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Intersectional Illumination of Neural Circuit Function

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In this issue of *Neuron*, Madisen et al. (2015) report the construction of several new transgenic mouse lines that apply intersectional genetic tools to achieve high levels of expression and cell-type specificity, providing a useful resource for future studies.

The development of molecular tools to anatomically map, functionally manipulate, and record the activity of genetically defined subpopulations of neurons has revolutionized neuroscience (Luo et al., 2008). It is now possible in a variety of organisms to deconstruct complex neural circuits into their constituent components and to study each part's anatomy, physiology, and function in isolation. Many neuroscientists believe that this reductionist approach will result in a mechanistic understanding of how brains compute, learn, and produce behavior. A necessary component of this approach is methods to target the expression of genes encoding these molecular tools to specific groups of neurons.

The most common method is to inject viral vectors that encode molecular tools. In the mouse, this is often used in conjunction with transgenic lines that express the site-specific recombinase Cre in specific cell populations. While offering high-level expression and spatial control, virally delivered tools suffer from several problems that can introduce significant uncontrolled variability into experiments: it is difficult even with stereotactic surgery to repeatedly infect exactly the same population of cells; viral titer varies from batch to batch, affecting the efficacy of infection and expression; and long-term viral infection may affect cell health. One solution to these problems is the use of transgenic mouse lines that heritably express a molecular tool in a specific pattern.

The simplest approaches use a genomic locus or promoter to directly express a molecular tool in a specific spatiotemporal pattern as a one-component transgenic (Table 1, left). Different approaches to generating one-component transgenic lines trade off simplicity for specificity. The simplest approach uses zygotic pronuclear microinjection of recombinant DNA that is then randomly integrated into the genome as a transgene with variable copy numbers. The transgene can contain just a short promoter or enhancer sequence directly driving a molecular tool gene or a more complex bacterial artificial chromosome (BAC) containing a molecular tool gene embedded in an endogenous gene's *cis*-regulatory elements to better mimic that gene's expression pattern (Gong et al., 2003). The most specific but also most labor-intensive method reproduces endogenous expression patterns by knocking the coding sequence of a molecular tool into single genomic loci through homologous recombination in embryonic stem (ES) cells.

One-component approaches suffer from several drawbacks. A major problem is the lack of flexibility: a separate mouse line has to be generated for each combination of molecular tool and targeted cell type. In addition, the endogenous *cis*-regulatory elements surrounding the transgene have a strong effect on the transgene's cell-type specificity, regulability,

Table 1. Comparisons of Different Transgenic Approaches to Access Cell Type

	One Component	Two Component	Three Component
Convenience	+++	++	+
Ease of construction	+++ (Random transgenes) ++ (BAC) + (Knockin)	+ (*Rosa26* knockin) ++ (RMCE at *Rosa26* or *TIGRE* loci[a]) +++ (integrase-mediated transgenesis)	+ (*Rosa26* knockin) ++ (RMCE at *Rosa26* or *TIGRE* loci[a]) +++ (integrase-mediated transgenesis)
Cell-type specificity	+ (Determined by promoter specificity and integration site)	++ (Determined by specificities of driver and responder lines)	+++ (Intersectional targeting[a])
Expression level	+ to +++ (Determined by promoter specificity and integration site)	++ (e.g., *Rosa26-CAG*) +++ (e.g., *TIGRE-TRE*[a])	++ (e.g., *Rosa26-CAG*) +++ (e.g., *TIGRE-TRE*[a])
Regulability	–	++ (e.g., *Rosa26-CAG-LSL*)	++ (e.g., *Rosa26-CAG-LSL*) +++ (e.g., *TIGRE-TRE-LSL*[a] + Cre line)
Flexibility	–	++	+++ (But limited by availability of tTA and Flp/Dre driver lines)

Abbreviations: BAC, bacterial artificial chromosome; *CAG*, CMV early enhancer/chicken beta-actin/rabbit beta-globin; *LSL*, *loxP-STOP-loxP*; RMCE, recombinase-mediated cassette exchange; *TIGRE*, tightly regulated response element; *TRE*, tetracycline response element; tTA, tetracycline-regulated trans-activator.
[a]New tools in Madisen et al. (2015).

and expression level in transgenes produced by all three methods. In particular, there is a great deal of variability in random transgenics due to differences in transgene copy number and in the genomic environment surrounding the insertion site. Random transgenes containing a short promoter fragment can yield high expression levels when used with a strong promoter (such as that of *Thy1*) and can trap specific populations of neurons (Feng et al., 2000) but are particularly susceptible to random integration effects. A more versatile approach is to decouple which molecular tool is utilized from where it is expressed.

The two-component approach (Table 1, middle) splits the responsibility for "where" and "what" into a driver transgene and a responder transgene. The previously mentioned Cre-driver lines are examples of driver transgenics: rather than directly expressing a molecular tool, these lines express Cre in specific patterns that determine in which cells a responder transgene can be expressed. Responder transgenes contain a molecular tool at a different locus under the control of a well-characterized promoter, often conferring ubiquitous high-level expression. For example, placing the *loxP-STOP-loxP* (*LSL*) sequence between a strong promoter and the coding sequence causes the target gene's expression pattern to mimic that of Cre but at high levels. A similar effect can be achieved with other site-specific recombinase systems such as Flp/*FRT*, or by placing a gene under the control of a tetracycline response element (*TRE*) so that it is regulated by the transcription factor tetracycline-regulated trans-activator (tTA).

The main advantages of two-component systems are flexibility, regulability, and potentially higher and more consistent expression. Different molecular tools can be expressed in the same population of neurons by simply breeding different driver and responder lines together. To generate new responder lines, genes containing molecular tools can be targeted to a known permissive locus that allows for recombinase- or tTA-regulated transgene expression in many cell types without positional effect. Finally, exogenous promoters can be used to express a molecular tool at consistently high levels in whichever cells the driver permits. This approach would appear promising but has suffered from several practical limitations.

First, and perhaps most importantly, many Cre-driver lines only coarsely define the cell type of interest and so could benefit from further refinement. Indeed, it is debatable whether the expression of a single gene is sufficient to define a cell type. It is possible to overcome this broader cell-type specificity through the use of intersectional approaches that make transgene expression dependent on the simultaneous presence of two site-specific recombinases or transcriptional activators driven by different genes. Although most applications use intersectional regulation to create a genetic AND gate, two recombinases or transcriptional activators can gate gene expression in the form of any Boolean logical operations—OR, NOT, XOR, etc. For example, in *Drosophila* intersectional targeting of split-Gal4 drivers can yield breathtaking levels of specificity, such as targeting of individual bilateral neurons with defined roles in sensory processing or behavior (Aso et al., 2014). This approach has been implemented in mice using combinations of viruses containing different recombinases and multiple-recombinase-regulated molecular tools (Fenno et al., 2014). However, investigators wishing to use transgenic mice were limited to whatever population happened to be targeted due to a paucity of intersectional responder lines.

Second, generating high-quality transgenic responder lines is currently difficult and expensive. Most existing Cre-responder mice utilize the permissive *Rosa26* locus in conjunction with a strong, ubiquitous *CAG* promoter (Zong et al., 2005), which is targeted through homologous recombination in ES cells. Homologous recombination is a low-efficiency process, making the generation of these mice slow and laborious. To increase the efficiency of genomic targeting, an approach based on recombinase-mediated cassette exchange (RMCE) was developed that allows for significantly

higher transgene integration efficiency into a single genomic locus in ES cells. An even more efficient approach utilizes an integrase for site-specific trangenesis in zygotic pronuclei but has not yet been widely used for responder mouse construction (Tasic et al., 2011).

Third, effectors and sensors such as ChR2 and GCaMP require high levels of expression that have historically been difficult to achieve using transgenics. Viruses can multiply in infect cells to create multiple simultaneously expressed molecular tool genes, whereas transgenes ideally only exist at one genomic locus to minimize positional effects. Even the *Rosa26-CAG* combination cannot drive sufficiently high gene expression for many applications. The tTA/*TRE* binary system can in principle yield higher levels of transgene expression through transcriptional amplification but is unreliable for inducible control of transgene expression because transgenes containing the *TRE* promoter tend to become silenced in many genomic loci, including *Rosa26* (Tasic et al., 2012). In short, new tools were necessary to make mouse transgenic tools reach their full potential for dissecting neural systems.

Madisen et al. (2015) has taken a large step toward overcoming these three limitations associated with mouse transgenic responder lines. They create several three-component systems (Table 1, right) that allow for stable molecular tool expression at high levels in any cell type, with highly specific targeting through the intersection of different genetic markers. These tools are built using RMCE to ease the development of additional responder lines in the future.

First, they develop new Flp/Cre and Dre/Cre (Dre is yet another site-specific recombinase) intersectional responder lines in the *Rosa26* locus, as well as new Flp and Cre driver lines targeting commonly studied neuronal populations such as Parvalbumin+ interneurons. These lines enable highly specific expression of various molecular tool genes in any cell type, although it should be noted that their use is currently limited by a paucity of available Flp and Dre driver lines compared to the abundance of Cre lines.

Second, they generate tTA/Cre responder mice to simultaneously achieve high levels of expression and intersectional targeting of neuronal populations specified by tTA and Cre expression. Madisen et al. (2015) show that a previously discovered permissive locus called *TIGRE* (Zeng et al., 2008) is suitable for use with the tTA/*TRE* and that tTA regulation of this locus yields significantly higher levels of expression than the widely used *Rosa26-CAG* promoter combination, comparable to that of virally encoded reagents.

Finally, they generate a variety of new responder lines that have the potential to be widely useful for the study of mouse development, function, and anatomy. These new lines allow for high expression of some of the latest sensors and effectors: the calcium sensor GCaMP6, the red-shifted optogenetic silencer Jaws, and as-yet less commonly used tools such as genetically encoded voltage and glutamate sensors.

What experiments do these new tools make possible?

We have only the barest notion of how the time varying activity of neurons wired into circuits produces behavior. Although anathema to some neuroscientists trained in the hypothetico-deductive tradition, a hypothesis-free approach might be useful in cracking open this black box. This approach has yielded great results in many other areas of biology. For example, the systematic application of forward genetic screens in yeast, *C. elegans* and *Drosophila* has revealed the basic logic and genetic players of many complex biological processes, from cell division to morphogenesis. A similar logic has recently been applied to study *Drosophila* neural circuits with spectacular results. By performing behavioral screens using large collections of fly lines labeling specific subsets of neurons, in combination with genetically encoded neuronal activators and silencers, investigators have revealed the involvement of individual neurons in specific behaviors such as aggression, mating, and locomotion, as well as the general anatomical and functional logic of such complex processes as associative learning (Venken et al., 2011; Aso et al., 2014). The new tools developed in Madisen et al. (2015) have the potential to enable two types of screens—behavioral and physiological—to be carried out in mice, albeit on a more limited scale.

By targeting optogenetic activators and silencers to specific populations of cells and expressing them at high enough levels to be effective, these new mice make the use of optogenetic "behavioral screens" possible in mice. These screens would systematically test the necessity and sufficiency of a specific type of cell in multiple brain regions in the context of behavior. Transgenic mice expressing ChR2 in all inhibitory interneurons have already been successfully utilized to perform a functional screen to determine which cortical regions are necessary for somatosensory-based decision making (Guo et al., 2014). That approach was not cell type specific, making it difficult to conclude much beyond the involvement of a certain brain region in a behavior. The activity of different types of neurons intermingled in the same brain area can produce dramatically different behaviors (Hong et al., 2014), highlighting the need for increased cell-type specificity in performing and interpreting causal manipulations.

The absence of any responder lines that express chemogenetic effectors (Sternson and Roth, 2014) remains an unfortunate lacuna in the transgenic toolbox. Tissue scatters and absorbs light, making it impossible to optogenetically modulate cells deep in the mouse brain without invasive surgery and difficult to activate or silence widely distributed neurons. Chemogenetic lines would better enable silencing or activation of specific cell types throughout an intact brain with systemic administration of the chemogenetic protein's ligand. These tools would particularly take advantage of the increased cell-type specificity that intersectional methods afford, since spatial targeting of cells to silence or activate would no longer be possible.

While causal tools are useful for delineating which cells are involved in a behavior, they do not reveal how those cells encode relevant information while performing computations. The new sensor lines in Madisen et al. (2015) will enable "physiological screens" that measure neural coding by specific types of neurons throughout the brain. In many cases, neural computation is the result of coordinated activity by large

ensembles of neurons. Much research in systems neuroscience has correspondingly come to focus on measuring the activity of ensembles of neurons during sensory processing and behavior. Advanced microscopes are now under construction in many labs that enable simultaneous imaging of thousands of neurons extended over a few millimeters of tissue or in multiple brain areas simultaneously in mice (Lecoq et al., 2014). At the extreme, it is now possible in transparent larval zebrafish to simultaneously record the activity of every neuron in the brain of a behaving animal (Keller and Ahrens, 2015). It has also become apparent that in many cases distinct neuronal cell types encode specific pieces of information. This specificity is perhaps most obvious in the retina, where different ganglion cell types encode different aspects of the visual scene but has also been observed in cortex (Gollisch and Meister, 2010; Kepecs and Fishell, 2014).

The high-expressing tTA/*TRE* GCaMP responder mice will enable large-scale recordings for the systematic study of neural coding by specific cell types during sensory processing and behavior in mice. For example, with these mice every neuron in dorsal cortex of mice is potentially optically accessible for recording. By restricting expression to specific types of cells one could determine the role of their population activity in behavior. Cell-type-by-anatomical-region brain activity maps could be constructed in different behavioral contexts, enabling the inductive inference of general rules describing how different neurons interact within local microcircuits and across distributed networks.

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