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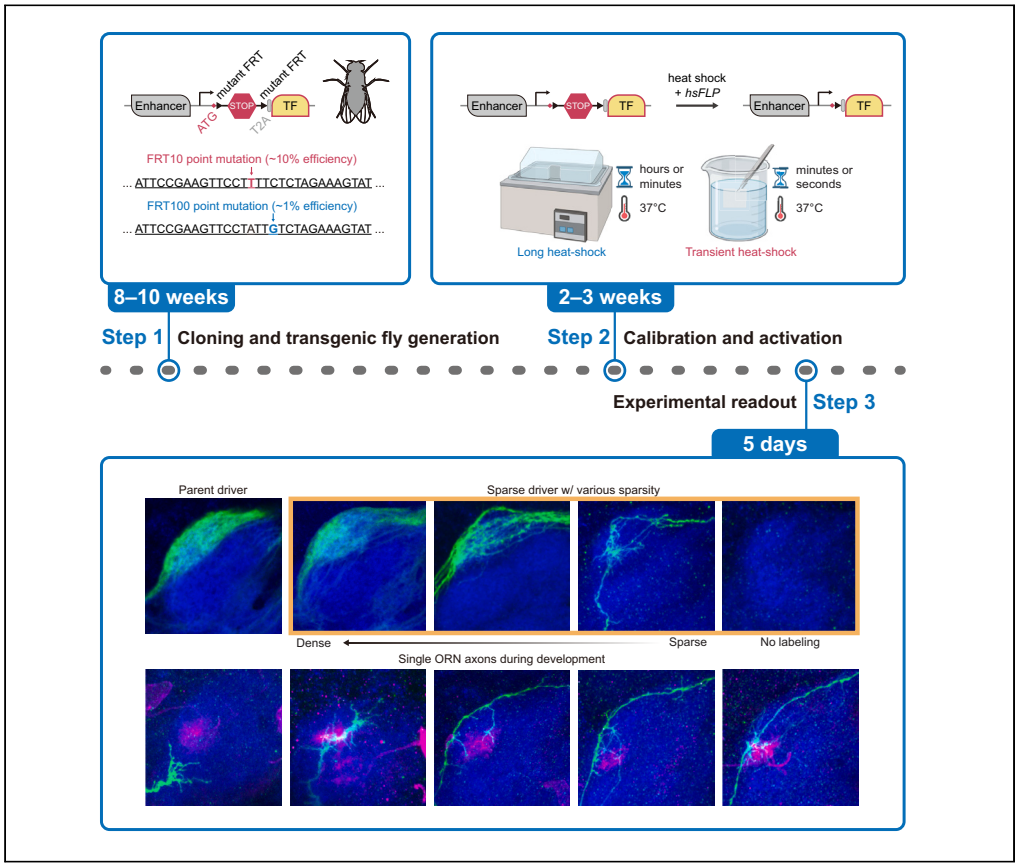
Protocol for cell-type-specific single-cell labeling and manipulation in *Drosophila* using a sparse driver system



Here, we present a protocol for cell-type-specific single-cell labeling and manipulation in *Drosophila* using a sparse driver system. We describe steps for generating constructs and fly lines, titrating heat-shocked durations for precise temporal control and desired sparsity, and co-expressing multiple transgenes for experiments. We demonstrate that this generalizable toolkit enables tunable sparsity, multi-color staining, single-cell trans-synaptic tracing, single-cell manipulation, and cell-autonomous gene function analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Instructions for generating sparse driver constructs and fly lines

Steps to titrate heat-shocked durations for precise temporal control and desired sparsity

Co-express multiple effectors/reporters for morphology, tracing, and gene manipulation

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Protocol for cell-type-specific single-cell labeling and manipulation in *Drosophila* using a sparse driver system

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SUMMARY

Here, we present a protocol for cell-type-specific single-cell labeling and manipulation in *Drosophila* using a sparse driver system. We describe steps for generating constructs and fly lines, titrating heat-shocked durations for precise temporal control and desired sparsity, and co-expressing multiple transgenes for experiments. We demonstrate that this generalizable toolkit enables tunable sparsity, multi-color staining, single-cell trans-synaptic tracing, single-cell manipulation, and cell-autonomous gene function analysis.

For complete details on the use and execution of this protocol, please refer to Xu et al.[1]

BEFORE YOU BEGIN

Background

Sparse neuron labeling and manipulation are powerful tools in neuroscience, allowing for detailed study of individual neurons within complex brain networks.[2] By targeting a small subset of neurons, researchers can label neuronal morphologies with fluorescent markers, trace synaptic partners with trans-synaptic tracing methods, and monitor real-time activity with GCaMPs. Additionally, when used to express genetic or optogenetic effectors, sparse manipulation enables the study of cell-autonomous gene functions or the dissection of specific neural circuits in behaviors.

However, sparse manipulation methods that rely on probabilistic gating of reporter or effector transgenes often struggle to co-express all desired transgenes in the same subset of neurons.[3–5] This issue arises because different reporter or effector transgenes may be activated stochastically in different cell subgroups, as recombination events are independent of each other. A potential solution is to use a stochastically expressed driver transgene that simultaneously controls multiple effectors or reporters, ensuring coordinated expression. The MARCM system[6] is one approach to achieve this. However, MARCM relies on cell division to lose a repressor transgene after mitotic recombination, such that the events cannot be initiated in postmitotic cells. Additionally, its dependence on repressor loss after mitotic recombination hinders its effectiveness in studying developmental events shortly after cell division due to residual repressor activity from mRNA and/or proteins produced before the mitotic recombination event.[7]

To address these limitations, we developed a sparse driver system to target single cells within specific neuron types, allowing simultaneous expression of multiple transgenes. Expression probability and desired sparsity are controlled by mutant *FRT* sites with reduced recombination efficiency and tunable FLP recombinase levels through variable heat-shock durations (Figure 1A). Point mutations

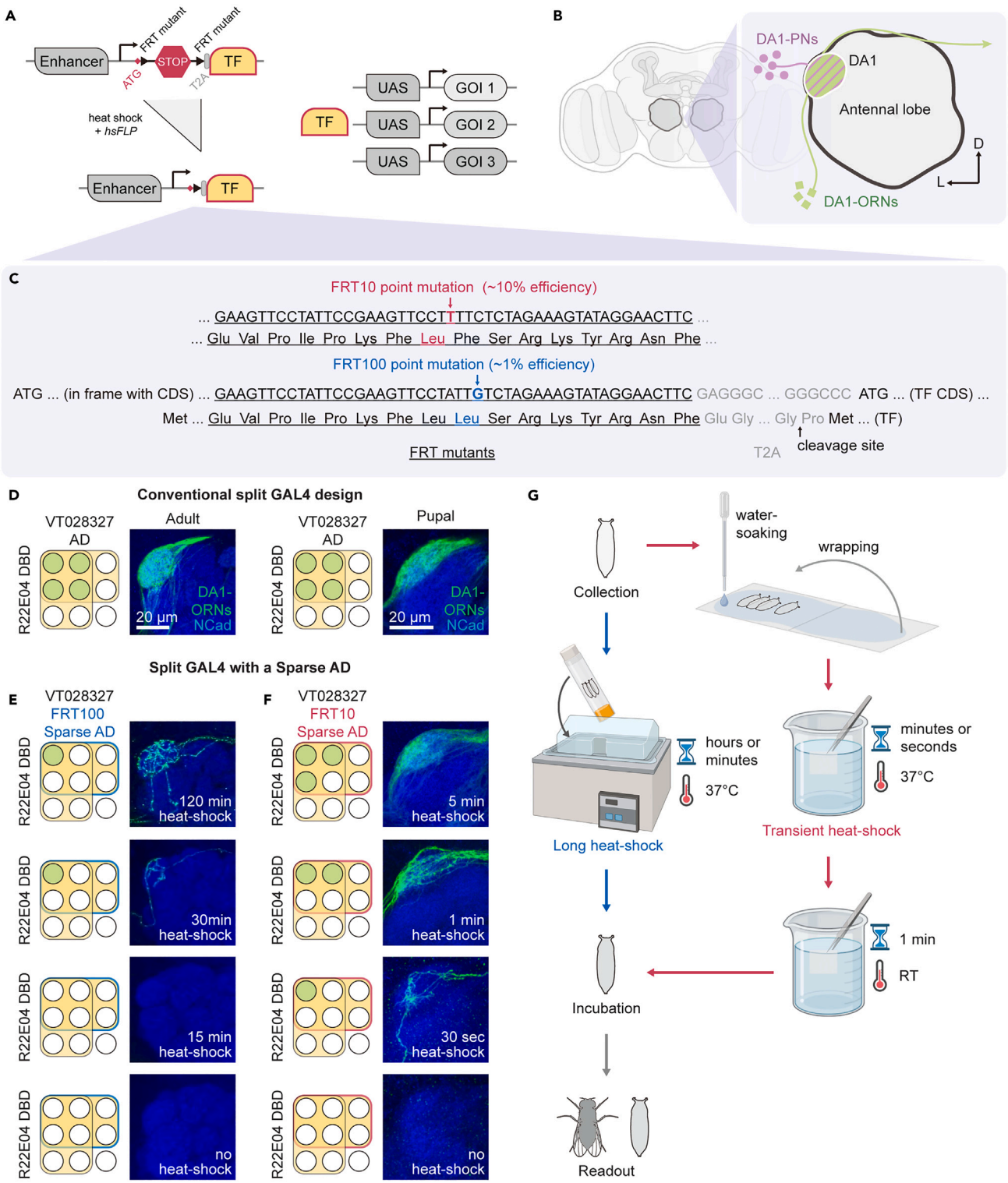

Figure 1. The sparse driver system and its demonstration in the *Drosophila* olfactory circuit

(A) The sparse driver system allows simultaneous expression of multiple transgenes in a subset of cells through stochastic TF (transcription factors) expression. The TF expression is gated by a pair of mutant *FRTs* (*FRT10* or *FRT100* sites) and a transcription termination sequence (shown as *STOP*). Heat-shock-induced stochastic FLP expression removes the *STOP* and enables TF expression in a fraction of cells, driving the co-expression of multiple genes of interest (GOI) in these cells.

Figure 1. *Continued*
(B) Adult *Drosophila* brain schematic highlighting antennal lobes and locations of the DA1 glomerulus. Left, DA1-ORN axons (green) synapse with DA1-PN dendrites (purple, contralateral projection omitted).
(C) Point mutations (the A→T mutation of *FRT10* or the C→G mutation of *FRT100*) in the *FRT-STOP-FRT* sequence can reduce FLP-*FRT* recombination efficiency by approximately 10- or 100-fold, respectively. Following recombination, the in-frame peptide derived from the mutant *FRT* and *T2A* sequences is excised during the translation of the TF.
(D) A conventional split GAL4 strategy to target DA1-ORNs in the adult or pupal antennal lobe.
(E) The Sparse^{FRT100}-AD-based split GAL4 enables different sparsity tuned by heat-shock time (from 0 to 120 min).
(F) The Sparse^{FRT10}-AD-based split GAL4 enables different sparsity tuned by heat-shock time (from 0 to 5 min).
(G) Two procedures for sparse driver activation.

in the *FRT-STOP-FRT* sequence (mutant *FRT10* or *FRT100* sites[8]) can reduce FLP-*FRT* recombination efficiency by about 10- or 100-fold, respectively (Figure 1C). The sparse driver system allows for more precise spatial and/or temporal control, enabling the dissection of cellular events and molecular mechanisms at single-cell resolution.

Drosophila olfactory circuit as a demonstration

In the *Drosophila* olfactory circuit, ~50 types of olfactory receptor neurons (ORNs) synapse with 50 types of second-order projection neurons (PNs) to form precise 1-to-1 matching at 50 discrete glomeruli (Figure 1B), providing an excellent model for investigating mechanisms of synaptic partner matching.

Driver, reporter, docking site, and mutant *FRT* sequence selection

Effective single-cell morphological characterization requires robust driver and reporter systems. Screening strong drivers and testing reliable reporters (e.g., using *UAS-myr-mGreenLantern* or increasing transgene copies) will improve the signal-to-noise ratio of the following sparse driver experiments. In principle, the sparse driver system works for common driver lines and transcription factors (TFs), e.g., GAL4, QF2, LexA, and their split versions.[9–11] The FlyLight Project[12–14] has generated extensive anatomical data and well-characterized GAL4, LexA, and Split-GAL4 drivers to visualize and manipulate individual cell types in the *Drosophila* nervous system. If no validated drivers exist for the desired cell type, start with the FlyLight Project database (https://www.janelia.org/project-team/flylight). Notably, since the genomic locations of plasmid docking sites significantly influence driver characteristics,[15] select docking sites with expression levels similar to or identical to the original driver for the sparse driver injection. We used the split-GAL4/UAS binary expression system for demonstration, specifically the *VT028327-p65.AD* as the parent driver for the sparse driver, along with *GMR22E04-GAL4.DBD*, to robustly target DA1-ORN single axons (Figure 1D).

> ***Note:*** The earliest expression time point of the sparse driver is controlled by heat-shock timing in experiments and restricted by the original driver's characteristics. For developmental research, characterize the expression intensities and patterns of the chosen drivers at different developmental stages before designing the sparse driver.

> ***Note:*** If the properties (e.g., targeted cell number, localization of targeted cells, or expression level) of the parent driver are not well-documented, we recommend testing both *FRT10-STOP-FRT10* (*Sparse^{FRT10}*) and *FRT100-STOP-FRT100* (*Sparse^{FRT100}*) to increase the likelihood of achieving the desired sparsity.

> ***Note:*** In principle, this protocol can be used in other tissues and different *Drosophila* species. Here, we used the *Drosophila melanogaster* olfactory system for demonstration.

Experimental model and subject details

Flies (*Drosophila melanogaster*) were raised on standard cornmeal medium in a 12 h/12 h light cycle at 25°C. For *Sparse^{FRT10}*, avoid 29°C to prevent any leakiness of *hsFLP*; for *Sparse^{FRT100}*, 29°C is

optional to enhance transgene expression. Details of genotypes used in this study and their sources are described in the key resources table.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Gateway LR Clonase II enzyme mix	Thermo Fisher Scientific	Catalog #: 11791020
pENTR/D-TOPO cloning kit	Thermo Fisher Scientific	Catalog #: K240020
Zero Blunt TOPO PCR cloning kit	Thermo Fisher Scientific	Catalog #: 450245
Phire Tissue Direct PCR master mix	Thermo Fisher Scientific	Catalog #: F170L
Q5 site-directed mutagenesis kit	New England Biolabs	Catalog #: E0554S
Q5 hot-start high-fidelity DNA polymerase	New England Biolabs	Catalog #: M0494S
NEBuilder HiFi DNA assembly master mix	New England Biolabs	Catalog #: E2621L
Antibodies		
Rat anti-Dncad (1:40)	Developmental Studies Hybridoma Bank	Catalog #: DN-Ex #8
Chicken anti-GFP (1:1,000)	Aves Labs	Catalog #: GFP-1020
Rabbit anti-HA (1:100)	Cell Signaling Technology	Catalog #: 3724S
Chemicals, peptides, and recombinant proteins		
JF646-HaloTag ligand	the Lavis lab	N/A
PBS (10X), pH 7.4	Thermo Fisher Scientific	70011-044
Paraformaldehyde 20% aqueous solution	Electron Microscopy Sciences	15713
Normal donkey serum	Jackson ImmunoResearch	017-000-121
Experimental models: Organisms/strains		
D. melanogaster: *GMR22E04-GAL4.DBD*	Jenett et al.[13]	BDSC: 69199
D. melanogaster: *VT028327-p65.AD*	Tirian and Dickson[14]	BDSC: 73064
D. melanogaster: *QUAS-mtdTomato-3xHA*	Potter et al.[16]	BDSC: 30004
D. melanogaster: *trans*-Tango	Talay et al.[17]	BDSC: 77123
D. melanogaster: *UAS-mCD8-GFP*	Lee and Luo[6]	DGRC: 108068
D. melanogaster: *hsFLP*	Golic and Lindquist[18]	N/A
D. melanogaster: *UAS-myr-mGreenLantern*	Wong et al.[19]	N/A
D. melanogaster: *Mz19-QF2^{G4HACK}*	Xu et al.[1]	N/A
D. melanogaster: *UAS-Halo-Moesin*	Xu et al.[1]	N/A
D. melanogaster: *UAS-V5-Ten-m*	Xu et al.[1]	N/A
D. melanogaster: *VT028327-FRT10-STOP-FRT10-p65.AD*	Xu et al.[1]	N/A
D. melanogaster: *VT028327-FRT100-STOP-FRT100-p65.AD*	this study	N/A
Recombinant DNA		
pUAS-FRT10-STOP-FRT10-mCD8-GFP	Li et al.[3]	N/A
pUAS-FRT100-STOP-FRT100-mCD8-GFP	Li et al.[3]	N/A
pCR-Blunt-TOPO	Thermo Fisher Scientific	Catalog #: K280020
UAS-Halo-CAAX	Sutcliffe et al.[20]	Addgene: 87645
VT028327-FRT10-STOP-FRT10-p65.AD construct	Xu et al.[1]	Addgene #232827
VT028327-FRT100-STOP-FRT100-p65.AD construct	this study	N/A
78H05-FRT100-STOP-FRT100-p65.AD construct	this study	Addgene #232828
VT028327-FRT10-STOP-FRT10-GAL4 construct	this study	Addgene #232829
pBP-Sparse^{FRT10}-p65.AD construct	this study	Addgene #232830
pBP-Sparse^{FRT10}-GAL4.DBD construct	this study	Addgene #232831
pHACK-Sparse^{FRT10}-p65.AD construct	this study	Addgene #232832
pHACK-Sparse^{FRT10}-GAL4.DBD construct	this study	Addgene #232833
pHACK-Sparse^{FRT10}-GAL4 construct	this study	Addgene #232834
Software and algorithms		
Zen	Carl Zeiss	RRID: SCR_013672

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
ImageJ	National Institutes of Health	RRID: SCR_003070
Illustrator	Adobe	RRID: SCR_010279

MATERIALS AND EQUIPMENT

Washing buffer		
Reagent	Final concentration	Amount
PBS (10X)	1X	50 mL
Triton X-100	0.3% [v/v]	1.5 mL
ddH_2O	N/A	Up to 500 mL
Total	**N/A**	**500 mL**
Store at 15°C–25°C for up to 6 months.		

Fixing buffer		
Reagent	Final concentration	Amount
Paraformaldehyde (PFA)	4% [v/v]	0.08 mL
Washing buffer	5% [v/v]	0.1 mL
PBS	N/A	Up to 2 mL
Total	**N/A**	**2 mL**
Freshly prepared before use.		

Blocking buffer		
Reagent	Final concentration	Amount
Normal Donkey Serum	5% [v/v]	0.1 mL
Wash buffer	N/A	1.9 mL
Total	**N/A**	**2 mL**
Store at 4°C for up to 3 weeks.		

STEP-BY-STEP METHOD DETAILS

Note: The selection of enhancers, mutant *FRT* sequences, and TFs are highly flexible for preparing the Enhancer-Sparse-TF construct. For simplicity, we use *VT028327-Sparse^{FRT10}-p65.AD* as an example.

Preparation of the Sparse-TF construct

Timing: 1 week

This section outlines the steps for constructing a Sparse-TF plasmid by integrating an *FRT10-STOP-FRT10* sequence to enable conditional TF expression. The construct ensures proper peptide removal after translation and can be modified based on the experimental design.

1. Select a plasmid backbone for the desired TF. For example, to generate the DA1-ORN sparse AD, *VT028327-Sparse^{FRT10}-p65.AD* (Addgene #232827), we used *pBPp65ADZpUw*.[15]
2. To generate the *FRT10-STOP-FRT10-T2A* sequence, PCR amplify *FRT10-STOP-FRT10* sequence and the *T2A* element from *VT028327-Sparse^{FRT10}-p65.AD* (Addgene #232827).

   Note: For *FRT100-STOP-FRT100-T2A* sequence, use *78H05-Sparse^{FRT100}-p65.AD* (Addgene #232828) as the template.

Note: Following recombination, the in-frame peptide derived from FRT10 and T2A sequences will be cleaved off from the TF after the translation.

Alternatives: Instead of inserting the *FRT10-STOP-FRT10-T2A* sequence in-frame with the TF coding sequence, an alternative approach is to insert the *FRT10-STOP-FRT10* (without the *T2A* sequence) between the *Drosophila* synthetic core promoter (DSCP) and the coding sequence. Ensure the Kozak sequence remains intact if present in the original construct.

3. Insert the *FRT10-STOP-FRT10* sequence and the *T2A* element after the start codon of the p65.AD (or other desired TFs) and keep them in-frame (Figure 1C).
4. Verify the Sparse^{FRT10}-AD construct, *pBP-Sparse^{FRT10}-p65ADZpUw*, by sequencing.

Note: For the use of p65.AD or GAL4.DBD with Sparse^{FRT10} sequence, *pBP-Sparse^{FRT10}-p65.AD* (Addgene #232830) or *pBP-Sparse^{FRT10}-GAL4.DBD* (Addgene #232831) are available for enhancer recombination.

Generation of the Enhancer-Sparse-TF construct

Timing: 1 week

This section describes the process for generating an Enhancer-Sparse-TF construct by amplifying enhancer sequences characterized by the FlyLight Project and assembling them into the Sparse-TF plasmid.

5. Collect primers of selected enhancers from the FlyLight Project (https://www.janelia.org/project-team/flylight).

Note: For example, for the enhancer *VT028327*, we first verified its expression in the developing and adult brain and obtained the primer sequences from the enhancer's website (https://flweb.janelia.org/cgi-bin/view_flew_imagery.cgi?line=VT028327).

6. Lysis the *w1118* strain or the corresponding Bloomington stock (e.g., BDRC #73064 for the VT028327 enhancer) with the Phire Tissue Direct kit (Thermo Fisher #F170L).
7. PCR-amplify enhancer fragments from the lysate using primers from the FlyLight Project.
8. Purify the PCR products and confirm their identity through agarose gel analysis and sequencing.
9. Assemble the verified enhancer fragment into the *pENTR/D-TOPO* vector, integrate it into the *pBP-Sparse^{FRT10}-p65ADZpUw* vector using the Gateway LR Clonase II Enzyme Mix, and generate *VT028327-Sparse^{FRT10}-p65.AD* construct.
10. Verify the Enhancer-Sparse^{FRT10}-AD construct, *VT028327-Sparse^{FRT10}-p65.AD*, by full-length plasmid sequencing.

Generation and maintenance of transgenic flies carrying the sparse driver

Timing: 6–8 weeks

This section outlines the generation of transgenic *Drosophila* flies by microinjecting DNA into early embryos and balancing the resulting progeny to isolate successful transformants.

11. Generate transgenic flies in-house using standard methods[21] or commercial injection services like BestGene (https://www.thebestgene.com/HomePage.do).
    a. Microinject DNA into early *Drosophila* embryos before cellularization.
    b. Cross G0 flies to a *white^{-}* balancer.
    c. Individually balance and verify all *white^{+}* progeny.

Note: The Bloomington stock of the parent driver *VT028327-p65.AD* uses docking site *attP40*. We selected the docking site *VK00027*, which has a similar expression to *attP40*, for *VT028327-Sparse^{FRT10}-p65.AD* and *VT028327-Sparse^{FRT100}-p65.AD*.

Note: Keep the sparse driver and *hsFLP* (or other FLP transgenes) in separate stocks. This prevents stochastic FLP expression-mediated recombination and avoids the loss of the *Sparse^{FRT10}* or *Sparse^{FRT100}* cassette.

Generation of experimental flies carrying desired transgenes

Timing: 2 weeks

This section describes steps and tips to obtain experimental offsprings with desired transgenes.

12. Cross parent flies with desired transgenes to get experimental offsprings.

Note: Genotype or functionally validate the sparse driver and other transgenes (e.g., reporters, effectors, *hsFLP*) before the final cross to reduce future troubleshooting difficulties.

Note: The crossing scheme is highly flexible for generating parent flies with the desired transgenes. Adjust the scheme based on the availability and genomic locations of genetic reagents, preferred balancers and markers, and any experimental requirements for a specific gender.

Optional: To assess phenotype in the pupal stage, if possible, use parents with homozygous transgenes or balancers that have markers identifiable in pupae (e.g., *Wee-P* and *Tb*). This is not required but can maximize the likelihood of obtaining the correct genotype in pupae.

Validation of the sparse driver and titration for desired sparsity

Timing: 1–2 weeks

This section describes the validation and titration process for achieving desired cell sparsity in *Drosophila* by adjusting heat-shock durations. Pupae are synchronized and subjected to varying heat-shock times, with stepwise adjustments to optimize labeling.

Note: This procedure requires *Drosophila* samples carrying all necessary transgenes (e.g., reporters, *hsFLP*, sparse driver, with its split partner driver included if required) to target sparse cells.

13. Transfer all adults to a new vial.
14. Remove any existing pupae from the original vial, then set a collection time window to synchronize the pupal stage.

Note: Adjust the collection time window based on the experiment. Longer windows allow more individuals per batch but increase variability in heat-shock-to-readout intervals, leading to inconsistent FLP expression and sparsity. For time-sensitive studies like development, shorter windows are recommended to ensure consistent gene activation timing.

15. After the time window, collect the correct pupae for each test condition (e.g., no heat-shock, 30 min, 1 h, and 2 h of heat-shock) and transfer them into separate vials.

Note: Place them on the wall of each vial and below the water bath water level to maximize the heat transfer efficiency.

16. Heat-shock vials in a 37°C water bath according to the specified durations (Figure 1G, left).
17. Wipe up vials and transfer them back to the incubator.
18. Collect pupae or adults at the desired stage. Dissect the flies, perform immunostaining (refer to the immunostaining section), and proceed with imaging.
19. Determine the optimal heat-shock duration within current conditions, then proceed to the sparse labeling and manipulation section;

Alternatives: If some heat-shock conditions fail to achieve the desired labeling sparsity, perform an additional round of titration with extended or refined conditions.

20. If all heat-shock conditions label a large subset of cells, perform another round of fine-tuning titration (e.g., 15 min, 10 min, 5 min, 1 min, or 30 s durations):
    a. For durations longer than 5 min, perform heat-shock in a 37°C water bath as previously described (Figure 1G, left; also see steps 15–18). Collect pupae or adults at the desired stage. Dissect the flies, perform immunostaining, and proceed with imaging (Figure 1E).
    b. For durations of 5 min or less (Figure 1G, right):
        i. Wrap pupae in a single layer of paper towel soaked with room-temperature water, ensuring no air bubbles to maintain efficient heat transmission.
        ii. Using forceps, immerse the "paper bag" in a 37°C water bath for the target duration, then cool in a room-temperature water bath for 60 s.
        iii. Transfer the pupae back to the vials and return them to the incubator.
    c. Collect pupae or adults at the desired stage. Dissect the flies, perform immunostaining, and proceed with imaging (Figure 1F).
    d. Determine the optimal heat-shock duration within adjusted conditions, then proceed to the sparse labeling and manipulation section.

Note: Room temperature = 18°C–22°C.

21. If none of the heat-shock conditions label any cells, refer to the troubleshooting section.

Note: The heat-shock-to-readout interval also affects the accumulated expression level of FLP. Therefore, the heat-shock duration should be adjusted when conducting comparative experiments across early development and adulthood.

Note: The total cell number targeted by the parent driver and the tissue depth will also influence the heat-shock duration required for achieving the desired sparsity.

Note: Since FLP-induced recombination and enhancer activation are independent processes, the heat-shock can be applied before the enhancer activation begins.

Optional: To increase the likelihood and speed of determining the optimal heat-shock duration, test both *Sparse*^{FRT10} and *Sparse*^{FRT100} in parallel.

Sparse labeling and manipulation

Timing: 1 week

This section outlines the sparse labeling and manipulation process in *Drosophila*, using heat-shock treatments to induce sparse transgene expression for targeted cell analysis. It enables single-cell

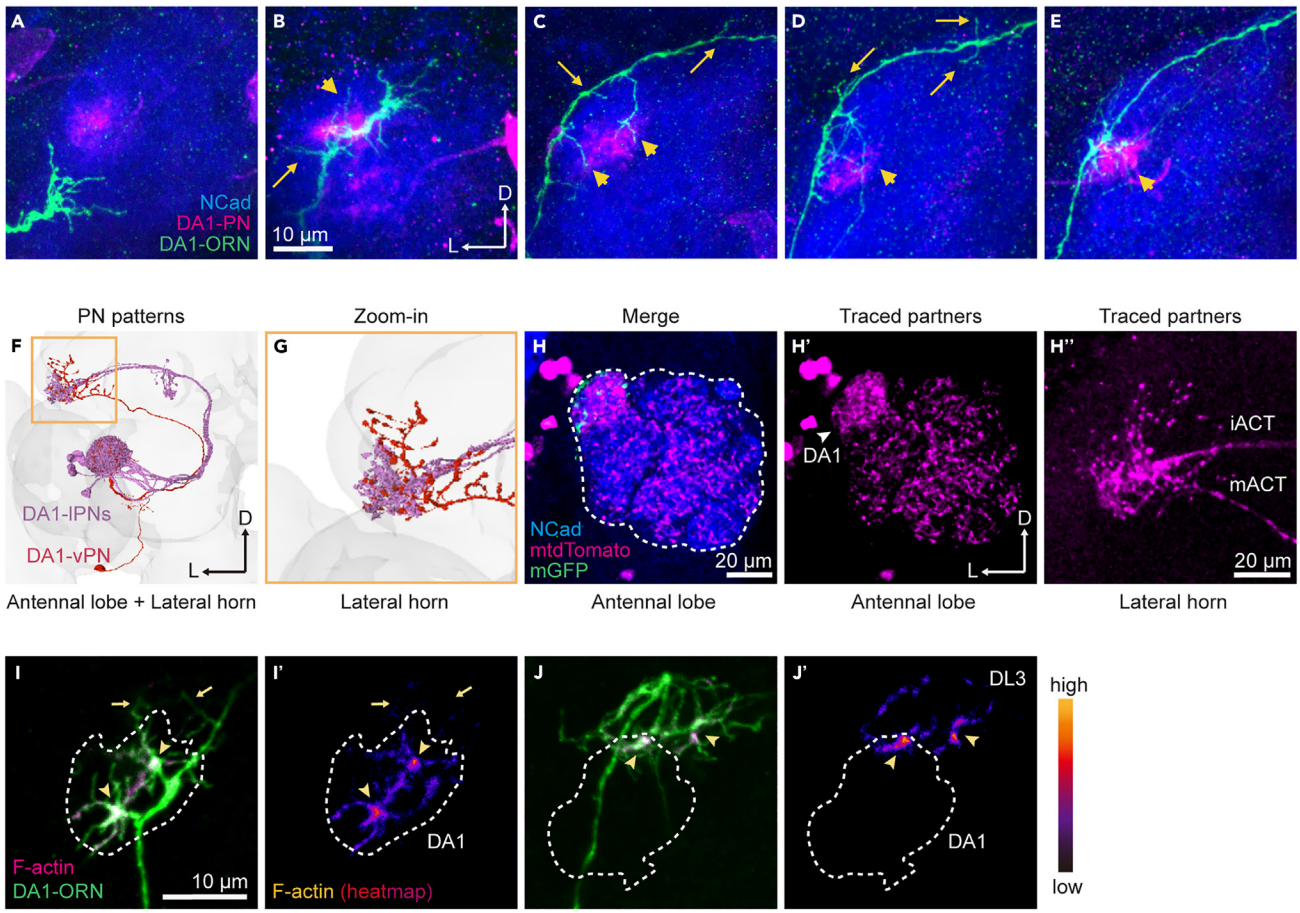

Figure 2. Sparse driver system in various applications

(A–E) Examples of single DA1-ORN axons (green) innervating the ipsilateral antennal lobe in different stages. Axonal exuberant branches that contact target DA1-PN dendrites (magenta) are eventually stabilized, showing a stabilization-upon-contact manner. Arrows, non-DA1-PN-contacting (OFF-target) branches; arrowheads, DA1-PN-contacting (ON-target) branches.

(F, G) FlyWire tracings of DA1-lPNs and DA1-vPN from the left hemisphere (F), with a magnified view at the lateral horn (yellow box), to visualize their stereotyped axon branching patterns (G).

(H-H″) Representative confocal images of *trans-Tango*-mediated trans-synaptic tracing from DA1-PNs. Green, ORN axons; magenta, postsynaptic neurons labeled by *trans-Tango*, which include dendrites of local interneurons and more intensely labeled DA1-PNs in the antennal lobe (H, H′) and DA1-PN axons in the lateral horn (H″). Dashed outlines, antennal lobe.

(I, I′) Representative confocal images of the F-actin distribution (I, magenta; I′, heatmap based on Halo-Moesin staining) in a control DA1-ORN axon (I, green). Arrows, non-DA1-PN-contacting subregion; arrowheads, F-actin hotspots. Dashed white traces outline DA1-PN dendrites.

(J, J′) Representative confocal images of the F-actin distribution in a Ten-m overexpressing DA1-ORN axon (targeting to the DL3 glomerulus). Labels are the same as I and I′. D, dorsal; L, lateral. NCad (N-cadherin) is a general neuropil marker.

resolution studies of neuronal structure, function, and connectivity through various labeling and tracing tools.

Note: This procedure requires *Drosophila* samples carrying all necessary transgenes (e.g., effectors, reporters, *hsFLP*, sparse driver, with its split partner driver included if required) to target sparse cells.

Note: In principle, the sparse driver system should allow membrane markers for morphology or live imaging studies, protein markers for subcellular localization studies, tracing tools for trans-synaptic tracing, GCaMPs for real-time activity monitoring, genetic and optogenetic

tools for gene and neuron manipulation, or combinations of the above at single-cell resolution.

Note: For demonstration, we used *VT028327-Sparse*$^{FRT10/FRT100}$*-p65.AD* and *GMR22E04-GAL4.DBD* to target DA1-ORN axons with different sparsity (Figures 1E and 1F), *UAS-myr-mGreenLantern* and *UAS-mCD8-GFP* to label the membrane, *Mz19-QF2*^{G4HACK} and *QUAS-mtdTomato-3xHA* to orthogonally mark DA1-PN dendrites (Figures 2A–2E), *trans*-Tango for trans-synaptic tracing (Figures 2H–H″), and *UAS-Halo-Moesin* to label F-actin (Figures 2I and 2I′).

Note: For a protocol outlining how to perform single ORN live imaging, please refer to Li and Luo (2021).

22. Transfer all adults to a new vial.
23. Remove any existing pupae from the original vial, then set a specific collection time window to synchronize the pupal stage.

Note: For the DA1-ORN development study, we use a 0–6 h APF (after puparium formation) window.

24. After a 6-h time window, collect the correct pupae and transfer them into a new vial.

Note: If necessary, select against pupal markers, e.g., *Wee-p* or *Tb*, to maximize the success rate; refer to generation of experimental flies carrying desired transgenes section.

25. For *VT028327-Sparse*^{FRT100}*-p65.AD* (Figure 1G, left):
    a. Heat-shock the vial in a 37°C water bath for 1 h.

Note: Place them on the wall of the vial and below the water bath water level to maximize the heat transfer efficiency.

    b. Wipe up vials and transfer them back to the incubator.
26. For *VT028327-Sparse*^{FRT10}*-p65.AD* (Figure 1G, right):
    a. Wrap pupae in a single layer of water-soaked paper towel, ensuring no air bubbles to maintain efficient heat transmission.
    b. Using forceps, immerse the "paper bag" in a 37°C water bath for 30 s, then cool in a room-temperature bath for 60 s.
    c. Transfer the pupae back to the vials and return them to the incubator.

Immunostaining

Timing: 5 days

This section describes an example immunostaining process for *Drosophila* brains.

27. Collect pupae or adults at the desired stage.
28. Dissect brains or other tissues in pre-cooled phosphate-buffered saline (PBS).
29. Fix them in 4% paraformaldehyde in PBS with 0.015% Triton X-100 (fixing buffer) for 15 min on a nutator at room temperature.

Note: If necessary, adjust fixation conditions to minimize background from over-fixation.

30. Wash the fixed brains with 0.3% Triton X-100 in PBS (washing buffer) four times, nutating for 15 min each time.
31. Block the brains in 5% normal donkey serum in PBST (blocking buffer) for 1 h at room temperature or overnight at 4°C on a nutator.

Note: Overnight = 8–24 h.

32. Dilute primary antibodies in the blocking buffer and incubate the brains with the antibodies for 36–48 h on a 4°C nutator.

Note: Primary antibodies used in immunostaining include rat anti-NCad (1:40; DN-Ex#8, Developmental Studies Hybridoma Bank), chicken anti-GFP (1:1000; GFP-1020, Aves Labs), and rabbit anti-HA (1:100, 3724S, Cell Signaling).

33. After incubation, wash the brains with washing buffer four times, nutating for 20 min each time.
34. Incubate the brains with secondary antibodies diluted in the blocking buffer, nutating in the dark for 24–48 h at 4°C.

Note: Donkey secondary antibodies conjugated to Alexa Fluor 405/488/568/647 (Jackson ImmunoResearch or Thermo Fisher) were used at 1:250.

35. Rewash the brains with washing buffer four times, nutating for 20 min each time.
36. Mount the immunostained brains with SlowFade antifade reagent and store them at 4°C until imaging.

HaloTag labeling

Timing: 1 day

This section describes an optional halotag labeling step for *Drosophila* brains.

37. Dissect fly brains in pre-cooled PBS and fix them in 4% paraformaldehyde in PBS for 10 min on a nutator at room temperature.

Optional: Fix the tissue using a standard fixation buffer (with 0.015% Triton X-100) if the sample is sticky inside the tube.

38. Wash the fixed brains with washing buffer for 5 min, repeating the wash thrice.
39. Incubate the brains with Janelia Fluor 646 HaloTag Ligand (0.5 μM in PBS) for 5 h or overnight at room temperature in the dark.
40. After incubation, wash the brains with washing buffer for 5 min, repeating three times.
41. If needed, proceed with the immunostaining protocol (refer to the immunostaining section, steps 31–36).

EXPECTED OUTCOMES

The example dataset includes sparse axons of DA1-ORNs in the developing or adult antennal lobe.

Axon labeling with different sparsity in the adult or pupal brain

Across a range of heat shock durations (15–120 min), DA1-ORN Sparse^{FRT100}-AD-based split GAL4 labeled no axons or sparse axons in the adult antennal lobe (Figure 1E). Over 0.5–5 min of heat shock, Sparse^{FRT10}-AD-based split GAL4 labeled a single axon, an intermediate subset, or a large subset of DA1-ORN axons (Figure 1F).

ORN-PN synaptic partner matching at single-axon resolution

The orthogonal labeling of the DA1 ORN-PN pair revealed that, during development, the DA1-ORN axon initially overproduces exuberant branches along the stem axon to expand the searching space for target selection (Figures 2A–2E). Over time, branches that contact the target PN dendrites are stabilized, while OFF-target branches are pruned.

Single-neuron trans-synaptic tracing

Trans-synaptic tracing of a single DA1-ORN axon labeled its contacting neurons, including DA1-PNs and local interneurons, in the antennal lobe (Figures 2H–2H″). The traced DA1-PNs exhibited a morphology similar to DA1-PNs reconstructed in the Flywire database (Figures 2F and 2G).

Single-axon manipulation and HaloTag staining

Co-labeling of membrane marker and F-actin marker in control DA1-ORN single axons revealed that contact with DA1-PN dendrites promoted local F-actin levels in target-contacting branches (Figures 2I and 2I′). Overexpression of Ten-m (tenascin-major),[1,22] a transmembrane protein instructing synaptic partner matching, in single axons led to axon mistargeting and promoted F-actin levels in the DL3 glomerulus (Figures 2J and J′).

Note: Data from Figures 1D, 1F, and 2 are reprinted with permission from Xu et al.[1]

Fly genotype	
Figure 1D	*UAS-dcr2, UAS-CD8-GFP / +; VT028327-p65.AD / +; GMR22E04-GAL4.DBD / +*
Figure 1E	*UAS-CD8-GFP, hsFLP / UAS-dcr2, UAS-CD8-GFP; ; GMR22E04-GAL4.DBD / VT028327-FRT100-STOP-FRT100-p65.AD*
Figure 1F	*UAS-CD8-GFP, hsFLP / UAS-dcr2, UAS-CD8-GFP; ; GMR22E04-GAL4.DBD / VT028327-FRT10-STOP-FRT10-p65.AD*
Figures 2A–2E	*UAS-CD8-GFP, hsFLP / UAS-dcr2, UAS-CD8-GFP; Mz19-QF2^{G4HACK}, QUAS-mtdTomato-3xHA / UAS-myr-mGreenLantern; GMR22E04-GAL4.DBD / VT028327FRT10-STOP-FRT10-p65.AD*
Figures 2H–2H″	*QUAS-mtdTomato-3xHA, UAS-CD8-GFP, hsFLP / + ; trans-TANGO / +; GMR22E04-GAL4.DBD / VT028327-FRT10-STOP-FRT10-p65.AD*
Figures 2I and 2I′	*UAS-CD8-GFP, hsFLP / UAS-dcr2, UAS-CD8-GFP; Mz19-QF2G4HACK, QUAS-mtdTomato-3xHA / UAS-myr-mGreenLantern; GMR22E04-GAL4.DBD / VT028327-FRT10-STOP-FRT10-p65.AD, UAS-Halo-Moesin*
Figures 2J and 2J′	*UAS-CD8-GFP, hsFLP / UAS-dcr2, UAS-CD8-GFP; Mz19-QF2G4HACK, QUAS-mtdTomato-3xHA / UAS-myr-mGreenLantern; GMR22E04-GAL4.DBD, UAS-V5-Ten-m /VT028327-FRT10-STOP-FRT10-p65.AD, UAS-Halo-Moesin*

LIMITATIONS

Although this protocol enables single-cell visualization and manipulation *in vivo*, it has a few limitations. First, the performance of the sparse driver system relies heavily on the properties of the parent driver. If the parent driver fails to target the desired cell population effectively, this protocol cannot robustly access single cells within that population *de novo*. Additionally, the sparse driver system has only been tested with enhancer lines from the FlyLight Project; GAL4-based enhancer trap lines and T2A-GAL4 knock-in lines remain untested. We have also deposited HACK[23,24]-based constructs (*pHACK-Sparse^{FRT10}-p65.AD*, Addgene #232832; *pHACK-Sparse^{FRT10}-GAL4.DBD*, Addgene #232833; *pHACK-Sparse^{FRT10}-GAL4*, Addgene #232834) for users who are interested in testing HACK-based sparse drivers. Second, while heat-shock at the pupal stage ensures controllable heat transmission, heat-shock at the larval or adult stage has not been tested. Third, the heat-shock promotor may respond to other stimuli, such as heavy metals, oxidative stress, UV radiation, hypoxia, inflammation, and certain chemical treatments. Therefore, this protocol may not be suitable for experiments involving these treatments.

TROUBLESHOOTING

Problem 1

No cells are labeled after sparsity titration (Related to steps 13–21).

Potential solution

If a 2-h heat-shock duration does not label any cells:

- Try extending the duration or performing multiple heat-shocks.
- Verify the genotype of experimental pupae, parents, and stocks to ensure all necessary components are present.
- Sequence the sparse driver stocks to confirm the presence of both mutations in either *FRT10-STOP-FRT10* or *FRT100-STOP-FRT100*.
- Functionally validate *hsFLP* by using *UAS-FRT-STOP-FRT-mCD8-GFP*. Double-check the characteristics of the original driver to confirm if it is active at the assay stage.
- Use stronger parent drivers (if available), improved reporters (e.g., *UAS-myr-mGreenLantern* and *UAS-Halo-CAAX*), and optimized fixation and staining conditions to enhance the signal-to-noise ratio.
- Increase the sample size of each condition.

Problem 2

Too many cells are labeled after sparsity titration (Related to steps 13–21).

Potential solution

If a 30-s heat-shock duration still labels too many cells:

- Try reducing the duration or decreasing the heat-shock temperature.
- Try the *FRT100-STOP-FRT100* sequence.
- Reduce the interval between the heat-shock and the readout time.
- Sequence the sparse driver stocks to ensure that the *FRT10-STOP-FRT10* or *FRT100-STOP-FRT100* have been correctly constructed.

Problem 3

Samples show a high background or weak signal (related to steps 27–36).

Potential solution

- Use stronger parent drivers (if available), improved reporters (e.g., *UAS-myr-mGreenLantern* and *UAS-Halo-CAAX)*, and optimized fixation and staining conditions to enhance the signal-to-noise ratio.

Problem 4

The probability of the same sparsity is inconsistent across batches (Related to steps 22–26).

Potential solution

- Ensure that intervals between the heat-shock and the readout time are consistent across batches.
- Confirm that transgenes are homozygous in the parent generation or strictly select against pupal markers, e.g., *Wee-p* or *Tb*, in the experimental generation.
- Perform consistent heat-shock durations across batches.
- Avoid stacking samples during heat-shock.
- If the heat-shock duration is 5 min or less, ensure no air bubbles in the "paper bag" and sufficient cooling time in the room temperature water bath.

- Divide large sample sizes into smaller batches (~30 pupae per bag) to prevent stacking and improve uniformity.
- Check the copy number of *hsFLP* transgene.

Problem 5

Animals are dying after long heat-shock (Related to steps 13–18).

Potential solution

- Weaker flies may need multiple shorter heat-shocks; try 2–3 heat-shocks of 30 min each, with 30-min recovery intervals.

Problem 6

Labeled cells in the control condition without heat-shock (Related to steps 13–21).

Potential solution

- Normally, in the absence of FLP, the sparse driver does not spontaneously recombine. Spontaneous recombination is typically caused by FLP leakage and accumulated expression. To address this, reduce the heat-shock-to-readout interval for all conditions (ensuring consistency in experimental timing), use the less sensitive *FRT100-STOP-FRT100*, or raise flies at 25°C.

Problem 7

The expression level of a transgene changes when co-expressed with varying numbers of other transgenes or between experimental and control groups (Related to steps 22–26).

Potential solution

- When a cell expresses multiple transgenes, they share the same driver TFs. If the driver cannot produce enough TFs for all transgenes, the TF dilution effect becomes apparent. To ensure consistency, maintain the same number of transgenes across conditions. Ideally, prepare a control transgene inserted into the same locus as the key transgene whose biological effect is to be examined.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Chuanyun Xu (chuanyun94@gmail.com).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Chuanyun Xu (chuanyun94@gmail.com).

Materials availability

Plasmids and *Drosophila* lines are available upon request from the lead contact.

Data and code availability

This study did not generate or analyze datasets or code.

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AUTHOR CONTRIBUTIONS

Methodology and investigation, C.X.; characterization and cloning assistance, Z.L.; writing, C.X. and L.L.; funding acquisition and supervision, L.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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