# A Genetic Toolkit for Simultaneous Generation of *LexA*- and *QF*-Expressing Clones in Selected Cell Types in *Drosophila*

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Kota Banzai, Ping Shen and Daichi Kamiyama
Department of Cellular Biology, University of Georgia, Athens, GA, USA.

**ABSTRACT:** Visualization and manipulation of defined motoneurons have provided significant insights into how motor circuits are assembled in *Drosophila*. A conventional approach for molecular and cellular analyses of subsets of motoneurons involves the expression of a wide range of *UAS* transgenes using available *GAL4* drivers (eg, *eve* promoter-fused *GAL4*). However, a more powerful toolkit could be one that enables a single-cell characterization of interactions between neurites from neurons of interest. Here we show the development of a *UAS>LexA>QF* expression system to generate randomly selected neurons expressing one of the 2 binary expression systems. As a demonstration, we apply it to visualize dendrite-dendrite interactions by genetically labeling *eve*$^+$ neurons with distinct fluorescent reporters.

**KEYWORDS:** *Drosophila*, mosaicism, gene expression, dendrites, motor neuron

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**CORRESPONDING AUTHOR:** Daichi Kamiyama, Department of Cellular Biology, University of Georgia, 1000 Cedar Street, Athens, GA 30602-0002, USA. Email: daichi.kamiyama@uga.edu

## Significance Statement

The study of how motor circuits are assembled in the *Drosophila* brain is limited by the genetic tools available to target and manipulate individual neurons. We present a genetic toolkit that allows us to generate *LexA*- and *QF*-expressing clones randomly in *eve*$^+$ motoneurons. Here, we demonstrate the utility of this system in visualizing dendrite-dendrite interactions between *eve*$^+$ clones at high resolution. We anticipate that this system will become an indispensable tool to study interactions between distinct clones in unique cell types.

## Introduction

Revealing the circuit principles underlying specific behavior is one of the primary goals of neuroscience research. Particularly in *Drosophila*, there is a vivid interest in dissecting the motor circuit.[1,2] We have previously focused on *eve*-positive (*eve*$^+$) neurons, aCC and RP2, which are segmentally repeated in the ventral nerve cord (VNC).[3-5] Both motoneurons innervate body wall muscles (Figure 1a, dots). These neurons also extend their dendrites to overlapping fields across segments in the VNC (Figure 1a),[6] suggesting dendrite-dendrite interactions between the neurons. However, little is known about how contacts between dendrites from neighboring neurons influence the formation of dendritic fields. A toolkit with which one can study interactions between *eve*$^+$ neurons would be of particular interest. Here, we describe a new system genetically manipulating these neurons by 2 binary expression systems (ie, *LexA/LexAop* and *QF/QUAS*).[7,8]

## Materials and Methods

### *Generation of transgenic flies*

The *pUCIDT>LexA>QF* plasmid was generated through synthesis and molecular cloning by Integrated DNA Technologies, Inc. To generate *pUAS>LexA>QF*, we PCR-amplified the *>LexA>QF* cassette and cloned it into the backbone of *pACUH* (Addgene # 58374) via the BglII and XbaI sites. To generate *QUAS-myr::mCherry*, we PCR-amplified the myristoylation sequence from *pJFRC19-13XLexAop2-IVS-myr::GFP* (Addgene # 26224) and placed it in the *pQUAST-mCherry* vector via the NotI and BamHI sites. After verifying these sequences, we generated transgenic flies using a transposon-based approach (Rainbow Transgenic Flies, Inc).

### *Cell culture*

*Drosophila* Schneider S2 cells were cultured at 25°C in an SFX-INSECT cell culture medium (HyClone). An *actin 5C-GAL4* driver, *pUAS>LexA>QF*, and fluorescent reporter plasmids (100 ng of each plasmid per well) were co-transfected by using Effectene (2.5 µl, QIAGEN) into an 8-well Lab-Tek II chambered #1.5 coverglass system (Nalge Nunc International).

### *Fly genetics and husbandry*

Flies were maintained at room temperature on standard cornmeal-agar food. Full genotypes of stock used in Figures 1a and 2b: (**1a**) *w;; eve-GAL4, UAS-CD8::GFP*, (**2b**, left) *w/w; LexAop2-mCD8::GFP, UAS-mCD8::Cerulean/UAS>LexA>QF; QUAS-myr::mCherry/eve-GAL4*, and (**2b**, right) *w/hs-Cre; LexAop2-mCD8::GFP, UAS-mCD8::Cerulean/UAS>LexA>QF; QUAS-myr::mCherry/eve-GAL4*. The genotype to test the viability of *LexA>QF* transgenic flies: *w/hs-Cre; LexAop2-mCD8::GFP, UAS-mCD8::Cerulean/UAS>LexA>QF; QUAS-myr::mCherry/elav-GAL4*.

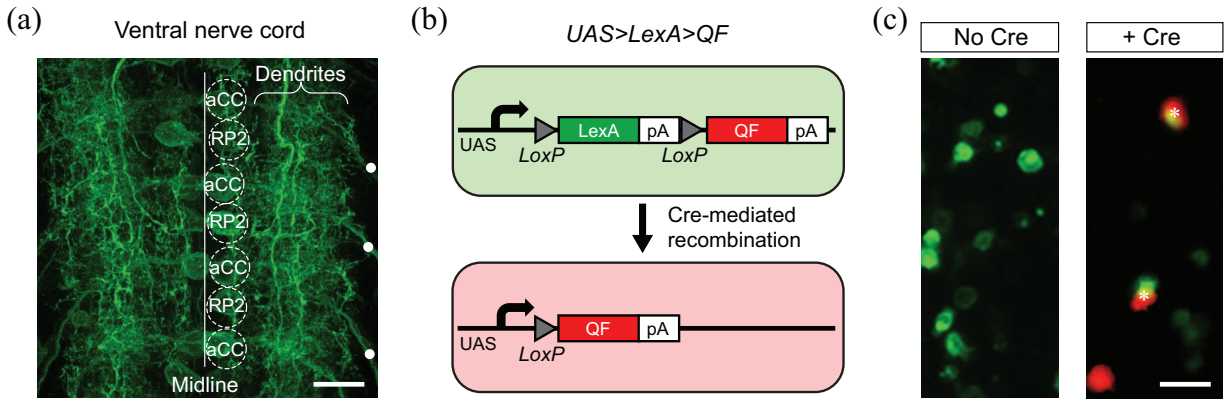


**Figure 1.** The>*LexA*>*QF* mosaic expression system: (a) Gross anatomy of *eve*⁺ neurons in the ventral nerve cord (VNC) at the 2 instar larva stage. Dots indicate the VNC exit points of *eve*⁺ neurons. (b) Schematic of the >*LexA*>*QF* system. Cre induces an excision event between *LoxP* sites. Before Cre action, only *LexA* is expressed. Cre-mediated recombination switches expression to *QF*. (c) S2 cells transiently transfected with *pUAS*>*LexA*>*QF*. Co-transfected with *pUAS-Cre*, these cells switch to *QF* expression. We use the *actin 5c* ubiquitous promoter to drive *GAL4*, which in turn activates the expression of genes under the control of *UAS*. We also co-transfect reporter plasmids, that is, *LexAop-GFP* (green) and *QUAS-RFP* (red). There are 2 cells shown in yellow*; the red and green cells contact each other. Scale bars: 10 µm.

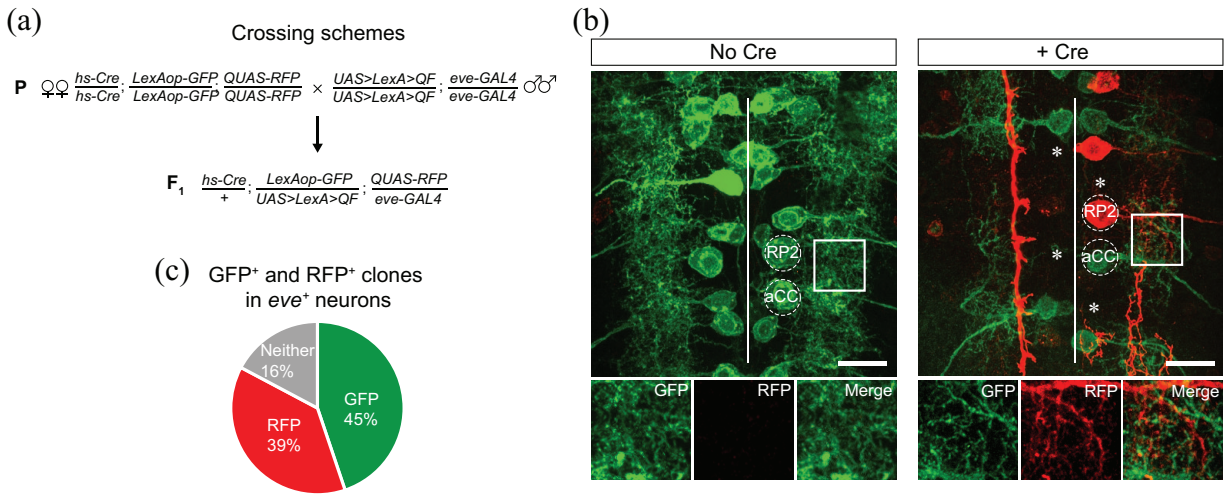


**Figure 2.** Fluorescent reporter expression in >*LexA*>*QF* larvae: (a) Example crossing schemes for the generation of *LexA* and *QF* neurons using *UAS*>*LexA*>*QF*. (b) Confocal projection of the VNC with Cre-mediated expression of *LexA* (green) or *QF* (red) in *eve*⁺ clones. Note that we see a variety of *FP* expression levels. Asterisks indicate *eve*⁺ neurons that express neither *LexA* nor *QF*. Insets show higher magnification of boxed regions. (c) Quantification of GFP⁺ and RFP⁺ clones in *eve*⁺ neurons. The pie chart shows the percentage of different clones in the same third instar larva. This experiment was replicated 5 times independently. Scale bars: 10 µm.

### *Immunohistochemistry*

Fly larvae were dissected in PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Brains were washed in TBS (1XPBS + 0.1% Triton X-100), blocked for at least 1 hour in blocking solution (1XPBS + 0.1% Triton X-100 + 1% bovine serum albumin), and incubated in primary antibodies at 4°C overnight. On day 2, brains were washed 3 times in TBS and then incubated in secondary antibodies in TBS for 2 hours at room temperature. After extensive wash in TBS, brains were mounted in 50% glycerol for slide preparation. See also Table 1 for antibodies used.

### *Confocal imaging*

Confocal images of neurons were captured using an inverted fluorescence microscope (Ti-E, Nikon) with a 10× or 100× objective (Nikon). The microscope was attached to the Dragonfly Spinning disk confocal unit (CR-DFLY-501, Andor). Images were recorded with an EM-CCD camera (iXon, Andor). All images in figures are maximum Z-projections of confocal images (FIJI/ImageJ, NIH).

## Results and Discussion

It has been demonstrated that *eve-GAL4* can drive the expression of *UAS* transgenes in every aCC and RP2 neuron.[9] Shown by GFP expression via *eve-GAL4*, GFP⁺ dendrites from *eve*⁺ neurons extensively overlap during larval stages (Figure 1a). Unless these neurons are labeled with different FPs in a mosaic manner, the use of this *GAL4* driver is challenging to investigate dendrite-dendrite interactions between these neurons. We thus design a new mosaic expression system (Figure 1b). This system exploits mitotic recombination by a Cre enzyme to

**Table 1.** Reagents we used in this study.

| *DROSOPHILA* STOCKS USED | | |
|---|---|---|
| GENOTYPE | SOURCE | IDENTIFIER |
| *y[1] w[*]; Pw[+mC]=eve-GAL4.RN2E* | Bloomington *Drosophila* Stock Center | BDSC: 7470 |
| *w[*]; P{GAL4-elav.L}CG16779* | Bloomington *Drosophila* Stock Center | BDSC: 8760 |
| *w*; Peve-GAL4.RN2P, P{UAS-mCD8::GFP.L}LL5* | Bloomington *Drosophila* Stock Center | BDSC: 7475 |
| *y[1] w[67c23] P{y[+mDint2]=Crey}1b; sna[Sco]/CyO; Dr[1]/TM3, Sb[1]* | Bloomington *Drosophila* Stock Center | BDSC: 34516 |
| *w[*]; P{y[+t7.7] w[+mC]=13XLexAop2-mCD8::GFP}attP40/CyO* | Bloomington *Drosophila* Stock Center | BDSC: 32205 |
| PLASMIDS USED | | |
| PLASMID | SOURCE | IDENTIFIER |
| *pUAS-Cre* | Addgene | Plasmid #50797 |
| *pJFRC18-8XLexAop2-mCD8::GFP* | Addgene | Plasmid #26225 |
| *pAC-GAL4* | Addgene | Plasmid #24344 |
| ANTIBODIES USED | | |
| ANTIBODY | SOURCE | IDENTIFIER |
| Rabbit anti-dsRed | Takara | Cat# 632496; RRID: AB_10013483 |
| Mouse anti-Fasciclin II | DSHB | Cat# 1D4, RRID: AB_528235 |
| Goat anti-rabbit Alexa 555 | Thermo | Cat# A-21429, RRID: AB_2535850 |
| Donkey anti-mouse Alexa 647 | Thermo | Cat# A-31571, RRID: AB_162542 |

generate 2 distinct clones under the control of *GAL4* drivers. A similar design has already been adopted in several techniques to generate mosaic neurons, including *UAS-dBrainbow*.[10] Whereas the Brainbow techniques can label a subpopulation of neurons with fluorescent reporters, they cannot be used to examine mosaic phenotypes in the context of genetic knockdown or over-expression. Thus, we sought to apply 2 transcriptional activators (ie, *LexA* and *QF*) to the mosaic system instead of fluorescent reporters. In this system, Cre-mediated recombination between 2 *loxP* sites leads to the excision of the *LexA* sequence, which subsequently induces *QF* expression (Figure 1b, bottom). By contrast, no recombination gives rise to *LexA* expression (Figure 1b, top).

To assess whether the *>LexA>QF* cassette functions in *Drosophila* S2 cells, we created the *pUAS>LexA>QF* expression construct and drove the expression by using a ubiquitous *actin 5C-GAL4* driver construct. We also employed 2 reporter plasmids for LexA and QF transcriptional activators, such as *LexAop-GFP* and *QUAS-RFP*, respectively. In the absence of Cre, we observed only $GFP^+$ cells (Figure 1c). This observation is consistent with the notion that the cassette retains the *LexA* sequence without *Cre* expression. For comparison, when we co-transfected *>LexA>QF* with *Cre*, we observed *RFP* expression from some cells (Figure 1c). Note that other cells also expressed *GFP* due to a lack of recombination (the *Cre* plasmid would not be introduced into these cells because of co-transfection failure; Figure 1c). Altogether, these experiments demonstrate that *pUAS>LexA>QF* enables the expression of these transcriptional activators in different groups of cells.

To test whether this expression system functions in *Drosophila in vivo*, we generated flies harboring *UAS>LexA>QF*, fluorescent reporters, and *eve-GAL4* (see also the crossing scheme in Figure 2a). Using the flies in the absence of *Cre* recombinase, 100% of $eve^+$ neurons expressed only *LexA* as assessed by fluorescent reporters (ie, *LexAop-GFP* and *QUAS-RFP*) (Figure 2b, left). When *Cre* was expressed, recombination induced *RFP* expression (Figure 2b, right). As described in the crossing scheme (Figure 2a), *Cre* expression was under the control of the *hsp70* promoter.[11] It has been reported that *loxP* sites can be recombined by *hs-Cre* with ~100% efficiency in cells at room temperature.[11] However, we empirically identified an optimal temperature (such as 16°C) at which *Cre*-mediated recombination happens in a mosaic pattern (Figure 2b, right). Among animals examined, the selection between these activators appears to be stochastic and independent in each neuron. To quantify the choice of these activators, we measured the number of $GFP^+$ and $RFP^+$ neurons. We found that both reporters were expressed at similar relative frequencies (Figure 2c).

We also noticed that a couple of neurons expressed neither GFP nor RFP in the same animals (Figure 2b, **asterisks**). We initially suspected that *LexA* or *QF* neurons were severely damaged due to the known toxicity of the activators.[12] However, 3 lines of evidence suggest that this is not the case: (1) the dendritic arbors of the $FP^+$ neurons (Figure 2b, right) were morphologically indistinguishable from the wild-type reported in the previous literature[6]; (2) these FP-negative ($FP^-$) neurons could successfully innervate the target muscles, and their axon terminals were fully differentiated (Figure S1); (3) In the presence of *Cre* recombinase, adult flies with the pan-neuronal expression of *>LexA>QF* under the control of *elav-GAL4* were viable (see Material and Methods for its genotype). These data indicate that the morphology and function of *LexA* or *QF* neurons are largely normal, yet additional investigation is required for understanding how the system generates the $FP^-$ neurons.

Nonetheless, as seen in Figure 2b, this system labels individual neurons whose dendritic branches can be visualized in great detail. Analogous results were also observed using a serotonergic neuron-specific *Trh-GAL4* (KB and DK, unpublished data). We thus believe that this expression system will be broadly applicable in its current form.

The *>LexA>QF* system provides a facile platform for studying the interaction between genetically manipulated neurons. A major advantage of this system over previous techniques such as dBrainbow is that we could examine phenotypes elicited by the expression of transgenes in given clones. For example, with the expression of RNAi transgenes (the crossing scheme is outlined in Figure S2a), we would investigate molecular mechanisms of how dendrites are established and patterned via dendrite-dendrite contacts. In such an experiment, one might choose room temperature (25°C) to enhance RNAi efficacy. Another way of recombination can circumvent the low-temperature requirement. In this case, one would use a different *Cre* variant such as a ligand-inducible DD-Cre (Cre is fused to the destabilizing domain from dihydrofolate reductase of E. coli).[13] The antibiotic trimethoprim (TMP) is a cell-permeable ligand for the DD domain. Adding TMP into fly food prevents DD-fusion proteins from rapid degradation.[14] By adjusting the TMP concentration rather than temperature, we would achieve mosaic expression of the Cre in an $eve^+$ population.

The *>LexA>QF* system could also get along with a variety of fluorescent reporters. In conjunction with the GFP reconstitution across synaptic partners (GRASP) system (see also the crossing scheme in Figure S2b), it would allow us to characterize the sites of dendrite-dendrite interactions. The recent advent of the split GFP system furthermore enables labeling of proteins endogenously expressed in subsets of neurons.[15] The super-folder GFP sequence is split between 10th and 11th β-strands ($GFP_{1-10}$ and $GFP_{11}$). These fragments do not individually fluoresce, but upon associating each other, they can reconstitute a GFP signal. Only the $GFP_{11}$ fragment is inserted into a specific genomic locus via a custom-design genome editing strategy or a genomic integration into an available MiMIC site. By introducing the expression of the large $GFP_{1-10}$ fragment into $eve^+$ clones, any clone expressing both fragments can be fluorescently labeled (see the crossing scheme in Figure S2c). This way, we could investigate how endogenous proteins dynamically localize during dendrite-dendrite interactions between $eve^+$ neurons.

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## Author Contributions

Kota Banzai: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing—review and editing. Ping Shen: Conceptualization, Writing—review and editing. Daichi Kamiyama: Conceptualization, Supervision, Writing—original draft, Writing—review and editing, Funding acquisition.

## Data Availability

The *pUAS>LexA>QF* plasmid and sequence have been deposited in Addgene. The *UAS>LexA>QF* transgenic line is available from the Bloomington *Drosophila* Stock Center.

## ORCID iD

Daichi Kamiyama https://orcid.org/0000-0002-1466-5295

## Supplemental Material

Supplemental material for this article is available online.

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