Expanding the power of recombinase-based labeling to uncover cellular diversity

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SUMMARY STATEMENT

New fluorescent indicator alleles utilize Cre, Flp, and Dre recombinases to label previously inaccessible cell populations. The complete architecture of two cell populations can be visualized in the same mouse.
ABSTRACT

Investigating the developmental, structural, and functional complexity of mammalian tissues and organs depends on identifying and gaining experimental access to diverse cell populations. Here, we describe a set of recombinase responsive fluorescent indicator alleles in mice that significantly extends our ability to uncover cellular diversity by exploiting the intrinsic genetic signatures that uniquely define cell types. Using a recombinase-based intersectional strategy, these new alleles uniquely permit non-invasive labeling of cells defined by the overlap of up to three distinct gene expression domains. In response to different combinations of Cre, Flp and Dre recombinases, they express eGFP and/or tdTomato to allow the visualization of full cellular morphology. Here, we demonstrate the value of these features through a proof-of-principle analysis of the central noradrenergic system. We label previously inaccessible subpopulations of noradrenergic neurons to reveal details of their three dimensional architecture and axon projection profiles. These new indicator alleles will provide experimental access to cell populations at unprecedented resolution, facilitating analysis of their developmental origin and anatomical, molecular, and physiological properties.
INTRODUCTION

Strategies and tools to label cells on the basis of gene expression provide the experimental framework for much of modern biology. By exploiting intrinsic genetic differences that uniquely define cell types, fluorescent labeling renders cells accessible for a wide variety of morphological, molecular, and physiological analyses. Although expression of fluorescent proteins can be controlled directly by cell-type specific promoters, recombinase-based strategies (Dymecki and Tomasiewicz, 1998; Zinyk et al., 1998) constitute the key technology that gives gene-based labeling much of its power and flexibility. The use of a cell-type specific recombinase driver allele in combination with an indicator allele encoding a fluorescent marker permits labeling on the basis of transient gene expression and, in the case of inducible recombinases, temporal control of labeling (reviewed in Jensen and Dymecki, 2014). Despite these refinements, however, labeling on the basis of a single gene defines relatively broad classes of cells (e.g. neurons synthesizing a specific neurotransmitter), and it is clear that this is only the tip of the cellular diversity iceberg.

In mice, access to more narrowly defined cell populations has been achieved using recombinase-based intersectional strategies, in which labeled cells occupy the narrow intersection of two broader gene expression domains (Awatramani et al., 2003). Intersectional labeling is accomplished via an indicator allele that is responsive to two distinct site-specific recombinases controlled by promoters of two different genes. Only those cells that have expressed both recombinases will express the marker encoded by the indicator allele. Additionally, some indicator alleles encode a second marker designed to label the subtractive population (Awatramani et al., 2003; Bang et al., 2012; Farago et al., 2006; Jensen et al., 2008; Yamamoto et al., 2009). The subtractive population consists of the balance of cells defined by one of the gene expression domains, minus the intersectional population (Farago
et al., 2006). A labeled subtractive population provides important context for the intersectional population and, in some experiments, may actually be the population of interest. Intersectional and subtractive labeling can be applied to analysis of any tissue, but it has proven particularly useful in studies of the highly complex mammalian brain. This versatile approach has revealed previously unappreciated genetic and developmental heterogeneity within important neuronal classes (Awatramani et al., 2003; Bang et al., 2012; Cocos et al., 2009; Farago et al., 2006; Jensen et al., 2008; Miyoshi et al., 2010; Robertson et al., 2013; Sakamoto et al., 2014).

Despite the power of the intersectional approach, however, significant gaps remain in the toolkit. As development proceeds, genetically defined cell lineages become progressively subdivided by subsequent gene expression. It is becoming clear that a two-gene intersectional strategy, particularly when applied to the nervous system, provides insufficient resolution to dissect this complexity (Huang, 2014; Poulin et al., 2014). Furthermore, although some existing dual-recombinase responsive indicator alleles have the capability of labeling cell bodies of the intersectional and subtractive populations (Awatramani et al., 2003; Bang et al., 2012; Farago et al., 2006; Jensen et al., 2008; Yamamoto et al., 2009), none are capable of labeling the axonal projections of the subtractive population. This restriction limits our ability to visualize the complete architecture of genetically defined neuronal populations.

Here, we describe a series of recombinase responsive indicator alleles that overcome these deficiencies and significantly extend the utility of the intersectional approach. We have generated a new triple-recombinase responsive fluorescent indicator allele that labels cell populations at the intersection of three independent gene expression domains. By taking advantage of the Dre/rox recombinase system (Sauer and McDermott, 2004), as well as the more commonly used Cre/loxP (Sternberg and Hamilton, 1981) and Flp/FRT (Andrews et al., 1985) systems, this allele offers unprecedented access to genetically defined cell types. A pair
of dual-recombinase responsive alleles allows labeling of Dre/Cre and Flp/Cre intersectional populations. Unlike any previously described indicator alleles, these three new alleles encode two fluorescent proteins that both fill cellular processes, allowing simultaneous tracing of axons of intersectional and subtractive populations. In addition, we have generated single-recombinase responsive derivative alleles, including the first to provide a fluorescent readout of Dre expression.

We have observed expression of these alleles in adult mice and embryos, within and outside the nervous system. In conjunction with new tissue clearing techniques, we have demonstrated the potential of our new indicator alleles by visualizing details of the heterogeneous architecture of the central noradrenergic system. The capability of these new alleles to label previously inaccessible intersectional and subtractive cell populations should prove valuable to a broad range of scientists, and their utility will only increase as more recombinase driver alleles are generated and characterized.

RESULTS

Generation of a new triple-recombinase responsive fluorescent indicator and derivative alleles

To ensure expression of our new triple-recombinase responsive fluorescent indicator allele, RC::RFLTG (Rosa CAG rox FRT loxp tdTomato eGFP), in a wide range of tissues and developmental stages, we used a synthetic CAG promoter (Niwa et al., 1991) and targeted the indicator construct to the Gt(ROSA)26Sor locus (Friedrich and Soriano, 1991) in embryonic stem cells (Fig. 1A). RC::RFLTG expresses tdTomato after Dre-mediated deletion of a rox-flanked transcriptional stop cassette and Flp-mediated deletion of a FRT-flanked stop cassette. eGFP is expressed following Dre-, Flp-, and Cre-mediated deletion of all stop
cassettes and the loxP-flanked tdTomato. To generate dual- and single-recombinase responsive alleles (Fig. 1A) and confirm that the stop cassettes can be efficiently recombined, we crossed RC::RFLTG mice with animals expressing Dre, Flp, or Cre recombinase in the germline. Following recombinase expression in the predicted combinations, we observed ubiquitous fluorescence in intact mouse embryos (Fig. 1B) and a variety of intact organs in adult mice (Fig. S1), confirming that the indicator alleles are broadly expressed both pre- and postnatally. When one or more transcriptional stop cassettes remained intact, no fluorescence was observed in intact samples (Figs 1B, S1).

To confirm the ability to detect eGFP and tdTomato expression at the level of individual cells we crossed our new alleles to recombinase driver lines with restricted expression patterns. In sections examined microscopically, we observed the expected eGFP and tdTomato fluorescence in a variety of neurons in embryos and adult brain (Fig. 2). In the absence of recombinase expression, we observed no tdTomato expression in brain and non-neuronal tissues. However, very faint eGFP was occasionally observed in a few granule cells of the hippocampal dentate gyrus and scattered large cells of the choroid plexus. The absence of tdTomato expression suggests that the faint eGFP does not result from ‘leaky’ transcription through the upstream stop cassettes. This low level expression was virtually undetectable when tissue was labeled using anti-GFP antibody and fluorescently coupled secondary antibody (Fig S2). Even under the most rigorous immunolabeling conditions using the most sensitive enzymatic detection method, it was fainter than in recombinase-expressing cells (Fig. S2). Therefore, it is not expected to limit the use of these new indicator alleles.

To address recent reports of occasional Cre mediated recombination of rox sites in transgenic mice and viral constructs (Fenno et al., 2014; Madisen et al., 2015), we performed a stringent test of this phenomenon. We crossed mice carrying our RC::RG allele, which contains a rox-flanked stop cassette (Fig. 1A), to ACTB-cre (Lewandoski et al., 1997) which
drives Cre expression in every cell of the mouse from the blastocyst stage or earlier. In
double heterozygous offspring, we counted eGFP-labeled cells in every fourth section across
the brain to determine the frequency of Cre-mediated recombination of the rox-flanked stop
cassette. We observed 2-7 labeled cells per mouse (n=4), indicating very low levels of
Cre/rox recombination. Dre-mediated recombination of loxP sites has not previously been
observed in transgenic mice (Madisen et al., 2015) but was reported in the context of highly
expressed viral constructs (Fenno et al., 2014). To confirm that our alleles do not exhibit this
cross-reactivity, we crossed the ubiquitously expressed CAG-Dre driver to a cre-responsive
eGFP indicator generated by germline deletion the rox- and FRT-flanked stop cassettes from
RC::RFLTG. No eGFP-labeled cells were observed (n=4 mice). Thus, the use of RC::RFLTG
and RC::RLTG for intersectional analyses using Cre and Dre will not be limited by cross-
reactivity of these two recombination systems.

Taken together, these results indicate that the promoter of RC::RFLTG and its
derivatives remains broadly active throughout development, and the stop cassettes are
capable of restricting fluorescent protein expression. Therefore, these alleles are useful for
labeling cells in any tissue of embryonic or adult mice for which appropriate recombinase
driver lines are available, revealing their anatomical location and distinct morphology, and
rendering them experimentally accessible.

**New dual-recombinase responsive indicator alleles offer significant advantages over
previously described alleles**

Several previously published Flp/Cre responsive indicator alleles label cell bodies of
intersectional and subtractive populations, but they are unable to label the axons of
subtractive populations (Awatramani et al., 2003; Bang et al., 2012; Farago et al., 2006;
Jensen et al., 2008; Yamamoto et al., 2009). The only previously published Dre/Cre
responsive indicator allele encodes a single fluorescent protein and is therefore incapable of labeling the subtractive population (Madisen et al., 2015). To determine whether our new alleles overcome these deficiencies we applied our new Flp/Cre dual-recombinase responsive allele, \( RC::FLTG \), to analysis of the central noradrenergic system.

We recently used an intersectional genetic fate mapping strategy to determine the embryonic origins of central noradrenergic neurons (Robertson et al., 2013). Using a Flp/Cre indicator allele (Bang et al., 2012), a noradrenergic specific Flp driver line (\( Dbh^{Flpo} \)), and available Cre driver lines, we identified four intersectional subpopulations of noradrenergic neurons that differ in their anatomical location and efferent projection pattern in the adult brain. However, a significant proportion of noradrenergic neurons were not included in any of the four intersectional populations. Therefore, their projections were not labeled by the indicator allele, and we were unable to account for all noradrenergic inputs at target sites.

Here, we use our new Flp/Cre responsive allele, \( RC::FLTG \), in combination with \( En1^{cre} \) (Kimmel et al., 2000) and \( Dbh^{Flpo} \), to visualize projections from all noradrenergic neurons. The intersection of \( En1 \) and \( Dbh \) expression domains (Fig. 3A) defines a subpopulation of noradrenergic neurons encompassing 99.8% of the functionally important locus coeruleus (LC) and a portion of the dorsal subcoeruleus (Robertson et al., 2013), according to an adult mouse brain atlas (Paxinos and Franklin, 2013). This intersectional subpopulation (hereafter designated the LC complex) is a major source of noradrenergic projections to the forebrain. The subtractive population consists of all remaining noradrenergic neurons.

In mice heterozygous for \( En1^{cre} \), \( Dbh^{Flpo} \), and \( RC::FLTG \), we observed fluorescently labeled cell bodies and projections from both the intersectional (\( En1^{cre}/Dbh^{Flpo} \), eGFP-labeled) and subtractive (\( Dbh^{Flpo} \) expression only, tdTomato-labeled) noradrenergic populations (Fig. 3). Co-labeling with antibodies against tyrosine hydroxylase (TH) and
norepinephrine transporter (NET) confirmed that \textit{RC::FLTG} is efficiently recombined by these recombinase drivers (Fig. S3). The eGFP and tdTomato labeling allowed us to readily observe the distinct axonal morphologies of the two populations and their relative contribution to target regions within the same animal. In the brainstem, we were able to observe, for the first time, inputs to the locus coeruleus from all other noradrenergic neurons (Fig. 3B). In the forebrain, we could determine that axons originating in the LC complex are a minority of all noradrenergic inputs in the basolateral amygdala, bed nucleus of the stria terminalis (BNST), and paraventricular hypothalamus (Fig. 3C). The difference between the thin axons originating from the LC complex and the coarser axons with larger varicosities originating from other noradrenergic neurons (Robertson et al., 2013) was striking when the two populations were colabeled in the same tissue (Fig. 3C).

In regions where axons from the LC complex predominate, \textit{RC::FLTG} allowed us to visualize inputs arising from all other noradrenergic populations. In the hippocampus, for instance, we observed a few tdTomato-labeled axons in every brain (n=5) that we examined (Figs 3C, S3). This result contrasts with our previous study, in which we observed only axons from the \textit{En1} -derived LC complex in the hippocampus due to our inability to label projections from all noradrenergic neurons. This minor input to the hippocampus must originate from noradrenergic neurons located outside of the LC. The very small percentage (~0.2%) of LC neurons not derived from the \textit{En1} expression domain is defined by early transient expression of \textit{Hoxa2-cre}, and that noradrenergic neuron population does not project to the hippocampus. Therefore, in \textit{En1}^{cre}; \textit{Dbh}^{Flpo}; \textit{RC::FLTG} brain, the tdTomato-labeled axons in the hippocampus arise from non-LC neurons not included in any of the four intersectional populations defined in our previous study (Robertson et al., 2013).

Taken together, these results confirm that the addition of a second fluorescent protein capable of filling cellular projections constitutes a significant improvement over existing
dual-recombinase indicators. The ability to label, in isolation, a genetically defined neuronal subpopulation and determine where it projects is a critical step in determining its function. Embedding an intersectional subpopulation in the context of a more broadly defined subtractive population permits, within a single animal, comparisons between different neuronal subtypes that previously would have required labor intensive analysis of multiple intersectional crosses using several different Cre driver lines.

**RC::RFLTG permits simultaneous intersectional labeling of cell bodies and axons from two neuronal subpopulations defined by overlapping expression of three genes**

The intersectional strategy is a powerful method for gaining access to neuronal populations defined by complex combinatorial genetic signatures, but it is clear that subtype diversity exceeds the discriminatory power of dual-recombinase responsive indicator alleles (Huang, 2014; Poulin et al., 2014). The characterization of additional recombinase systems such as Dre/rox now offers the possibility of capturing a larger proportion of the genetic heterogeneity in complex organs. To demonstrate the ability of our new triple-recombinase responsive indicator, **RC::RFLTG**, to label cells defined by the intersection of three gene expression domains we again turned to the central noradrenergic system.

As we have shown above, genetic access to the LC complex is achieved via a shared history of *En1* and *Dbh* expression. However, it is well established that locus coerulceus neurons are heterogeneous with respect to axon projection profile, electrophysiological characteristics, and expression of neuropeptides (Chandler and Waterhouse, 2012; Chandler et al., 2013; Chandler et al., 2014; Holets et al., 1988; Olpe and Steinmann, 1991; Xu et al., 1998). Until now, it has not been possible to selectively label subpopulations of the LC complex. With its ability to label cells defined by the intersection of three gene expression domains, **RC::RFLTG** now provides access to these subpopulations and the capability to map
their axonal projections to reveal potential functional differences.

To label two subpopulations of the LC complex based on neuropeptide expression, we utilized a Cre transgene driven by the promoter of the neuropeptide galanin (Gal) (Gong et al. 2007). This transgene is expressed in a subset of locus coeruleus neurons, but also elsewhere in the noradrenergic system and more widely in the brain (McCall et al., 2015; N. Plummer and P. Jensen, unpublished observations). Our initial analysis of the Gal-cre-expressing noradrenergic neurons in Gal-cre; DbhFlpo; RC::FLTG mice demonstrated projections to brain regions that receive axons from the LC complex and from other noradrenergic populations. However, labeling of the entire noradrenergic system in these mice made it impossible to determine which projections arise from the LC complex (Fig. S4). Thus, a triple-recombinase approach was required to visualize projections from the Gal-cre-expressing and Gal-cre-negative subpopulations of the LC complex in isolation from all other cells.

To accomplish this goal, we bred mice heterozygous for RC::RFLTG, Gal-cre, DbhFlpo (Robertson et al., 2013), and En1Dre (N. Plummer, J. De Marchena, and P. Jensen, manuscript in preparation). Although the expression patterns of these three recombinase drivers are very different, spatially and temporally, their intersection exclusively defines a subpopulation of noradrenergic neurons within the LC complex (Fig. 4). The subtractive population consists of the remaining neurons of the LC complex that do not express the Gal-cre transgene, and all neurons outside the LC complex are unlabeled.

In hindbrains of En1Dre; DbhFlpo; Gal-cre; RC::RFLTG quadruple heterozygotes, we observed Gal-cre-expressing (eGFP-labeled) and Gal-cre-negative (tdTomato-labeled) noradrenergic neurons intermingled throughout the LC complex (Fig. 4). As with RC::FLTG, co-staining with TH and NET antibodies confirmed that RC::RFLTG is efficiently recombined by the recombinase drivers (Fig. S3). Next, we examined forebrain sections in
order to observe the axonal projections from these two noradrenergic subpopulations and
determine if they project to distinct target sites. Labeled axons from both subpopulations
were visible running together in the medial forebrain bundle. Surprisingly, we observed eGFP
and tdTomato axons intermingled at all forebrain target sites (Figs 4, S5). These results
suggest that galanin may be required in all regions innervated by the LC complex.

These experiments demonstrate the unique ability of our triple-recombinase
responsive indicator allele to label in isolation subpopulations of cells that have never before
been accessible. Compared to existing indicator alleles, RC::RFLTG is capable of deeper
analysis of the complex gene expression patterns underlying different cell types, and in
combination with our new dual-recombinase responsive alleles, it represents a significant
expansion of the capabilities of the intersectional approach.

New indicator alleles are compatible with new tissue clearing techniques

Recently described techniques for rendering organs from mice and other animals
optically transparent have made it practical to visualize the intact three dimensional structure
of fluorescently labeled cell populations, particularly neurons (Chung et al., 2013; Ertürk et
al., 2012; Hama et al., 2011; Ke et al., 2013; Renier et al., 2014; Yang et al., 2014). Not only
do these methods provide valuable information about the anatomical distribution and long-
range connectivity of the labeled populations, but quantitative methods such as cell counting
can be applied without labor intensive and error prone reconstruction from many different
sections. To realize the full experimental utility of our new alleles, it is important that they be
compatible with these techniques.

To test the compatibility of our alleles with the passive clarity technique (PACT)
(Yang et al., 2014) and our ability to visualize complex neuronal populations in three
dimensions, we cleared whole $\text{Dbh}^{\text{Flpo}}; RC::\text{FLTG}$ embryos at E12.5 and three-millimeter
thick slices of brain from adult $En1^{Dre}; Dbh^{Flpo}; Gal-cre; RC::RFLTG$ mice. In the embryos, we visualized the three dimensional structure of the $Dbh$-expressing sympathetic ganglia. The intact, bilaterally symmetric sympathetic chains were readily observed running on each side of the spinal column (Fig. S6). In the brain slices, the full morphology of the intact LC complex, including the $Gal-cre$-expressing and $Gal-cre$-negative subpopulations was visible (Fig. 5 and Movie S1). These neurons clearly form an unbroken continuum extending from the densely packed locus coeruleus to the more dispersed neurons in the dorsal subcoeruleus. Fluorescently labeled cells in 40-µm thick virtual sections extending at least 500 µm deep in the tissue slice (Fig. 5) could be visualized as clearly as cells in 40-µm thick free floating sections (Fig. 4). In cleared cerebral cortex, we were able to observe axons from both the $Gal-cre$-expressing and $Gal-cre$-negative subpopulations (Fig. 5). These results confirm that the combination of PACT and intersectional labeling using $RC::RFLTG$ or its derivative alleles represents a powerful new method for visualizing the architecture of genetically defined cell types.

**DISCUSSION**

The experiments described here demonstrate that $RC::RFLTG$ and its derivative alleles are a valuable toolkit of fluorescent indicators for noninvasive labeling of cell populations defined by one, two, or three gene expression domains. Our proof-of-principle application of $RC::FLTG$ and $RC::RFLTG$ to analysis of the central noradrenergic system confirms the value of key features of these alleles. There are no molecular markers known to uniquely label subpopulations of noradrenergic neurons, so investigation of noradrenergic neuron heterogeneity, particularly axon projection patterns, depends either on genetic intersectional labeling (Robertson et al., 2013) or strategies for retrograde labeling by injection of dyes or viruses at target sites (Chandler and Waterhouse, 2012; Chandler et al.,
2013; Chandler et al., 2014; Schwarz et al., 2015). The ability of RC::FLTG to label axons of the entire noradrenergic system, subdivided between an intersectional and a subtractive population, reveals diversity of noradrenergic projections at target sites. As demonstrated in the hippocampus, our new allele allowed observation of previously undetected projections, without requiring the labeling of new intersectional populations. RC::RLTG offers the same features as RC::FLTG but adds to the flexibility of the intersectional approach by allowing dual-recombinase labeling with Dre and Cre.

As the first fluorescent indicator allele responsive to three recombinases, RC::RFLTG provides experimental access to previously inaccessible cell populations and thus is capable of detecting greater cellular diversity than any published indicator allele. This unique capability allowed us, for the first time, to visualize two locus coeruleus subpopulations distinguished by Gal-cre-expression in isolation from all other cells. Wide-ranging evidence indicates the importance of galanin in noradrenergic neurons and possible functional differences between galanergic and non-galanergic LC neurons (Sevcik et al., 1993; Miller et al., 1999; Sciolino et al., 2015). However, despite evidence that the LC contains neurons with distinct projection profiles (Chandler and Waterhouse, 2012; Chandler et al., 2013, Chandler et al., 2014), the subpopulations distinguished by Gal-cre expression show no gross difference in their axonal targets. This surprising result is compatible with the recently reported finding that specifically activating the Gal-cre-expressing LC subpopulation has the same effect on aversive behavior as activating the whole LC (McCall et al., 2015).

In addition to revealing the anatomic distribution and axonal projection patterns of neuronal populations, RC::RFLTG and its derivative alleles will render labeled cells accessible for morphometric analyses, electrophysiological recording, gene expression profiling, and sorting from acutely dissociated tissue. With the rapidly expanding use of Dre and Flp recombinases, our new Dre and Flp responsive alleles, RC::RG and RC::FG, are
important additions to the genetic toolkit. These single-recombinase responsive alleles label with eGFP all cells that have a history of Dre or Flp expression, permitting rapid characterization of the driver lines used in more complex intersectional experiments. \textit{RC::RLTG} and \textit{RC::FLTG} also function as Dre or Flp single recombinase indicators, respectively, with readout of tdTomato. Therefore, our alleles permit flexibility in the choice of fluorophores for experiments combining the indicator alleles with other fluorescent molecules. \textit{RC::RLTG} and \textit{RC::RG} are the first indicator alleles to provide a fluorescent readout of Dre expression.

Increased use of intersectional strategies for labeling and functional manipulation of restricted cell populations (Duan et al., 2014; Fenno et al., 2014; Hermann et al., 2014; Kim et al., 2009; Madisen et al., 2015) will undoubtedly lead to development of an array of new Dre and Flp driver lines to rival the existing inventory of Cre lines. However, in the absence of mouse lines with the desired recombinase expression pattern, one or more of the recombinases can also be delivered virally, rendering \textit{RC::RFLTG} and its derivatives broadly useful for further dissection of cell types that have been defined by dual- or single-recombinase strategies. Combining our new indicator alleles with stereotaxic injections of a recombinase-expressing viral vector will permit access to subsets of genetically defined cell populations that are distributed among more than one anatomical location. Alternatively, retrograde-transported viruses (Kato et al., 2011; Soudais et al., 2001) can be used in the nervous system to subdivide populations on the basis of axonal target sites.

In summary, we have generated, characterized, and demonstrated the use of a new suite of mouse lines for labeling genetically defined cell populations at any stage of development. The expression of eGFP and tdTomato from these alleles is capable of revealing cell morphology, including the axonal projection profile, of two neuronal subpopulations simultaneously, and they are compatible with tissue clearing techniques.
While these features make our new alleles particularly useful in neuroscience research, they will be important tools for dissection of developmental and genetic complexity in a wide range of biological systems.

MATERIALS AND METHODS

Generation of new mouse strains

The pRC-RFLTG targeting vector was generated by insertion of a CAG promoter, rox-flanked His3-SV40 stop cassette, FRT-flanked His3-SV40 stop cassette, loxp-flanked tdTomato-stop, and eGFP cDNA into the \( \text{Gt(ROSA)26Sor} \) targeting vector pAi9 (Madisen et al., 2010) after digestion with PacI and FseI (see Supplementary Materials and Methods). Linearized pRC-RFLTG was electroporated into G4 embryonic stem cells (George et al., 2007) obtained from the Lunenfeld-Tanenbaum Research Institute, Mt Sinai Hospital, Toronto, CA. Homologous recombinants were identified by long range PCR using the Expand Long Range dNTP Pack (Roche Diagnostics, Indianapolis, IN) and primers specific for the 5’ and 3’ ends of the recombinant locus. Southern blots probed for the neomycin resistance gene were used to confirm absence of random integrations. The karyotypes of recombinant clones were assessed, and several clones were transfected with pPGKPhiC31obpa (Raymond and Soriano, 2007) to remove the attB-attP-flanked Neo cassette. The clones were then injected into B6(Cg)-\( Y \text{tr}^{-2}/J \) blastocysts to produce chimeric mice, and male chimeras were bred to female C57BL/6J mice to establish the \( \text{RC::RFLTG} \) mouse line.

The \( \text{RC::FLTG} \) derivative strain was established by breeding \( \text{RC::RFLTG} \) chimeras with B6;129-Tg(CAG-dre)1Afst mice (Anastassiadis et al., 2009) (\( \text{CAG-Dre} \)) which express Dre recombinase in the germline. \( \text{R/C::RLTG} \) was established by crossing \( \text{RC::RFLTG} \) with
B6.Cg-Tg\(ACTFlpe\)9205Dym/J mice (Rodriguez et al., 2000) \(ACTB-Flpe\) which express Flp recombinase in the germline. \(RC::RG\) and \(RC::FG\) mice were generated by crossing B6;FVB-Tg\(ACTB-cre\)2Mrt mice (Lewandoski et al., 1997) \(ACTB-cre\) with \(RC:RLTG\) and \(RC::FLTG\), respectively. Each derivative strain was maintained by back-crossing to C57BL/6J mice. \(RC::RFLTG\) (Stock No. 026930), \(RC::RLTG\) (Stock No. 026931), and \(RC::FLTG\) (Stock No. 026932) mice are available from the Jackson Laboratory, Bar Harbor, ME.

**Experimental crosses**

To test expression of \(RC::RFLTG\) and its derivatives in various adult tissues and during embryonic development, we first deleted both the rox- and FRT-flanked stop cassettes by crossing \(RC::RFLTG\) with an animal hemizygous for both \(CAG-Dre\) and \(ACTB-Flpe\). In offspring inheriting all three transgenes, tdTomato demonstrates expression of the recombined indicator allele. Mice carrying the recombinant allele were then crossed with \(ACTB-cre\) hemizygotes by timed mating, and embryos were collected at E11.5 to demonstrate expression of eGFP after cre-mediated excision of the tdTomato cassette. Breeding \(En1^{Dre}\); \(Dbh^{Flpo}\); \(Gal-cre\); \(RC::RFLTG\) quadruple heterozygotes required several crosses. First, B6;129-\(Dbh^{tm1(Flpo)Pjen}\) mice (Robertson et al., 2013) \(Dbh^{Flpo}\) were crossed with \(RC::RFLTG\) mice, and offspring were back-crossed to generate animals heterozygous for \(Dbh^{Flpo}\) and homozygous for the indicator allele. Mice heterozygous for the \(En1^{Dre}\) knock-in allele (N. Plummer, J. de Marchena, and P. Jensen, manuscript in preparation) were crossed with STOCK Tg\(Gal-cre\)K187Gsat hemizygotes (Gong et al., 2007) \(Gal-cre\); MMRRC Stock #031060-UCD), and offspring were back-crossed to \(En1^{Dre}\) to generate animals hemizygous for \(Gal-cre\) and homozygous for \(En1^{Dre}\). Finally, \(Dbh^{Flpo}\); \(RC::RFLTG\) mice were crossed with \(En1^{Dre}\); \(Gal-cre\) mice to generate the \(En1^{Dre}\); \(Dbh^{Flpo}\); \(Gal-cre\); \(RC::RFLTG\) quadruple
heterozygotes and control genotypes. En1^cre; Dbh^Flpo; RC::FLTG and Hoxb1^cre; Dbh^Flpo; RC::FLTG triple heterozygotes were generated by crossing B6.Cg-En1^tm2(cre)Wrst heterozygotes (Kimmel et al., 2000) or B6;129-Hoxb1^tm1(cre)Og heterozygotes (O'Gorman, 2005) (Jackson Laboratory Stock #012373) with mice heterozygous for Dbh^Flpo and homozygous for RC::FLTG. B6.Cg-Pvalb^tm3.1(dreo)Hze/J heterozygotes (Madisen et al., 2015) (Pvalb^Dre; Jackson Laboratory Stock #021190) were crossed with RC::RLTG to generate double heterozygotes.

All mice were maintained on a 12/12-hour light/dark cycle with food and water ad libitum, and either singly or group housed. Both male and female mice were examined. Unless otherwise indicated, all mice examined were adults 5-weeks or older. Sample sizes were not pre-determined statistically but are similar to those reported previously (Bang et al., 2012; Robertson et al. 2013). Investigators did not collect data blind to genotype, because the fluorescent labeling in each cross and genotype is so distinctive that they would be easily recognized. All mouse experiments were performed with approval of the NIEHS Institutional Animal Care and Use Committee.

**Imaging of native fluorescence from new indicator alleles**

For imaging native eGFP and tdTomato fluorescence, embryos were fixed overnight by immersion in 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS) at 4 °C. Adult mice were anesthetized with sodium pentobarbital and transcardially perfused with PBS followed by 4% PFA in PBS. After dissection, the brains and other organs were postfixied overnight in 4% PFA at 4 °C and rinsed in PBS before equilibration in 30% sucrose in PBS for 48 hours at 4 °C. Before sectioning on a Leica CM3050 S cryostat (Leica Biosystems, Buffalo Grove, IL), the cryoprotected samples were embedded in Tissue Freezing Medium (General Data Company, Cincinnati, OH). 40-µm thick free-floating
sections were mounted on microscope slides and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA) for imaging.

**Antibodies and Immunohistochemistry**

For immunohistochemistry, mice were perfused and sections collected as described above. Immunofluorescent labeling was performed on 40-µm thick free-floating coronal or sagittal sections. tdTomato-expressing neurons were detected using rabbit anti-dsRed primary antibody (1:1000; Cat.# 632496, Clontech Laboratories, Mountain View, CA) and Alexa Fluor 568 goat anti-rabbit secondary antibody (1:1000; Cat.# A11036, Life Technologies, Grand Island, NY), and eGFP-expressing neurons were detected using chicken anti-GFP primary antibody (1:10,000; Cat.# ab13970, Abcam, Cambridge MA) and Alexa Fluor 488 goat anti-chicken secondary antibody (1:1000; Cat.# A11039, Life Technologies) (Robertson et al., 2013). For tissue cleared by the passive clarity technique (see Supplementary Materials and Methods), the rabbit anti-dsRed (1:500) and chicken anti-GFP (1:1000) primary antibodies were detected using Alexa Fluor 568 donkey anti-rabbit F(ab’)_2 fragments (1:500; Cat.# Ab175694, Abcam) and Alexa Fluor 488 donkey anti-chicken F(ab’)_2 fragments (1:500; Cat.# 703-546-155, Jackson ImmunoResearch Laboratories, West Grove, PA). Noradrenergic identity of labeled axons was confirmed using mouse monoclonal anti-NET (1:1000, clone NET-05; Cat.# 1447-NET, PhosphoSolutions, Aurora, CO) (Matthies et al., 2009) and Alexa Fluor 633 goat anti-mouse secondary antibody (1:1000; Cat.# A21052, Life Technologies). Noradrenergic identity of fluorescently labeled neuronal cell bodies was confirmed using mouse monoclonal anti-TH (1:500, clone 185; Cat.# GTX10372, GeneTex, Irvine, CA) (Lundgren et al., 2011) and Alexa Fluor 633 goat anti-mouse secondary antibody. After staining, sections were mounted on microscope slides for imaging. For 4’,6-diamidino-2-phenylindole (DAPI) staining of nuclei, slide-mounted sections were incubated in 1 µg/ml
DAPI in PBS for one minute, followed by two five-minute washes in PBS prior to cover slipping.

For the highest sensitivity detection of faint eGFP expression in dentate gyrus and choroid plexus cells, we used immunoperoxidase labeling on 40-µm free-floating sections. We used the chicken anti-GFP antibody (1:10,000) in conjunction with a biotinylated goat anti-chicken secondary antibody (1:500; Cat.# BA-9010, Vector Laboratories). To detect immunoreactivity, we used the Vectastain Elite ABC kit and Vector SG substrate (both Vector Laboratories). To allow for maximal staining, the sections were incubated in the Vector SG solution for 15 minutes, the longest incubation time recommended by the manufacturer. For comparison of staining in noradrenergic neurons, dentate gyrus, and choroid plexus, free-floating sections from the same brain were stained in the same experiment and then imaged using identical exposure settings.

Assessment of cross-reactivity between Cre/loxP and Dre/rox recombinase systems.

To assess the frequency of Cre-mediated recombination of the rox-flanked stop cassette, we generated mice hemizygous for \textit{ACTB-cre} and heterozygous for \textit{RC::RG}. In these mice, eGFP indicates recombination of rox sites by Cre. To assess Dre-mediated recombination of loxP sites, we crossed \textit{RC::RFLTG} with \textit{CAG-Dre} and \textit{ACTB-Flpe} to excise the rox- and FRT-flanked stop cassettes. After backcrossing to C57BL6/J, the mice were again crossed to \textit{CAG-Dre}. In these mice, eGFP indicates recombination of loxP sites by Dre. After perfusion, each brain was completely sectioned into four sets of 40-µm thick sections spanning the entire brain, and immunofluorescent labeling was performed as described above. eGFP-expressing cell bodies were counted in one full set of sections for each brain.
Digital Image Processing

Images of whole embryos and adult organs were collected on a Zeiss SteREO Lumar.V12 stereomicroscope (Carl Zeiss Microscopy, Thornwood, NY). Immunoperoxidase labeled sections were imaged on a Zeiss Axio Imager Z2. Images of fluorescently labeled sections were collected on a Zeiss LSM 710 or 780 inverted confocal microscope. After z-stacks were converted to maximum intensity projections using Zen 2012 Black Software (Carl Zeiss), the images were modified only by adjusting brightness and contrast to optimize the full dynamic range of the fluorescence signal of the entire image using Photoshop software (Adobe Systems, San Jose, CA). The location of imaged neurons was determined by reference to a mouse brain atlas (Paxinos and Franklin, 2013). Adult brain tissue cleared by PACT was imaged on a Zeiss LSM 780 microscope, and images of locus coeruleus cell bodies were compiled into three-dimensional structures and processed (background subtraction and attenuation correction) using Imaris Software (Bitplane). Images of axon fibers from PACT cleared tissue were also acquired on a Zeiss LSM 780, after which images were first processed with ImageJ software (US National Institutes of Health) by simply multiplying the image times itself to increase the fluorescence signal above the background and then run through a smoothing filter before being imported into Imaris (Bitplane) where 3D volume rendering was performed. E12.5 embryos cleared by PACT were imaged on a Zeiss LSM 780, and images were imported into Imaris for cropping and 3D rendering.
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AUTHOR CONTRIBUTIONS

NWP and PJ designed the targeting vectors. NWP generated all new mouse lines. NWP, IYE, SDR, and PJ characterized the new lines. JdM provided unpublished reagents. NWP and CJT collected and processed digital images. NWP and PJ wrote the manuscript with input from other co-authors.
REFERENCES


genetic tracing of the input-output organization of a central noradrenaline circuit. 


Fig. 1. A suite of recombinase-responsive fluorescent indicator alleles permits labeling of cells defined by expression of up to three different genes.

(A) The triple-recombinase responsive fluorescent indicator allele, $RC::RFLTG$ (Rosa CAG rox FRT loxP tdTomato eGFP), was generated by targeting intron 1 of the $Gt(ROSA)26Sor$ locus. Rox, FRT and loxP flanked transcriptional stop cassettes in $RC::RFLTG$ (Jackson Laboratory Stock No. 026390) prevent expression of fluorescent protein in the absence of
recombinase activity. The allele will express tdTomato in all cells with a history of Dre and Flp expression, and eGFP in all cells with a history of Dre, Flp, and Cre expression. $RC::RLTG$ (Jackson Lab Stock No. 026931) indicates history of Dre expression (tdTomato readout) and intersectional Dre/Cre expression (eGFP readout). $RC::FLTG$ (Jackson Lab Stock No. 026932) indicates history of Flp expression (tdTomato readout) and intersectional Flp/Cre expression (eGFP readout). $RC::RG$ and $RC::FG$ indicate history of Dre or Flp expression, respectively (eGFP readout). CAG, synthetic CAG promoter (Niwa et al., 1991); STOP, His3-SV40 stop cassette (Sauer, 1993); WPRE, woodchuck hepatitis virus posttranscriptional regulatory element (Zufferery et al., 1999); poly(A), bovine growth hormone polyadenylation signal. (B) tdTomato expression, but no eGFP, is observed in E11.5 embryos after $RC::RFLTG$ is recombined by ubiquitous Dre and Flp expression ($RC::RFLTG; CAG-Dre; ACTB-Flpe$, n=14). eGFP expression is observed after ubiquitous Dre, Flp, and Cre expression ($RC::RFLTG; CAG-Dre; ACTB-Flpe; ACTB-Cre$, n=7). Images show native fluorescence. Scale bar: 2 mm.
Fig. 2. eGFP and tdTomato expression from new indicator alleles permit visualization of individual cells.

Various recombinase lines were used to drive expression of eGFP or tdTomato from \textit{RC::FLTG}, \textit{RC::RLTG}, and \textit{RC::RFLTG} in a variety of neuronal subtypes. \textbf{Left}, eGFP-labeled petrose ganglion in E14.5 embryo (n=2) heterozygous for \textit{RC::FLTG}, \textit{Hoxb1^{cre}} (O’Gorman, 2005), and \textit{Dbh^Flpo} (Robertson et al., 2013). \textbf{Center}, tdTomato-labeled purkinje neurons in mouse heterozygous for \textit{RC::RLTG} and \textit{Pvalb^Dre} (Madisen et al., 2015) (n=4). \textbf{Right}, eGFP- and tdTomato-labeled neurons in the locus coeruleus of mouse heterozygous for \textit{RC::RFLTG}, \textit{En1^{Dre}}, \textit{Dbh^Flpo}, and \textit{Gal-cre} (Gong et al., 2007) (n=10). Images show native fluorescence. Scale bar: 83 µm (cranial ganglion), 53 µm (purkinje neurons), or 100 µm (locus coeruleus).
Fig. 3. Simultaneous labeling of axons from two genetically related neuronal subpopulations permits direct comparison of their projection profiles.

(A) Intersectional labeling of two subpopulations of noradrenergic neurons in En1Cre; DbhFlpo; RC::FLTG mice (n=5) is summarized in schematic sagittal views of the adult mouse brain. Cells with history of En1Cre (blue) or DbhFlpo (yellow) expression occupy domains that overlap in the anterior hindbrain. Noradrenergic neurons with history of both En1Cre and DbhFlpo expression are labeled with eGFP after deletion of the FRT-flanked transcriptional stop cassette and loxP-flanked tdTomato-stop in RC::FLTG. Noradrenergic neurons
originating outside the *Enl* domain express only *Dbh*\(^{F\text{lo}}\), resulting in deletion of the FRT-flanked stop cassette and labeling with tdTomato. Cells that do not express *Dbh*\(^{F\text{lo}}\) are unlabeled due to the presence of the FRT-flanked stop cassette, regardless of Cre expression history. (B) The eGFP-labeled dendritic field of the locus coeruleus seen in coronal section (white box, enlarged on right) receives heavy input (tdTomato-labeled axons) from other populations of noradrenergic neurons. (C) At target sites of noradrenergic projections seen in coronal sections, the proportion of axons originating from the locus coeruleus complex relative to those originating from all other noradrenergic neurons can be observed. The projections from the LC complex are a minority of all noradrenergic axons in the bed nucleus of the stria terminalis (BNST; lateral division, ventral part), paraventricular hypothalamus, and basolateral amygdala. In the hippocampus, rare but consistently observed tdTomato-labeled axons reveal, for the first time, noradrenergic inputs arising from neurons outside the LC complex. In the insular cortex, basolateral amygdala, BNST, and paraventricular hypothalamus, the eGFP-labeled axons from the LC complex are seen to be finer with smaller varicosities compared to the tdTomato-labeled axons of other noradrenergic axons (Robertson et al. 2013). The blue boxes on the line diagrams show the approximate location of the imaged axons. Images show immunofluorescence. Scale bar: 50 µm (locus coeruleus dendritic field and all axon images) or 130 µm (locus coeruleus cell bodies).
**Fig. 4.** Labeling cells defined by the expression of three genes increases cellular resolution, providing access to previously inaccessible cell populations.

Schematic sagittal views of the adult mouse brain summarize expression of recombinases and fluorescent proteins in $En1^{Dre}$; $Dbh^{Flpo}$; Gal-cre; RC::RFLTG quadruple heterozygotes (n=10). Neurons with history of $En1^{Dre}$ (purple), $Dbh^{Flpo}$ (yellow), or Gal-cre (blue) expression occupy domains that overlap in the anterior hindbrain. eGFP expression is restricted to noradrenergic neurons with history of $En1^{Dre}$, $Dbh^{Flpo}$ and Gal-cre expression, which results in deletion of tdTomato and all transcriptional stop cassettes. tdTomato expression is restricted to neurons with history of $En1^{Dre}$ and $Dbh^{Flpo}$ expression, without Gal-cre, which results in deletion of the rox- and FRT-flanked stop cassettes. In sections,
*Gal-cre*-expressing (eGFP) and *Gal-cre*-negative (tdTomato) noradrenergic neurons can be seen intermingled throughout the locus coeruleus. Labeled axons from both populations can be observed in the medial forebrain bundle and at cortical target sites. Locus coeruleus and medial forebrain bundle are shown in a sagittal section, rostral-left. The insular cortex is shown in a coronal section. Images show immunofluorescence. Scale bar: 100 µm (locus coeruleus, sagittal view), 38 µm (medial forebrain bundle, sagittal view), 22 µm (insular cortex, coronal view).
Fig. 5. Neurons and their axonal projections labeled by expression of eGFP and tdTomato from RC::RFLTG can be observed within intact brain tissue cleared by the passive clarity technique.

**Left:** In cleared 3 mm thick coronal slices of $Enl^{Dre}; Dbh^{F loro}; Gal-cre; RC::RFLTG$ quadruple heterozygous brain (n=3), the shape of the LC complex, including the spatial relationship between the dense locus coeruleus and more dispersed subcoeruleus regions, can be seen in a 3D reconstruction. **Center:** Gal-cre-expressing (eGFP) and Gal-cre-negative (tdTomato) neurons of the locus coeruleus are visible in a maximum intensity projection (MIP) compressed along the rostrocaudal axis of a 40 µm thick region beginning 460 µm from the rostral surface of the tissue. **Right:** Neurons of the dorsal subcoeruleus are visible in a 40 µm MIP beginning 280 µm from the rostral surface. The two MIPs are viewed with the medial side of the subpopulation to the left, and their approximate locations are indicated by
vertical lines on the 3D reconstruction. **Bottom:** In a 220 \( \mu m \) MIP from a 3 mm slice of forebrain viewed on the z-y plane, axons from both the *Gal-cre*-expressing and *Gal-cre*-negative subpopulations can be observed running in a rostrocaudal orientation within the cortex. Images show immunofluorescence. Scale bar: 200 \( \mu m \) (entire subpopulation), 86 \( \mu m \) (locus coeruleus and subcoeruleus), 30 \( \mu m \) (cortex).