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A zinc-finger fusion protein refines Gal4-defined neural circuits

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Abstract
The analysis of behavior requires that the underlying neuronal circuits are identified and genetically isolated. In several major model species—most notably Drosophila—neurogeneticists identify and isolate neural circuits with a binary heterologous expression-control system: Gal4–UASG. One limitation of Gal4–UASG is that expression patterns are often too broad to map circuits precisely. To help refine the range of Gal4 lines, we developed an intersectional genetic AND operator. Interoperable with Gal4, the new system’s key component is a fusion protein in which the DNA-binding domain of Gal4 has been replaced with a zinc finger domain with a different DNA-binding specificity. In combination with its cognate binding site (UASG) the zinc-finger-replaced Gal4 (Zal1) was functional as a standalone transcription factor. Zal1 transgenes also refined Gal4 expression ranges when combined with UASGZ, a hybrid upstream activation sequence. In this way, combining Gal4 and Zal1 drivers captured restricted cell sets compared with single drivers and improved genetic fidelity. This intersectional genetic AND operation presumably derives from the action of a heterodimeric transcription factor: Gal4–Zal1. Configurations of Zal1–UASZ and Zal1–Gal4–UASGZ are versatile tools for defining, refining, and manipulating targeted neural expression patterns with precision.

Introduction
For the analysis of neural circuits and behavior, neuroscientists use transgenic techniques to isolate neuronal groups with precision. Neurogeneticists working with the vinegar fly Drosophila melanogaster have developed a sophisticated, versatile toolkit that includes a foundational transcriptional system for mapping and manipulating neural circuits: Gal4–UASG [5]. This system typically uses two fusion transgenes: endogenous fly enhancer sequences are placed upstream of the yeast transcription factor Gal4; effector transgenes are fused to Gal4’s upstream activation sequence (UASG). This arrangement places the effector under the in trans transcriptional control of the enhancer [5]. The Gal4–UASG method has been used for cell-specific genetic rescue, gene overexpression, reporter expression, RNA-interference screens, optogenetic physiology, and many other applications [3, 18]. While this tool is vitally useful, one challenge to dissecting neuron–behavior relationships has been that Gal4-linked enhancers often capture more cells than are functionally relevant. To improve the precision of transgene expression, neural circuit analysis uses a variety of molecular strategies to produce AND and NOT genetic logic, producing expression refinements by intersection. Intersectional methods use either a repressor of Gal4, a targeted recombinase system, a leucine-zipped split-Gal4, or a combination. The native Gal4 repressor, Gal80, is used as a genetic NOT operator to exclude expression from a subset of cells captured by a driver [25]. The flippase (Flp) recombinase specifically excises genomic sequences flanked by flippase recognition target (FRT) sites. In the Flp-out method, Flp is transiently expressed under the control of a heat shock promoter to both generate AND and NOT operations [26]. Stochastic single-cell specificity can be achieved with the ‘mosaic analysis with repressible cell marker’ (MARC) technique [16]. Flp–FRT is also used in the ‘Flippase-induced intersectional Gal80/Gal4 repression’ (FINGR) intersectional method [4], wherein stable, elevated levels of Flp are expressed from an enhancer to add or remove Gal80 expression from a subset of Gal4 driver
cells with some stochasticity [24]. The split-Gal4 method uses a bipartite Gal4 variant, in which a heterodimerization leucine zipper joins the DNA-binding and activation domains; it is active as a transcription factor when both components are expressed in the same cell, producing AND logic between the two half-drivers [17]. A non-intersectional approach to improving cell set specificity uses driver lines constructed with small enhancer fragments instead of large upstream regions [12, 13, 21]. Such genomic fragments contain fewer enhancer modules, so they tend to express in more restricted anatomical ranges: an estimated 4- to 10-fold greater specificity compared with enhancer traps [21].

In light of the extensive Gal4 resources currently available, we aimed to develop an tool that would refine existing Gal4 lines. The DNA-binding domain of Gal4 is a zinc finger that can be substituted with another domain, conferring novel DNA-binding affinity in vitro [22]. We implemented and tested a zinc finger variant of Gal4 that works both as a standalone binary transcription system and as a genetic AND operator in combination with existing Gal4 lines. Using several enhancer sequences associated with particular neurotransmitter systems, we demonstrated that the variant transcription factor -termed Zinc finger-replaced Gal4 (Zal1) can drive expression from a corresponding upstream activating sequence, termed UASZ. When co-expressed in the same cells, Gal4 and Zal1 were active in the presence of a hybrid upstream activation sequence that contained asymmetric binding sites (UASGZ) for the Gal4-Zal1 heterodimer. This method allowed targeting of expression to neurons in which both transcription factor types are expressed. The Zal1-Gal4-UASGZ system will enable the refinement of existing Gal4 lines to isolate precise neuronal types.

Results
Ternary UAS expression system design
Gal4 binds to its cognate upstream activating DNA motif, referred to here as UASG (Fig. 1a). Gal4 can be used to drive specific expression of a responder transgene (e.g. green fluorescent protein, GFP) in defined cell types such as specific Drosophila neurons (Fig. 1b). Pomerantz and colleagues previously designed a transcription-factor fragment that fused the first two zinc fingers of mouse transcription activator EGR1 (previously referred to as ZIF268) with the linker and dimerization domains of Gal4 [22]. In an in vitro study, they showed that the resulting truncated fusion protein, zinc finger Gal4 dimerization 1 (ZFGD1), bound to DNA containing its corresponding UAS (here termed UASZ), a palindromic site with inverted EGR1 finger-binding sites (Fig. 1c). Using the same fusion design as ZFGD1, we generated a gene encoding a full-length transcription factor, zinc finger-replaced Gal4 (Zal1), to be used in vivo to activate genes placed downstream of a UASZ tandem repeat (Fig. 1d). Since heterodimeric ZFGD1 proteins assemble in vitro and specifically bind to hybrid UAS sites in DNA [22], we anticipated that a full-length heterodimeric Gal4/Zal1 transcription factor would form in vivo, bind hybrid sites in the genome (Fig. 1e), and activate a UASGZ-controlled responder in cells where Gal4 and Zal1 are co-expressed (Fig. 1f).

VGlut-Zal1 drives broad UASZ-GFP expression
The vesicular glutamate transporter (VGlut) enhancer was previously used to make a VGlut-Gal4 enhancer-fusion construct; it captures a large—though non-comprehensive— set of glutamatergic cells [10]. Following a similar method, transgenic flies were prepared to carry Zal1 fused to the same VGlut enhancer region [10]. Progeny of VGlut-Zal1 crossed with UASZ-GFP expressed GFP throughout the brain (Fig. 2a–b). The VGlut-Zal1 pattern differed from that of VGlut-Gal4 (Fig. 2c–d). These differences could arise from the expression variation that can arise from genomic insertion sites [19], driver vector design, and possible differences in activity between the two transcription factors. These results demonstrate that Zal1 is functional in the Drosophila brain, albeit with different expression from VGlut-Gal4.

Co-expressed Zal1 and Gal4 drive expression from a hybrid UAS
To explore the utility of Zal1 for expression refinement, we made flies carrying VGlut-Gal4, VGlut-Zal1, and a responder transgene UASZ-GFP. We hypothesized that a heterodimer of the two transcription factors would drive expression of GFP through the UASZ hybrid binding sequence. Flies carrying all three transgenes showed GFP expression in many cells, indicating that the Gal4-Zal1 heterodimer did form in vivo and was functional at the UASZ sites (Fig. 2e–f). There were qualitative differences between GFP expression in the Zal1-Gal4-UASZG homodimer brains and the respective monomer-expressing brains, possibly arising from differences in transgene design. These data verify that Zal1 and Gal4 can activate transcription from a hybrid UASGZ, and thus have the potential to drive expression at an intersection.

VGlut homodimeric lines do not activate non-cognate UAS sites
The specificity of intersectional expression patterns from Zal1-Gal4 combinations is predicated on the specificity of binding to their respective UAS sites: broad cross-reactivity would make an OR operation instead. Possible cross-reactivity was examined in non-cognate UAS/transcription factor controls. A VGlut-Gal4 line were crossed
with a UASZ-CD8::GFP (UASZ-GFP) reporter line. Confocal images revealed almost no GFP expression in the brain, indicating that VGlut-Gal4 by itself does not drive expression from a UASZ responder (Fig. 2g). Similarly, a VGlut-Zal1 line was evaluated by crossing it with UASG-GFP; brain expression in the progeny of these crosses was weak (Fig. 2h), indicating that cross-reactivity is minimal. As previously reported for their in vitro counterparts [22], the present results show that in vivo Zal1 and Gal4 interact with their cognate UAS sites specifically.

To exclude the possibility that homodimeric factors were inappropriately active at the hybrid UASGZ sites, VGlut-Gal4 flies were crossed with UASGZ-GFP. Green fluorescence was low (Fig. 2i), indicating that Gal4 activation from tandem UASGZ sites is poor. Similarly, we examined whether VGlut-Zal1 alone drove robust expression from UASGZ-GFP (Fig. 2j): it did not.

**VGlut-Zal1 restricts the expression breadth of Gal4 lines**

The VGlut-Gal4-dependent activity of VGlut-Zal1 at UASGZ suggested that VGlut-Zal1 could be useful to restrict the cellular range of existing Gal4 transgenes. To test this idea, we examined enhancer trap lines with and without VGlut-Zal1. The Orco-Gal4 line drives expression in a majority of olfactory receptor neurons [15], sending axonal projections to the antennal lobe (Fig. 2k). When Orco-Gal4 was combined with VGlut-Zal1 and UASGZ-GFP, green fluorescence was absent (Fig. 2l). This result likely reflects that VGlut-Zal1 and the cholinergic olfactory-receptor neurons have no overlap. Another line, OK107, drives expression in the mushroom body, the pars intercerebralis and the antennal lobe (Fig. 2m). When this line was crossed with glutamatergic Zal1, the mushroom body and pars intercerebralis were absent: only some antennal-lobe cells and a few dorsal cells remained (Fig. 2n). The same type of experiment was performed on 16 Gal4 enhancer-trap lines [11]. Compared with these lines’ own generally broad expression ranges, the distributions in combination with Vglut-Zal1 were sharply more limited (Additional file 1: Figure S1A–P). Several of the intersectional brains displayed almost no GFP+ cells (NP6235, NP2002), suggesting that Zal1-Gal4 does not produce broadly mistargeted or ectopic responder expression (Additional file 1: Figure S1K & Q).

**Gal4-Zal1 activation is susceptible to Gal80 repression**

We aimed to determine whether the Gal4-Zal1 dimer was repressible by Gal80. The NP4683 enhancer trap line expresses in several areas, including the antennal lobe, mushroom body, the ellipsoid body, subesophageal zone (SEZ) and the ventral nerve cord (VNC) (Additional file 2: Figure S2A). As with other lines, VGlut-ZAL1 intersection produced a reduced expression range; it excluded the mushroom body and antennal lobe expression, but retained GFP in the ellipsoid body, SEZ and VNC (Additional file 2: Figure S2B). The tsh-GAL80 driver represses GAL4 expression in the thoracic and abdominal nervous system [8]. In flies carrying both the ternary system and tsh-GAL80 (tsh-Gal80/UASGZ-GFP; VGlut-ZAL1/NP4683), the ellipsoid body remained brightly GFP+, but the SEZ and VNC expression was diminished (Additional file 2: Figure S2C). These data are compatible with the idea that the Gal4-Zal1 dimer is repressible by Gal80.
These qualitative observations show that Zal1-UASGZ is interoperable with both Gal4 and Gal80, and can limit and refine the expression range of existing lines. However, the glutamatergic system is a challenging target for quantitative analyses of expression: the cells are numerous; and the transporter is predominantly present at the nerve terminals—the α-VGLUT antibody labels cell bodies weakly, rendering their identification and quantification
inaccessible (data not shown). Therefore, we turned to other neurogenic systems to quantify Zal1 performance.

**Crz-Zal1 drives expression in Corazonergic neurons**

As we observed qualitative differences in GFP expression in the intersected heterodimer brains and the respective monomer expressing brains, we aimed to quantify Zal1 performance, for that, we used the Corazonin (Crz) neuropeptide system. The anatomy of these cells is tractable: a Crz-Gal4 line is available; Crz is expressed in just 6–8 cells per hemisphere; and an α-Crz antibody can be used for Crz+ cell identification [6]. To analyze Crz-Zal1 brain expression for comparison with Crz-Gal4, we fused Zal1 to the Crz enhancer region [6]. Control brains carrying the non-cognate driver–responder combinations displayed either GFP levels that were undetectable (UASZ, UASG), or weak (UASGZ, Fig. 3m–p). This expression in Crz-Zal1 > UASGZ-GFP may be due to a mild affinity of Zal1 for the 20 binding half-sites in UASGZ-GFP. In cognate, single-driver combinations, both Crz-Zal1 and Crz-Gal4 drove strong expression in numerous optic-lobe cells, the ventral nerve cord and in ~7 Corazonergic dorsal protocerebral neurons (Fig. 3a–h). This suggested that the two driver types similar patterns. We crossed both drivers with the UASGZ-GFP hybrid reporter, and found that the resulting brains had expression patterns nearly identical to the single-driver lines (Fig. 2i–l). Excluding broad ectopic expression in the optic lobes, Crz-Gal4 has 67% ectopic cells (~15 cells) in the non-optic-lobe brain (Fig. 3a–d, see arrow, Fig. 4). However, this ectopic expression was excluded when Crz-Gal4 and Crz-Zal1 were intersected (Fig. 3 j-l, Fig. 4), indicating that while both the Zal1 and Gal4 drivers have similar extensiveness within Crz+ cells, Crz-Zal1 has better fidelity—and establishes that a Zal1 driver can be used to refine a Gal4 driver pattern. These data further verify the hypothesis that Zal1 is useful as an effective intersectoral transactivator, and support the idea that Zal1 can be used to improve Gal4 driver fidelity.

**Trh-Zal1 drives expression in serotonergic cells**

We tested Zal1 in a third context: the serotonergic system. Serotonin synthesis relies on the Tryptophan hydroxylase (Trh) gene; in a Gal4 fusion, the Trh enhancer region drives expression in nearly ~90 serotonergic cells [1]. We prepared a Trh-Zal1 line and assessed expression in controls: Trh-Gal4 combined with UASZ-GFP was inactive; Trh-Zal1 crossed with UASG-GFP had no measurable expression; and green fluorescence in Trh-Gal4 > UASGZ-GFP flies was undetectable (Fig. 5m–o). Trh-Zal1 > UASGZ-GFP single-driver brains displayed off-target expression in a few cells, presumably from homodimeric Zal1 activation from the hybrid UASGZ sites (Fig. 5p, see arrows).

Compared with the controls, the three cognate driver–responder lines revealed expression patterns that were broad and strong. Trh-Gal4 > UASG-GFP expression includes a majority of brain 5-HT+ cells (Fig. 5a–d); 36 [95CI 32.5, 39.8] cells per hemisphere across nine clusters, with 85.7% fidelity and 90% extensiveness (Fig. 6a). Expression in Trh-Zal1 > UASGZ-GFP brains were 87.5% extensive, expressing in ~26 serotonergic cells per hemisphere (25.5 [95CI 22, 31]) across five serotonergic clusters (Fig. 5e–h), with <2 ectopic cells: 95.7% fidelity (Fig. 6a). The double-driver combination Trh-Gal4 + Trh-Zal1 > UASGZ-GFP expressed in ~24 cells per hemisphere across five cell clusters (Fig. 5i–l), representing 82% extensiveness and 100% fidelity (Fig. 6a). These results further verify Zal1’s interoperability with Gal4 for intersectional neurogenetics.

**Trh-Zal1–Gal4 combinations improve expression fidelity**

While the Vglut-Zal1 experiments showed that Zal1 can operate with enhancer-trap lines to limit expression, we were not able to quantify the resulting fidelity. Using an α-5-HT antibody that robustly stains fly serotonergic cell bodies, we aimed to test whether Trh-Zal1 could be used to refine low-fidelity serotonergic Gal4 lines. A visual scan of the FlyLight Gal4 collection [12] found possible serotonergic-driving candidate lines. Subsequent immunostaining of these lines identified four lines expressing in some serotonergic cells (Fig. 7a–d). However, these lines included numerous non-5-HT neurons that were densely packed and highly abundant (especially in the optic lobe). Such broad and ectopic expression would confound the interpretation of behavior from such lines; it also prevented quantification of these lines’ serotonin fidelity. Combining these drivers with Zal1-UASGZ greatly reduced range while improving fidelity (Fig. 7a–d). For example, R22H10-Gal4 drives intense fluorescence in non-serotonergic central-complex cells (Fig. 7a); the Trh-Zal1 AND operation on this driver excluded central-complex expression almost completely. The double-driver combination retained expression in a majority of verified serotonergic neurons: 75% [95CI 71, 80] (Fig. 7a). Overall, counting cells in the four lines found that the mean Gal4-Zal1 intersectional 5-HT+ fidelity was 77% [95CI 65, 92] (Fig. 6b). These data verify the hypothesis that Zal1 intersection is useful to refine Gal4 driver specificity.

**Discussion**

Elucidation of the anatomical and genetic complexity of the brain will require a range of progressively sophisticated tools. Here, we present a method to refine the expression range of existing Gal4 lines. At their non-cognate

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Raghu et al. Molecular Brain (2018) 11:46

Page 5 of 12
Fig. 3 A combination of Crz-Zal1 and Crz-Gal4 drives expression in corazonergic cells. Maximum intensity projection (MIP) images of brain immunofluorescence. 

a-d MIP images of (A) of a UASG-mCD8GFP/+;Crz-Gal4/+ brain stained with α-GFP (green) and α-DLG (magenta) antibodies.

b An image of a Crz-Gal4/UASZ-mCD8GFP brain stained with α-GFP (green), (C) and α-Crz antibodies (magenta) and (D) combined image.

e-h UASZ-mCD8GFP/+; Crz-Zal1/+ brains stained with α-GFP, (e) α-DLG (magenta) and (h) α-Crz. 

i UASZ-GZ-mCD8GFP/+;Crz-Gal4/+; Crz-ZAL1 brains stained with α-GFP and α-DLG (magenta) antibodies.

j A Crz-Gal4/UASGZ-mCD8GFP brain stained with α-GFP, k α-Crz, and l combined image.

m Control brains were stained with α-GFP and α-Crz. 

Crz-Gal4 is inactive at non-cognate UASZ sites in Crz-Gal4; UASZ-GFP brains. n Crz-Zal1; UASG-GFP brains stained with α-GFP showed no green fluorescence. o Crz-Gal4; UASGZ-GFP brains showed no fluorescence. p Crz-Zal1; UASGZ-GFP showed weak expression in a few Crz cells (arrows indicate expression). Scale bar represents 200 μm; dorsal is up.
The system has several limitations. First, Zal1 has weak off-target activity at a 20 × UASGZ responder; this means that the combined Gal4-Zal1 expression pattern will include low expression some cells from the Zal1 set. This issue necessitates that behavioral experiments include Zal1 > UASGZ control flies, to check whether an effect arises either from the non-cognate expression or the intersectional expression. Note that this control will include low expression some cells from the Zal1 set.

In conclusion, this new expression system provides a versatile tool for the examination of neuronal function, most importantly, for the refinement of Gal4 drivers. Zal1 promises to be a useful method for mapping neural circuits.

Methods

Replacement of the zinc finger in Gal4 with EGR1 domains

A Gal4 derivative was generated by fusing DNA sequences corresponding to the first two zinc fingers of the mouse transcription activator EGR1 (previously called ZIF268) with DNA coding for residues 41–881 of Gal4, a sequence that includes Gal4's linker and dimerization domains, as well as the transcriptional-activation regions (Fig. 1a–c). Codon-optimized DNA coding for residues 2–59 of EGR1 were synthesized (Genscript Ltd) with an upstream DNA linker that included a KpnI restriction site and 210 base pairs of Gal4 sequence that included an RsrII site. This section was digested and ligated into the pBPGA-L4.2Uw-2 vector [20], replacing the first 40 residues of Gal4 while leaving the domains necessary for dimerization and activation intact; this construct was labeled pSVRZal.

Construction of VGlut-, Trh-, and Crz-Zal1 driver lines

To generate drivers that would express Zal1 in glutamatergic cells, serotoninergic, and corazonin (Crz) positive cells, the VGlut, Trh, and Crz enhancer regions were subcloned upstream of Zal1 to generate
VGlut-Zal1, Trh-Zal1 and Crz-Zal1 lines respectively. In the case of VGlut-Zal1, a 5.5-kb piece of DNA [10] immediately upstream of the Vglut translation start site was used. For generating Trh-Zal1 and Crz-Zal1 lines, the same enhancer fragments which have been used to prepare Trh-Gal4 [1] and Crz-Gal4 [6] were amplified using PCR and subcloned into pSVRZal. For Trh-Zal1, the 1.6 kb promoter region [1] immediately upstream of the Trh transcriptional start site was used. For Crz-Zal1, a 434 bp promoter region [6] upstream of the putative Crz transcription start site was used. All lines were inserted into the attP2 sites on the 3rd chromosome (BestGene, Inc) of w^{1118} flies.
Construction of UASZ and UASGZ responders

The recognition site of ZFGD1 is a 25-base-pair sequence comprising two inverted six-base-pair EGR1 partial binding sites separated by spacer DNA sequence [22]. Following the convention set by ‘UASG’, we refer to this palindromic site (AAGCTT-[CGCCCAGAAGAGTCC TATGGGCGAG × 4]-GACGTC) as ‘UASZ’. Four UASZ sites were introduced using HindIII and AatII sites into the vector pJFRC7-20XUAS-IVS-mCD8::GFP, replacing the original UASG sequences [20] to produce pSVR-4XUASZ-IVS-mCD8::GFP. Four tandem sites were used, as longer repeats of UASZ proved intractable to synthesis subcloning. A non-palindromic, hybrid binding site that combined the recognition half-sites of Gal4 and Zal1 was also synthesized, termed UASGZ (AAGCTT-[CCGG AGTACTGTCCTATGGGCGAG × 20]-GACGTC). To make a GFP responder construct, the UASGZ sites were introduced into the pJFRC7-20XUAS-IVS-mCD8::GFP vector, replacing the original UASG sites using HindIII and AatII sites to generate pSVR-20XUASGZ-IVS-mCD8::GFP. Earlier attempts with 5× UASGZ Vglut-Zal1 construct produced only weak expression (data not shown). Here synthesis of 20× of tandem sites was successful, an arrangement suitable to maximize expression via the Gal4-Zal1 heterodimer. Both transgenes were targeted to the attP40 sites on the 2nd chromosome.

Fly stocks and transgenesis

Drosophila melanogaster flies were grown on standard medium at 23 °C–25 °C. Transgenic animals were generated with the PhiC31-mediated protocol (Bestgene Inc). For brevity, flies transformed with pJFRC7-20XUAS-IVS-mCD8::GFP are referred to as ‘UASG-GFP’; flies with pSVR-4XUASZ-IVS-mCD8::GFP are referred to as ‘UASZ-GFP’; and flies with pSVR-20XUASGZ-IVS-mCD8::GFP are referred to as ‘UASGZ-GFP’. The VGlut-Gal4 line was a gift from Aaron DiAntonio. Trh-Gal4 (BL#38389) was procured from the Bloomington stock center. Crz-Gal4 was a gift from Jae H. Park (The University of Tennessee). The Gal4 lines from the Janelia collection were obtained from Bloomington; NP enhancer trap lines were obtained from the Kyoto Stock Center of the Drosophila Genetic Resource Center.

Immunohistochemistry

Brains were dissected from anesthetized female flies 3–5 days after eclosion and fixed in 4% paraformaldehyde for 30 min at room temperature. Brains were washed for 45–60 min in PBT (phosphate buffered saline with 1% Triton X-100 at pH 7.2). For antibody staining, the samples were further incubated in PBT containing 2% normal goat serum (sc-2043, Santa Cruz Biotechnology) and primary antibodies overnight at 4 °C. Primary antibodies were removed by several washing steps (5 × 20 min in PBT) and secondary antibodies were added prior to a second overnight incubation at 4 °C. Secondary antibodies were removed with several washing steps (5 × 20 min) and then finally in PBS (5 × 20 min). Stained brains were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and recorded with confocal microscopy. The following primary and secondary antibodies were used: Alexa Fluor 488 rabbit α-GFP-IgG (A-21311, Molecular Probes, 1:200 dilution), chicken α-GFP (ab13970), rat α-mCD8 (MCD0800, Caltag Laboratories, Chatujak, Bangkok, Thailand), rat α-5-HT (MAB352, Merck), mouseα-DLG1 (4F3 α-DISCOs LARGE

Fig. 6 Genetic intersection of Trh-Zal1 with Trh-Gal4 and enhancer trap lines results in high-fidelity expression. a. A Venn plot displays α-GFP+ expression as a percentage of α-5-HT+ cells. Left Trh-Gal4 drives expression in 90% of serotonergic neurons, along with 17% of expression in ectopic cells; Center similarly, Trh-Zal1 drives expression in ~88% of serotonergic cells with ectopic expression in 4% of 5-HT+ cells. Right The Trh-Gal4/Trh-Zal1 combination drives expression in ~82% of serotonergic cells with no expression in ectopic cells. The total-count mean of 5-HT+ cells ranged from 30 to 34 per brain hemisphere. b. The R22H10-Gal4 + Trh-Zal1 combination has 51% extensiveness within the antibody stain, with 75% fidelity. The R33C03-Gal4 + Trh-Zal1 combination: 59% extensiveness and 71% fidelity. R70A11-Gal4 + Trh-Zal1 combination: 49.5% extensiveness and 91% fidelity. R89A09-Gal4 + Trh-Zal1 combination: 47.5% extensiveness and 69.5% fidelity. The total-count mean of 5-HT+ cells ranged from 35 to 42 per brain hemisphere.

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Brains were dissected from anesthetized female flies 3–5 days after eclosion and fixed in 4% paraformaldehyde for 30 min at room temperature. Brains were washed for 45–60 min in PBT (phosphate buffered saline with 1% Triton X-100 at pH 7.2). For antibody staining, the samples were further incubated in PBT containing 2% normal goat serum (sc-2043, Santa Cruz Biotechnology) and primary antibodies overnight at 4 °C. Primary antibodies were removed by several washing steps (5 × 20 min in PBT) and secondary antibodies were added prior to a second overnight incubation at 4 °C. Secondary antibodies were removed with several washing steps (5 × 20 min) and then finally in PBS (5 × 20 min). Stained brains were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and recorded with confocal microscopy. The following primary and secondary antibodies were used: Alexa Fluor 488 rabbit α-GFP-IgG (A-21311, Molecular Probes, 1:200 dilution), chicken α-GFP (ab13970), rat α-mCD8 (MCD0800, Caltag Laboratories, Chatujak, Bangkok, Thailand), rat α-5-HT (MAB352, Merck), mouseα-DLG1 (4F3 α-DISCOs LARGE
Neuroanatomical comparison of cell sets in NP and GMR lines

With either UASG-GFP or in combination with VGluT-Zal1; UASGZ-GFP, the following enhancer-trap lines were subjected to α-GFP and α-DLG staining: Orco-Gal4, OK107, NP0517, NP0588, NP3363, NP2002, NP2417, NP3008, NP4683, NP6235, NP6330, NP0318, NP2351, NP3156, NP0527, NP0615, NP0741, NP2252, NP0563, NP3055, and NP0564. With either UASG-GFP—or in combination with Trh-Zal1; UASGZ-GFP—the following GMR module-trap lines were subjected to α-GFP and α-5-HT staining: R89A09, R70A11, R53C03, and R22H01-Gal4.

Microscopy

Serial optical sections were taken in 0.5 μm steps at 1024 × 1024 pixel resolution using a confocal laser scanning microscope.
Calculation of driver extensiveness and fidelity

To quantify the quality of different drivers and their combinations, we defined two metrics: extensiveness (E) and fidelity (F). Extensiveness was measured as how completely a transgenic marker (M⁺) covers the range of cells identified by an antibody (Ab⁺) for the cognate protein of the driver’s source gene.

\[ E = \frac{(M^+ \cap Ab^+ \text{ cells/all Ab}^+ \text{ cells})}{100} \]

Fidelity was defined as the percentage of marker-positive cells that also immunostained for the cognate protein product.

\[ F = \frac{(M^+ \cap Ab^+ \text{ cells/all } M^+ \text{ cells})}{100} \]

Extensiveness is a desirable property for drivers that aim at capturing a complete set of cells of one neurotransmitter class; however, extensiveness is undesirable for mapping the functions of individual subsets (or individual cells). Fidelity is an unambiguously desirable characteristic.
Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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