from that depicted in (B) only in that the second stop cassette is now

the (essentially random) genomic site of insertion of the Gal80 transgene [39]. Such 'enhancer-trap' Gal80 lines can be helpful in restricting expression when defined promoters with expression patterns that intersect the primary pattern of interest are unavailable. In practice, however, identifying enhancer-trap Gal80 lines with useful overlapping patterns can be challenging. Identification based on the loss of a phenotype of interest is not possible, since the cells responsible for the phenotype are subtracted from the pattern, and direct characterization of Gal80 expression patterns has been hindered by the lack of a sensitive antibody against Gal80. Functional, tagged versions of Gal80 have been reported [40] but have not yet found general use in *Drosophila*.

Subtractive restriction in recombinase-based systems

Farago *et al.* [9••] have implemented a recombinase-based strategy for subtractive restriction that is conceptually similar to the intersectional technique also developed by Dymecki's group. The basic principle of this strategy, illustrated in Figure 3B, involves incorporating the transgene of interest into the second stop cassette. Removal of the first cassette by recombinase activity in the P1 pattern activates transcription of the transgene, but wherever this pattern overlaps with that of P2, the second recombinase excises the transgene and abolishes expression. Transgene expression is thus limited to those cells of P1 not included in P2. The strategy actually used in the fate-mapping studies of the mouse nervous system by Farago *et al.* [9••], was, in fact, somewhat more elegant than the one just described in that it combines both intersectional and subtractive restriction in the same mouse as shown in Figure 3C. For subtractive restriction in this system, as in the recombinase-based intersectional gene targeting system, the two promoters that direct recombinase expression do not have to be active at the same time.

Conclusions and prospects

Techniques for restricting the expression of transgenes to subsets of cells within a group of interest are clearly in their infancy. The most recent methods have been successfully implemented only once and will require further validation. Research in the near term will sort out not only which of these methods are most fruitful, but also which can be successfully coupled to other techniques to provide even higher degrees of spatial and/or temporal resolution of transgene expression. In *Drosophila*, the compatibility of the various Gal4-based methods of intersectional and subtractive restriction should allow them to be used in combination to achieve higher level restriction. In mice, combining dual recombinase approaches with the Tet-on/off system or with conditional Cre constructs (see reference [41]) also may enable refined spatial and temporal control of transgene expression.

Cross-fertilization of approaches between genetic model organisms also should foster progress. The dual recombinase techniques developed in mice can, in principle, be implemented in *Drosophila*, and the Gal80 and Split Gal4 techniques developed in *Drosophila* should be adaptable for use in zebrafish, if not in mice, where the Gal4-UAS system has gained little traction (but see reference [42]). Moreover, approaches that are conceptually similar to the Split Gal4 system may prove tractable in other organisms. Two split Cre approaches already have been tested *in vitro* [43,44], and a third recently has been used to mediate recombination in transgenic mice [45].

Finally, further techniques probably will be needed as neurobiologists probe the formation and function of the nervous system at ever finer levels of cellular resolution. Tools that should lend themselves to second generation methods of restriction include split inteins [46] and chemical inducers of dimerization [47], both of which naturally serve the needs of intersectional techniques and have spawned innovative recent applications [48•]. New recombinases, such as that from phage ΦC31 [49] also promise to increase the combinatorial power of recombinase-based techniques, which can be incrementally augmented simply by adding more stop cassettes in front of a transgene, each capable of excision by a unique recombinase. In addition, subtractive techniques may benefit from the introduction of novel methods of negative regulation, involving adaptations of the lacO/lacI repressor system [50], inducible protein degradation [51], or use of RNAi or miRNA constructs.

As more techniques for restricting transgene expression emerge, the question of which type of restriction best suits an experimenter's needs will continue to be dictated by the type of observation or manipulation required. In general, subtractive methods are most useful when the subset of neurons to be excluded is known and a defined promoter that expresses within them exists. Intersectional techniques, on the contrary, can be used to positively select cells that contribute to developmental or physiological processes, a feature that should prove increasingly useful in identifying and characterizing unknown neuronal substrates of such processes. These are early days, and as existing techniques are tested and new ones emerge, it will be good to have more, rather than fewer, choices. The good news for neurobiologists is that in the field of restriction, the number of choices is growing rapidly.

(Figure 2 Legend) followed by the sequence encoding the second transgene. Subtractive restriction thus occurs in the same manner as depicted in (B), as indicated by the left-hand pathway and in gray at the bottom. Similarly, intersectional restriction occurs in the same manner as depicted in Figure 2C, as indicated by the right-hand pathway and in black at the bottom.

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- • of special interest
- •• of outstanding interest

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This paper elegantly extends the intersectional 'dual recombinase' method introduced by the Dymecki group in reference [29], to permit both subtractive and intersectional restriction of reporter transgene expression. As in the first paper, the authors target groups of embryonic progenitor cells to elucidate the specification of structures in the mouse brain. Cell fates at the intersection were tracked by monitoring the expression of EGFP and WGA (from a bicistronic gene), and within the exclusive ('unsubtracted') group by nLacZ. Development of this system to permit expression of more general effectors should be possible (as outlined in reference [5]). The CAG-driven 'dual responsive indicator transgene' was knocked into the ROSA26 (R26) locus to insure the broadest possible expression.

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This paper provides a good example of the construction and *in vivo* application of a split effector molecule. The authors fuse caspase subunits (from both worms and humans) to anti-parallel leucine zippers that permit them to associate if expressed in the same cell. They illustrate intersectional cell killing in *C. elegans* by selectively ablating touch receptor neurons.

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This paper introduces a technique for reversibly silencing neuronal activity using a glutamate-and ivermectin (IVM)-gated chloride channel encoded by two subunits. As the authors note, intersectional neuronal silencing should be achievable if the two subunits are targeted to different but overlapping groups of neurons.

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This paper describes the development and deployment *in vivo* of a split luciferase molecule. A notable feature is the use of a previously developed 'conditional splicing system' (CPS) based on an artificially split intein, the splicing activity of which is contingent upon the binding of the small molecule, rapamycin. The authors demonstrate that rapamycin can rapidly trigger the reconstitution of active luciferase by ligation of two inactive luciferase fragments in *Drosophila* neurons that express the *timeless* gene. Intersectional luciferase targeting should be possible using this system.

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As described in detail in the text, this paper introduces a fully functional and versatile technique for intersectional restriction of transgene expression in *Drosophila*, based on the use of a Split Gal4 molecule. The method is particularly promising because it can interface with other Gal4-based technologies, including existing UAS-transgenes, Gal80 and Gal80ts lines, and UAS-STOP-transgene lines that use the 'flp-out' technique for further restriction of expression.

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