Cell-type-specific Labeling of Endogenous Proteins Using the Split GFP System in *Drosophila*

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Running head: Labeling of Endogenous Proteins using $GFP_{1\text{-}10/11}$ in Flies

i. Abstract

Accurate identification of the locations of endogenous proteins is crucial for understanding their functions in tissues and cells. However, achieving precise cell-type-specific labeling of these proteins has been challenging *in vivo*. A notable solution to this challenge is the self-complementing split green fluorescent protein ($GFP_{1-10/11}$) system. In this paper, we present a detailed protocol for labeling endogenous proteins in a cell-type-specific manner using the $GFP_{1-10/11}$ system in fruit flies. This approach depends on the automatic reconstitution of the GFP_{1-10} and GFP_{11} fragments, creating a fluorescence signal. We insert the GFP_{11} fragment into a specific genomic locus while expressing its counterpart, GFP_{1-10}, through an available Gal4 driver line. The unique aspect of this system is that neither GFP_{1-10} nor GFP_{11} alone emits fluorescence, enabling the precise detection of protein localization only in Gal4-positive cells. We illustrate this technique using the adhesion molecule gene *teneurin-m* (*Ten-m*) as a model, highlighting the generation and validation of GFP_{11} protein trap lines via Minos-mediated integration cassette (MiMIC) insertion. Furthermore, we demonstrate the cell-type-specific labeling of Ten-m proteins in the larval brains of fruit flies. This method significantly enhances our ability to image endogenous protein localization patterns in a cell-type-specific manner and is adaptable to various model organisms beyond fruit flies.

ii. Key Words

Split GFP, GFP_{1-10}, GFP_{11}, Cell-type-specific Labeling, Endogenous Proteins, Drosophila

1. Introduction

Fluorescent proteins (FPs) have become a cornerstone in cell and developmental biology. Observing the behaviors of proteins tagged with FPs in live specimens provides critical insights into various biological processes, including macromolecular assembly, protein trafficking, and protein translation [1]. Typically, these experiments involve overexpressing the protein of interest in cells or tissues of model organisms. While this overexpression strategy is highly effective, it can lead to artifacts due to the use of basal or tissue-specific promotors, often differing from the transcriptional regulation of target genes. These artifacts include protein mislocalization and aggregation, abnormal organelle morphology, and the mistiming of cellular signaling events [2, 3]. To mitigate these issues, we employ endogenous gene tagging, where an FP's coding sequence is inserted into a specific genomic locus [4]. This allows the FP-fused proteins to be expressed under native transcriptional and translational controls.

Historically, full-length FPs have been widely used in various applications. More recently, the split $GFP_{1-10/11}$ system has emerged as a valuable tool for live-cell protein imaging, especially in tissue imaging, due to its binary nature, which facilitates cell-type-specific labeling [5-9]. In this system, the super-folder GFP sequence is divided between β-strand 10 and 11 (GFP_{1-10} and GFP_{11}) [10]. The GFP_{11} fragment, a 16-residue peptide, fluoresces only when in the presence of its counterpart, GFP_{1-10}, as they can self-assemble to form a functional GFP. Neither GFP_{1-10} nor GFP_{11} alone is fluorescent. Our previous research has used this system to investigate the adhesion molecule Ten-m in Drosophila, previously known for its expression in the nervous system but whose synaptic functions required further exploration [11, 12]. We have developed a knock-in line of *ten-m* tagged with GFP_{11} using the Minos-mediated integration cassette (MiMIC) technique, a state-of-the-art method for precise genomic integration. By using a specific MiMIC insertion line that contains a *MiMIC* fragment, we can replace the *MiMIC* with the GFP_{11} fragment through Recombinase-Mediated Cassette Exchange (RMCE). This replacement has enabled the creation of *ten-m* knock-in lines tagged with GFP_{11}, allowing us to visualize the endogenous localization of Ten-m in the central nervous system (CNS). An additional advantage of this approach is the multimerization

capability of GFP_{11}, enabling the tagging of a gene with multiple GFP_{11} repeats (e.g., GFP_{11x7}) for enhanced fluorescent signals. Using this technique, we enhanced the Ten-m signal and achieved cell-type-specific labeling in various neuron types by expressing GFP_{1-10} in specific cells of the Drosophila brain.

In this chapter, we present a detailed protocol for this innovative imaging technique, focusing on its application to the *ten-m* gene locus in *Drosophila*. We detail the procedures for knock-in generation via MiMIC insertions, including the creation and injection of the plasmid and the subsequent crossing schemes. We emphasize the importance of confirming the directionality of GFP_{11} insertion for successful protein labeling. Lastly, we discuss the process for achieving cell-type-specific labeling of Ten-m proteins in the larval brains of fruit flies. This method can be adapted to label a wide range of proteins in a cell-type-specific manner.

2. Materials

1. DNA Preparation

- *GFP_{11}* DNA with linker/BamHI site (synthesized by company, Integrated DNA Technologies)
- BamHI (New England Biolabs Inc, R0136S)
- Protein trap RMCE plasmids in all 3 reading frames: Phase 0 (RRID: DGRC_1298), Phase 1 (RRID: DGRC_1306), Phase 2 (RRID: DGRC_1314)
- InFusion-HD Cloning System (Takara Bio USA, Inc., #638946)
- DH5α competent *E. coli* cells (Thermo Fisher Scientific, 18265017)
- Zymo Research Zyppy Plasmid Miniprep Kit (Zymo Research, #11-308)
- Nano spectrophotometer (Implen)

2. Injection

- MiMIC protein-trap flies of interest: *y[1] w[*]; Mi{y[+mDint2] =MIC}Ten-m[MI07828]* (RRID: BDSC_44887)
- ΦC31 integrase (RRID: BDSC_ 34771)
- Fly cage (Genesee Scientific #59-100)
- Standard grape juice plates
- *GFP_{11}* and *GFP_{11x7}* plasmids from DNA preparation step in the appropriate reading frame (RRID: Addgene_172068, Addgene_172067). Ten-m is found is phase 1 thus it is possible to use the AddGene plasmids for *GFP_{11}* insertions. See Note 4 about other phase insertions.
- Capillary glass with filament (Harvard Apparatus #30-0032)
- Needle puller (Narishige #PN-31)
- Spin filtration column (Co-Star #8160)
- 18 mm x 18mm cover glass (Corning #2845-18)
- Glass slide (Eisco #BI0082)
- Needle holder (Narishige H-7)

- Upright microscope with LED light source (Nikon Eclipse Ci)
- 100% Ethanol (Sigma #E7023)
- Mineral oil
- FemtoJet 5247 Microinjector (Eppendorf, Discontinued)
- Dumont #5 forceps (Fine Science Tools #11252–20)
- Standard food vial

3. Line Production and Validation

- Balancer adult flies (*yw/Y; TM3, Sb[1]/TM6B, Tb[+]*, RRID: BDSC_3720)
- Microcentrifuge tubes
- PureLink™ Genomic DNA Mini Kit (Invitrogen, K1820-01)
- GFP_{11} Tag-specific primers: GFP11.for (CGTGACCACATGGTCCTTCATGAGT) and GFP_{11}.rev (TGTAATCCCAGCAGCATTTA). See Note 5 for GFP_{11x7} primer sequences to use in validation.
- MiMIC-specific primers: MiL-F (GCGTAAGCTACCTTAATCTCAAGAAGAG). See Note 6 for MiL-R sequence needed for detailed orientation validation.
- For PCR reaction: Taq 2x Master Mix (New England Biolabs Inc, M0270L).

4. Tissue-specific Reconstitution

- GFP_{11} knockin line (generated in the previous step)
- Gal4 driver for cell-type of interest: *elav-Gal4* (RRID: BDSC_8765 or BDSC_8760), *dlip2-Gal4* (RRID: BDSC_35716), eve-Gal4 (RRID: BDSC_7473), *ato-Gal4* (RRID: BDSC_9494)
- *UAS-GFP*$_{1\text{-}10}^{sec}$ (for extracellular GFP_{11}; RRID: BDSC_93190) or *UAS-GFP*$_{1\text{-}10}$ (for intracellular GFP_{11}; RRID: BDSC_93189)

5. Imaging

- 100 x 1.45 NA oil immersion objective (Nikon).
- Inverted fluorescence microscope (Nikon) with a Dragonfly Spinning disk confocal unit (Andor). Any other confocal microscopes equipped with a 488-nm laser are suitable.

- Glass slide
- 22x22 mm square cover glass, #1.5 (Corning)
- Mounting media (1x PBS)
- Fiji software (NIH). Any other image processing software can be used.

3. Methods

3.1 Pre-experiment Reagent and Fly Preparation

3.1.1 Preparation of DNA for Transgenic Flies

The MiMIC insertion occurs randomly within the genome. The MiMIC insertion site, in relation to the open reading frame of the target gene, is a crucial consideration in selecting the appropriate backbone vector for RMCE, ensuring the correct translation of the gene product without a frameshift. Our gene of interest in this chapter, *ten-m* found in Phase 1, has been successfully tagged with GFP_{11} by insertion into the RMCE Plasmid Phase 1. If the gene of interest is found in other phases (i.e., Phase 0 or 2), it is possible to target GFP_{11} into those genes using their respective RMCE plasmids as previously published (See Note 1 about selecting target protein for GFP_{11} tagging).

1. We sourced the GFP_{11} coding DNA with the linkers and *BamHI* sites from an oligo synthesizer company (i.e., Integrated DNA Technologies). See Figure 1 for the plasmid design.
2. Digest the backbone vector (RMCE Plasmid Phase 1) with *BamHI* using standard procedures supplied by the manufacturer.
3. Run the digested vector on an agarose gel and isolate the DNA through gel purification and determine the recovered DNA concentration following purification for the cloning step with a nano spectrophotometer.
4. Using InFusion-HD cloning system, fuse the synthesized DNA into each RMCE plasmid backbone.
5. Transform the reaction product into DH5α competent *E. coli* cells.

6. Using a miniprep kit purify DNA from 5-10 colonies and check for insertion using a follow-up restriction digestion reaction.
7. Validate the new constructs in each open reading frame by sequencing prior to injection. The generated plasmid can be used to insert GFP_{11} into any MiMIC lines found in phase 1.
8. Following initial amplification, purify the GFP_{11} plasmid using a miniprep kit. Ensure the final concentration of DNA is at 100 ng/µl prior to microinjection using a nano spectrophotometer.

3.1.2 Fly Embryo Preparation and Needle Preparation for DNA Injection

For proper integration of external DNA (such as the GFP_{11} fragment) into the fly genome, it is essential to prepare the appropriate progeny before injection. For RMCE, the flies must carry the phiC31 integrase to allow for the GFP_{11} cassette to exchange out the original MiMIC-containing cassette. This step includes setting up a genetic cross and training the adult flies to lay eggs.

1. Start preparation 2-3 days prior to the experiment by setting up a fly cage with grape juice plates. Cross the virgin females carrying the phiC31 integrase on the X chromosome to the males carrying the MiMIC insertion in the Ten-m locus on the third chromosome. Each cage should maintain a male-to-female ratio of 1:5, encompassing a total of 100 adult flies per cage.
2. Transfer the flies into a cage and cover it with a grape juice plate. Maintain at 25°C (or room temperature) for approximately 3 days to allow the flies to synchronously lay eggs.

3.2 Knock-in Experiment

3.2.1 Injection of DNA Construct into Flies

The primary consideration is the proper execution of the injection process to maximize the number of survivors after injection. This is crucial for yielding 'founders' that transmit the GFP_{11} cassette to the next generation via germline transmission. Typically, more than 1-10% of the embryos become adults after injection.

1. On the day of injection, pull the needles using a needle puller with the heater level set to 81.6 and magnet level set to 45.5. The size of the needle tip should be empirically set because the glass will chip when touching the embryo.
2. Get the DNA ready for injection by passing it through a spin filtration column.
3. Load 1.5 µl of DNA per needle using Micro-loader pipette tip.
4. Replace the grape juice plate with a fresh one on the cage and collect embryos for one hour. This way you can ensure collection will be at stage 2, when the perivitelline space is observable.
5. Replace the plate every hour to continue collecting embryos for one hour each.
6. Collect embryos on a mesh, using a thin paintbrush to gently pick them up and place them on an 18mm x 18mm cover glass adhered on a slide with a drop of mineral oil.
7. Using the brush, align approximately 80 embryos per cover glass horizontally with the ventral side touching the glass while maintaining a moist condition. You can add a moist KimWipe strip to ensure embryos remain moist.
8. Position the slide on the stage, attach the DNA loaded needle to the capillary holder, and position both under the microscope with the tip of the needle facing the posterior end of the embryo.
9. Apply 100% Ethanol to cover the embryos completely, ensuring improved visibility for accurate observation.
10. Set the Pi (injection pressure) to 300 hPa (hectopascal), the Ti (injection time) to 0.1 second, and Pc (compensation pressure) to 300 hPa on the Eppendorf Microinjectors FemtoJet for injection. Using the hand control of the microinjector, inject the loaded plasmid into the embryos.
11. After the injection, carefully remove the cover glass from the slide and transfer it using forceps to a vial containing food.
12. Repeat steps 7 to 11 until you have injected up to 1,000 embryos.
13. Incubate the vial at 25°C until embryos reach adulthood, for about 9-10 days.

<u>3.2.2 Establishing Stable Transgenic Fly Stock</u>

Once DNA injection leads to adult flies, amplifying their progeny is crucial for establishing a stable transgenic line, a process aided by tracking the presence or absence of marker genes, such as yellow. Typically, approximately 10% of the injected adults become founders, producing progeny with a yellow-body color. This progeny can then be established as a stable line for studying GFP reconstitution in various tissue types.

1. Collect the resulting adults from section 3.2.1 and use an appropriate balancer for the third chromosome such as *yw; TM3, Sb[1]/TM6B, Tb[+]* or *yw/Y; TM3, Sb[1]/TM6B, Tb[+]*. (See Note 2 for establishing stable lines)
2. Collect the transgenic male flies with the absence of the yellow marker that has a balancer or a dominantly marked chromosome and cross with the balancer virgin females.
3. Intercross the balanced flies to establish stock. See Figure 3 for an example crossing scheme.

3.3 Post-genome Editing Analysis

3.3.1 PCR Analysis for Validation of Knock-in Event

After selecting progeny with a yellow-body color, we validate the successful knock-in event and the correct orientation of the GFP_{11} insertion through PCR analysis. By using standard DNA extraction and PCR reactions, we can assess the presence and orientation of the insertion prior to *in vivo* testing. It is important to note that after RMCE, the GFP_{11} fragment can be inserted in either direction – 5' to 3' or 3' to 5' - thus, validation of the orientation is required.

1. Using the manufacturer's protocol, extract DNA of 5 adult transgenic flies from F1 generation (PureLink™ Genomic DNA Mini Kit, Invitrogen). While we used the same volumes and concentrations of the reagents, we find that elution with 20 µl of Elution Buffer is sufficient in the final step.
2. Next, for the PCR reaction prepare PCR master mix with 10 µl reaction volume for each candidate. See Materials for specific primer sequences used.

a. Master mix recipe: 1 µl DNA, 5 µl Taq 2x Master Mix, 3 µl milliQ water, 0.5 µl per primer in each reaction listed.

   i. MiL-F and GFP_{11}.for

   ii. MiL-F and GFP_{11}.rev

3. Set the cycling conditions as follows:

   a. Initial denaturation at 95° for 30 seconds

   b. 35 cycles at 94° for 30 seconds, 67° for 1 minute, and 68° for 30 seconds

   c. Final extension at 68° for 5 minutes

d. Hold at 10 ºC.

4. Run PCR products on agarose gel. See Figure 2 for an example PCR validation for assessment of orientation (See Note 6 for detailed interpretation of results)

3.3.2 Protein Labeling in Specific Cell Types within Flies

The functional integration of *GFP_{11}* into a specific genomic locus and the reconstitution of those split GFP fragments can be validated using fluorescence microscopy. Further, increasing the number of *GFP_{11}* repeats can be tested for localizing low expression level proteins.

1. To achieve cell-specific reconstitution, express the *UAS-GFP_{1-10}* construct under a tissue- or cell-specific Gal4 driver (See Note 3 for tagging protein on the extracellular domain). This step can be done in parallel to the transgenic fly generation (section 3.3), to save time.
2. Using this stable line, cross it to the GFP_{11} knock-in line generated to visualize reconstituted $GFP_{1-10/11}$ signal to track protein localization.
3. Age the progeny of these flies to desired developmental stage (e.g., 2nd or 3rd instar larvae).
4. Dissect larval brains or the tissue of interest, if necessary.
5. Mount the tissue onto a clean glass slide using appropriate mounting media (e.g., 1x PBS).
6. Using a confocal microscope with an appropriate objective lens (e.g. 100x), acquire the image of reconstituted $GFP_{1-10/11}$ with a 488 nm laser.

7. If the reconstitution signal is weak due to a single copy of the GFP_{11}, it is possible to amplify the signal by generating a transgenic fly with the GFP_{11x7} construct by repeating sections 3.1-3.5 (see Notes 4 and 5) See Figure 4 for comparison of GFP_{11} and GFP_{11x7} tags in the larval brain.
8. Use ImageJ or other image analysis software to analyze the localization data. See Figure 5 for a representative confocal image endogenous Ten-m labeled with split GFP at 100x magnification in different cell-types.

4. Notes

1. The protocol we describe takes advantage of the MiMIC insertion collection generated as a part of the Gene Disruption Project (GDP) which allows for RMCE [13-15]. There are 17,500 MiMIC collection lines reported to be generated as part of the GDP. Of these lines, 1,860 of them have the MiMIC insertion in the intron positioned between the coding exons (also known as coding intron). These are the insertions that can be used to trap proteins with genetic tags such as the GFP_{11} [7] or a full-length *GFP* cassette as previously reported [13].

   Since *Minos* integrates randomly into the genome, it is important to consider where MiMIC is inserted as it may disrupt the gene function and prevent normal protein localization. According to the pilot studies, the GFP-tagged proteins through RMCE into a coding intron does not disrupt gene function in ~75% of the cases, thus allowing studies of endogenous localization [14, 15]. To assess whether protein function or localization is affected in these lines a complementation experiment or immunostaining experiment may be needed. The MiMIC fly stock information is maintained under the GDP website. To select a target protein, the information about the type of insertion site whether in the coding intron or elsewhere, as well as information for ordering flies through the BDSC can be found through the following link: https://flypush.research.bcm.edu/pscreen/mimic.html. CRISPR-mediated knock-in strategies can be incorporated to precisely modify target proteins, expanding the range of experimental possibilities for genes that are unavailable for protein trapping [16].

2. It is recommended to screen at least 10 independent candidates for 5 positive knock-in events which will guarantee 2-3 lines with proper insertion orientation.
3. To achieve cell-specific endogenous protein labeling with reconstituted GFP, there are 3 separate components that need to be integrated into the genome: tissue- or cell-specific Gal4, *UAS-GFP$_{1-10}$* and the GFP_{11}-tagged protein line generated by RMCE. If the protein is inserted into the membrane and is tagged on the extracellular domain, the secretion peptide tagged version of *GFP$_{1-10}$* should be used (*GFP$_{1-10}^{sec}$*) (RRID: BDSC_93190). If the protein in tagged on the intracellular domain, cytosolic *GFP$_{1-10}$* should be used (RRID: BDSC_93189).
4. If *GFP$_{11x7}$* will be used for tagging genes in phase 1, the modified plasmid is available through AddGene (RRID: Addgene_172067). Otherwise, it is possible to generate the *GFP$_{11x7}$* plasmid by repeating the steps described in section 3.1.
5. For validation of the GFP_{11x7} construct insertion in section 3.2.1, we used the following primers: GFP_{11x7}.for (CGTGACCACATGGTCCTTCATGAGTATGTAAATGCTGCTGGGATTACA) and GFP_{11x7}.rev (GGTGATACCGGCAGCATTGACATATTCGTGCAGGACCATAT) as the appropriate primer sequences.
6. If gene and original MiMIC insertion are in the same orientation, then the successful RMCE should screen positive for PCR reactions 2 only. If gene and original MiMIC insertion are in opposite orientations, then the successful RMCE should screen positive for PCR reactions 1 only. For more detailed validation PCR reactions should also be run with the MiL-R (CGCGGCGTAATGTGATTTACTATCATAC) primer. More thorough description of such approach can be found in the original manuscript [13].

5. Concluding remarks

A collection of approximately 1,800 MiMIC insertions, which are inserted in coding introns of specific genes, is now available [14, 15]. This resource enables the potential creation of a vast library of GFP_{11}-tagged genes, facilitating the study of the functions of proteins encoded by these genes. The generation of

this library opens a new era in *in vivo* proteomic analysis. In this chapter, we detailed the protocol for creating a knock-in line of GFP_{11}, using the genomic locus of Ten-m as an example. We demonstrated cell-type-specific labeling using various $GFP_{1\text{-}10}$ expression lines. These proteins can be imaged in unfixed tissues and cells. Furthermore, by combining other colors of split FP systems, it is possible to investigate the interactions between Ten-m and other molecules [7]. This protein-labeling strategy offers a new capability to study protein behaviors with unprecedented resolution in vivo and is likely to be extended to other organisms, including vertebrates.

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Figures

Linker GFP$_{11}$

GlyGlySerGlyGlySerGlyGlySerGlyGlySerMetArgAspHisMetValLeuHisGluTyrValAsnAlaAlaGlyIleThr
GGAGGTTCCGGTGGAAGCGGAGGTAGCGGCGGATCCATGCGTGACCACATGGTCCTTCATGAGTATGTAAATGCTGCTGGGATTACA

Linker

GlyGlyGlyGlySerGlyGlyGlySerGlySerGlySerGlyGlySerGlyGlySerGlyGlySer
GGTGGCGGCGGAAGTGGAGGTGGAGGCTCGGGCGGATCCGGAGGTAGCGGTGGAAGCGGAGGTTCC

Figure 1. Diagram of the GFP_{11} Plasmid Construction. This illustrates the sequence of the GFP_{11} plasmid, highlighting the specific insertion of the *GFP_{11}* coding sequence (green) into an established protein-trap plasmid. The insertion was facilitated using the *BamHI* sites (red) located between the peptide linkers. These peptide linkers, composed of a series of Glycine-Glycine-Serine amino acid residues, provide flexibility between the GFP_{11} fragment and the protein being tagged.

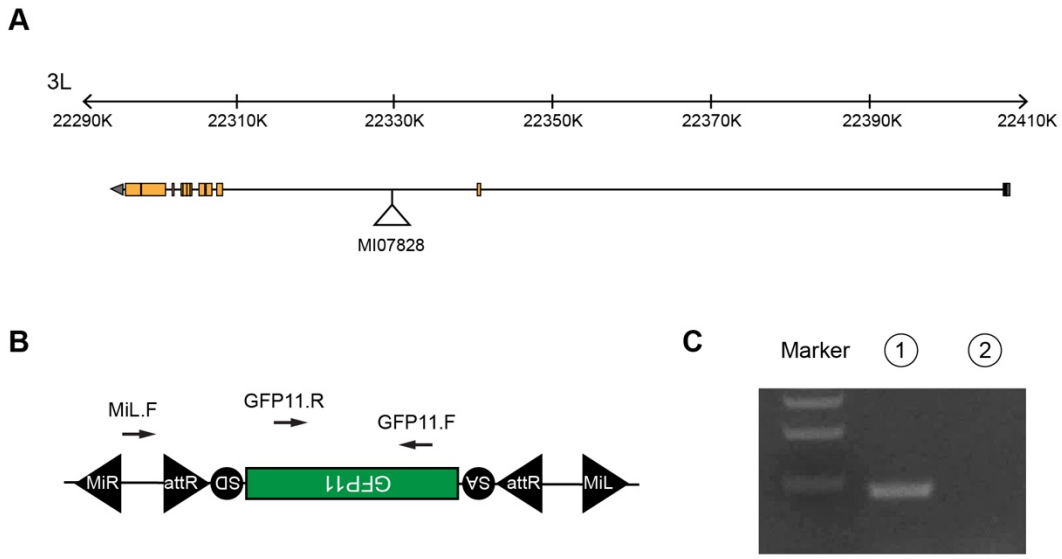

Figure 2. Assessment of GFP_{11} Insertion Orientation in the Ten-m MiMIC Insertion. (**A**) Schematic of a MiMIC insertion (MI07828) in the *ten-m* gene. (**B**) Representation of the RMCE process with *attP* sites, leading to the substitution of the target sequence with the GFP_{11} sequence from our plasmid construct. This process allows for the integration of GFP_{11} in two possible orientations, with the figure showing the preferred orientation. SA (splice acceptor) and SD (splice donor). This diagram also highlights the use of three specific primers for PCR reactions, which enable the determination of the GFP_{11} integration orientation. (**C**) An agarose gel image displays the PCR products, indicating that GFP_{11} has been inserted in the desired orientation. The numbers above the gel lanes correspond to the primer pairs used: 1 - MiL.F and GFP_{11}.for; 2 - MiL.F and GFP_{11}.rev.

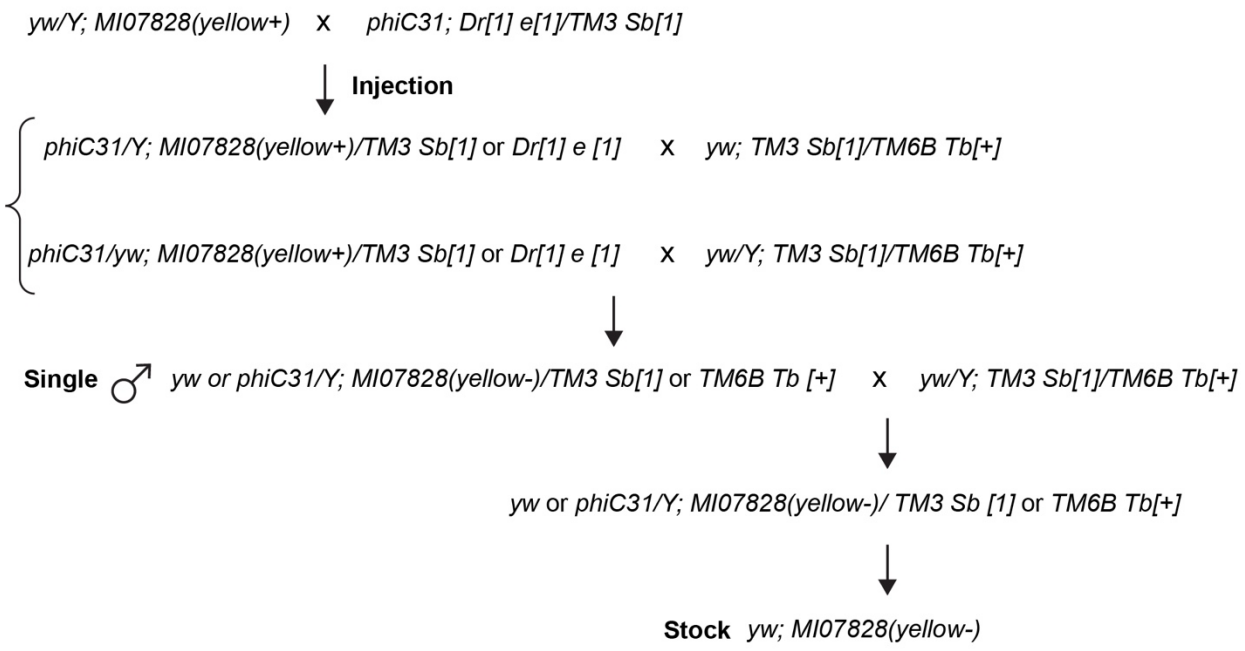

Figure 3. Crossing Schemes for GFP_{11} Line via MiMIC Insertion Using RMCE. This figure illustrates the specific crosses performed to establish the *GFP_{11}* line.

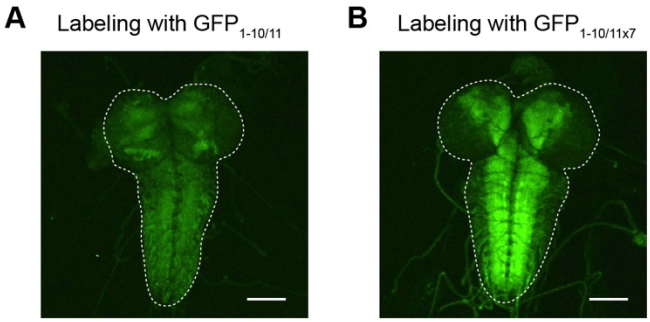

Figure 4. Reconstituted GFP Signals from GFP_{11x1} and GFP_{11x7}. Representative images display the reconstituted signals from GFP_{11} (A) and GFP_{11x7} (B). To visualize Ten-m, we crossed either the *GFP_{11}* or the *$GFP_{11 \times 7}$* strain with a pan neuronal *GFP_{1-10}* expression line. Scale bars: 100 µm.

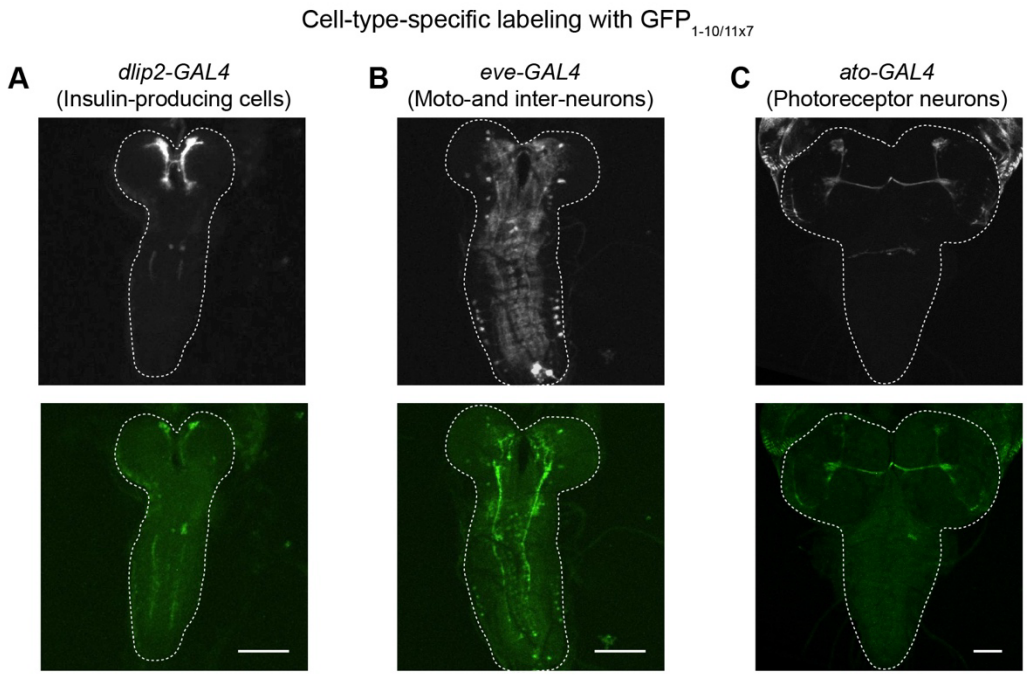

Figure 5. Cell-Type–Specific Labeling of Ten-m Proteins. Representative images demonstrate cell-type–specific fluorescence of a CD4 membrane marker (Top) and Ten-m (Bottom). The $GFP_{11 \times 7}$ protein trap line of ten-m was crossed with various $GFP_{1–10}$ expression lines. The following Gal4 lines were used to drive *UAS-*$GFP_{1–10}$ expression: (A) *dlip2-Gal4*, (B) *eve-Gal4*, and (C) *ato-Gal4*. Dotted lines mark the boundaries of the larval brains. Scale bars: 100 μm.

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