A protocol for mosaic analysis with a repressible cell marker (MARCM) in *Drosophila*

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Mosaic analysis with a repressible cell marker (MARCM) is a genetic technique used in *Drosophila* to label single cells or multiple cells sharing a single progenitor. Labeled homozygous mutant cells can be generated in an otherwise unlabeled heterozygous animal. Mutant or wild-type labeled cells can also be made to express one or more transgenes. Major applications of MARCM include (i) lineage analysis, (ii) investigating gene function in single or small populations of cells and (iii) neuronal circuit tracing. Our laboratory uses MARCM primarily to label and genetically manipulate neurons; however, this protocol can be adapted to any cell of interest. The protocol involves generating two fly stocks with the necessary genetic elements for MARCM analysis and subsequently generating MARCM clones. Labeled clones can be followed in live and fixed tissues for high-resolution analysis of wild-type or genetically manipulated cells.

INTRODUCTION

Mosaic analysis has been used widely in *Drosophila* to analyze gene functions. Techniques such as flippase (FLP) recombinase/FLP recombination target (FRT) system-mediated mitotic recombination[1], and the further ability to genetically mark homozygous mutant clones and wild-type twin spots[2], have allowed scientists to study mutant tissues in the background of a phenotypically wild-type heterozygous organism. These techniques allow for the positive marking of homozygous wild-type clones as well as heterozygous tissues where recombination has not occurred, but leave homozygous mutant clones unmarked. However, for various cell types, such as neurons, it is often more useful to positively mark a single neuron or a small subset of neurons in order to distinguish their complex processes from the numerous surrounding cells and to increase the resolution of phenotypic analysis.

Overview of the mosaic analysis with a repressible cell marker (MARCM) system

The MARCM system is a method that positively marks a small population of wild-type or mutant cells. The principle of the MARCM system[3,4] is schematically depicted in **Figure 1**. Mosaic analysis using this technique relies on generating homozygous mutant cells from heterozygous precursors via mitotic recombination. It combines the GAL80 repressor protein with the *Drosophila* GAL4 transcription factor–upstream activator sequence (UAS) binary expression system[5] and the FLP/FRT system to genetically label clones. In the GAL4–UAS system, expression of GAL4

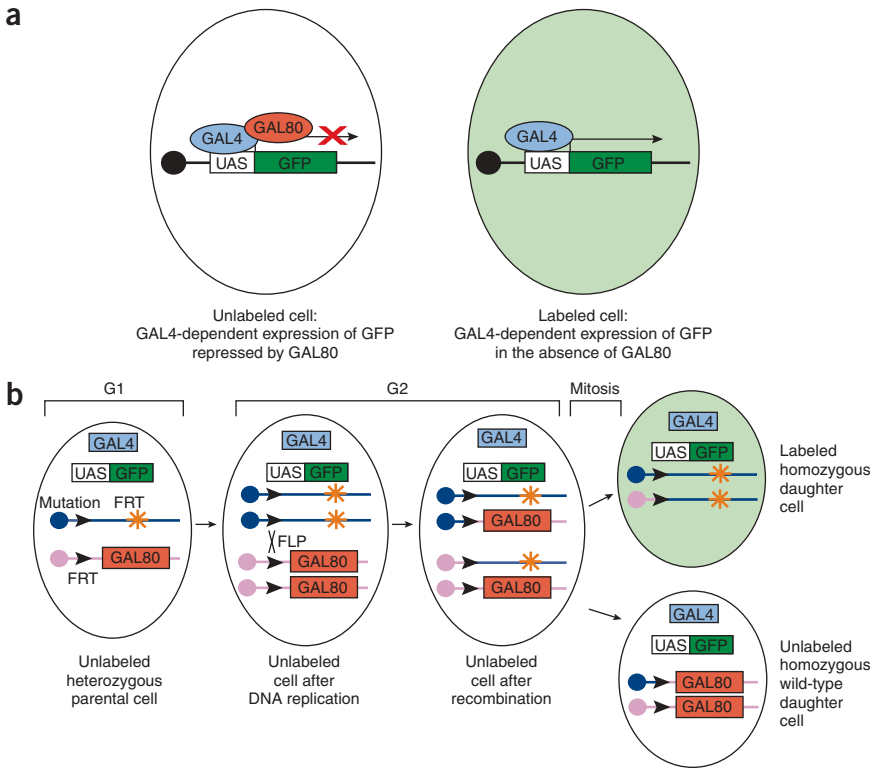

Figure 1 | Schematic representation of the GAL4–UAS system with GAL80 and the MARCM genetic system. (**a**) In cells containing the GAL80 protein, GAL4-dependent expression of a UAS–gene (GFP) is repressed. By contrast, cells containing GAL4 but lacking GAL80 will express the UAS–gene (GFP). In this schematic, genes are denoted by colored boxes whereas proteins are denoted by colored ovals. (**b**) MARCM requires (i) two *FRT* sites located at the same position on homologous chromosomes, (ii) *GAL80* located distal to one of the *FRT* sites, (iii) *FLP recombinase* located anywhere in the genome, (iv) *GAL4* located anywhere in the genome except distal to the *FRT* site on the *FRT, GAL80* recombinant chromosome arm, (v) *UAS–marker* located anywhere in the genome except distal to the *FRT* site on the *FRT, GAL80* recombinant chromosome arm, and optionally (vi) a mutation distal to *FRT*, in *trans* to but not on the *FRT, GAL80* recombinant chromosome arm. Site-specific mitotic recombination at *FRT* sites (black arrowheads) gives rise to two daughter cells, each of which is homozygous for the chromosome arm distal to the *FRT* sites. Ubiquitous expression of GAL80 represses GAL4-dependent expression of a *UAS–marker (GFP)* gene. Loss of GAL80 expression in homozygous mutant cells results in specific expression of GFP. Adapted from refs. 3,4.

causes transcriptional activation of a marker gene under the control of a UAS promoter in the same cell. In the MARCM system, the activity of GAL4 is repressed by the GAL80 protein (**Fig. 1**), resulting in unmarked cells that are heterozygous for both GAL80 and a mutation. After FLP/FRT-dependent mitotic recombination, homozygous mutant cells lack GAL80 and hence possess an active GAL4 that can activate reporter genes, such as *UAS–GFP*.

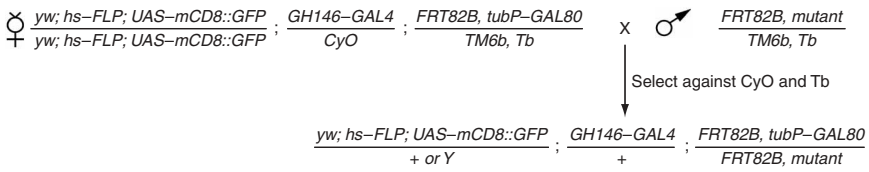

Figure 2 | Cross between a MARCM-ready and a *FRT, mutant* fly for MARCM analysis. After MARCM-ready and *FRT, mutant* stocks are established, only one cross is required to generate flies with the potential to contain MARCM clones. Crossing these two stocks together and selecting against the balancer chromosomes will result in progeny ready for MARCM analysis.

We typically generate 'MARCM-ready' flies that contain *FLP recombinase*, an *FRT* site, *GAL4*, tubulin 1 promoter (tubP)–*GAL80* and a *UAS–marker*. These flies are ready to cross to a line containing the corresponding *FRT* and mutation of interest for MARCM analysis (**Fig. 2**). For further details of the genetic elements required and important design considerations, see **Box 1**. It is also possible to add some of the MARCM components, such as the *GAL4*, *FLP* or *UAS–marker*, with the *FRT, mutant* fly. These choices will depend upon how easily the various components can be combined into a single fly line.

MARCM variations

We provide here the standard MARCM procedure. However, there are a number of variations of the system that expand the utility of the standard MARCM. The protocol remains the same, and the changes are mostly in the genetic composition of the transgenes used.

Reverse MARCM. Rather than introducing the mutation in *trans* to the *FRT, tubP–GAL80* chromosome arm, as in standard MARCM, the mutation is introduced onto the *FRT, tubP–GAL80* chromosome arm itself. This results in labeled homozygous wild-type cells, and unlabeled homozygous mutant and heterozygous cells. If the cell-division pattern is known, one can predict the presence of the unlabeled homozygous mutant clone based on the labeled homozygous wild-type clone. This can be used to assess non-cell-autonomous functions of candidate genes[6,7].

MARCM + cell lethal. A strong or null mutation in a gene that is required for cell viability or proliferation can be introduced on the *FRT, tubP–GAL80* chromosome arm. This results in the selective elimination of cells that are homozygous for the cell lethal mutation. This maximizes the contribution of labeled homozygous mutant cells, and is particularly effective when clones are generated in a highly proliferating tissue with a strong FLP (e.g., ey-FLP[8]; see ref. 6 for an example).

Dual-color MARCM. Lai and Lee[9] have further introduced LexA–lexAop, which is a second independent binary expression system. They have generated GAL80 repressible and irrepressible LexA transcription factors. The combination of LexA–lexAop and GAL4–UAS allows for the separate modification of gene expression

BOX 1 | GENETIC ELEMENTS AND IMPORTANT DESIGN CONSIDERATIONS FOR MAKING MARCM-READY FLY STOCKS

FLP recombinase

FLP is an enzyme that catalyzes double-stranded DNA breaks and recombination at *FRT* sites. *FLP* can be present anywhere in the genome. One copy is often sufficient, but more copies or a more strongly expressed transgene can be used for a higher clone frequency. The promoter used will determine in which cells FLP will be expressed to generate homozygous labeled clones. *FLP* under the control of a heat-shock promoter is useful for temporally controlling recombination. In addition, individual heat-shock protocols can be varied for finer control. *FLP* under the control of a tissue-specific promoter will be expressed based upon the promoter used and generally results in larger clones. *UAS–FLP* cannot be used to generate MARCM clones, as tubP–GAL80 will repress FLP expression.

FRT site

An *FRT* site is a DNA sequence that, when in the presence of FLP, will break and swap distal chromosome arms with the corresponding *FRT* site. *FRT* sites are located close to the centromere on a chromosome arm of interest. A MARCM-ready stock will contain one *FRT* site, whereas the corresponding *FRT* site will be present in the *FRT, mutant* stock.

GAL4

GAL4 is a protein from yeast that activates transcription by binding to *UAS* (that is, a DNA sequence preceding a transgene to be expressed). *GAL4* can be anywhere in the genome except on the same chromosome arm as *tubP–GAL80*. A tissue-specific GAL4 will label only homozygous cells within its expression.

tubP–GAL80

GAL80 is a protein from yeast that binds to and represses the activity of GAL4. tubP–GAL80 is expressed ubiquitously and has been shown to potently repress the activity of GAL4; thus, it is used in all MARCM studies[3]. Stocks are available from Bloomington containing *tubP–GAL80* recombined distally to *FRT* sites on the X, 2L, 2R, 3L and 3R chromosomes (**Table 1**).

UAS–marker

The marker typically encodes a fluorescent protein, such as GFP or red fluorescent protein (RFP). The *UAS–marker* can be present anywhere in the genome except on the same chromosome arm as *tubP–GAL80*. GAL4-dependent marker expression is repressed by tubP–GAL80 in heterozygous cells; it is expressed only in homozygous cells lacking tubP–GAL80.

and/or the marking of cells with high resolution in two different populations.

Applications of MARCM

Major applications of MARCM include (i) lineage analysis, (ii) investigating gene function in single or small populations of cells, and (iii) neuronal circuit tracing. In our laboratory (http://www.stanford.edu/group/luolab), we have used these properties to study the contribution of cell lineage to neuronal wiring[10,11], to follow neuronal circuits[12], and to study gene function in growth-cone signaling[13], axon pruning[14] and neuronal wiring specificity[15]. We typically use a membrane-targeted GFP marker (*UAS–mCD8::GFP*[3]) that strongly labels neuronal processes, in order to generate Golgi-like single-neuron resolution[16].

MARCM has also been used to study many other biological processes in *Drosophila*, such as spermatogenesis[17], asymmetric cell division in the adult sensory organ precursor[18], planar cell polarity[19] and tumor metastasis[20]. A conceptually analogous method called mosaic analysis with double markers (MADM) has recently been developed in mice[21].

Limitations of MARCM

The MARCM system can only be used reliably to label single cells 24–48 h after the induction of mitotic recombination because of GAL80 protein perdurance. Perdurance of maternally contributed GAL80 also limits the efficacy of the MARCM system for studying early embryonic development. These limitations could, in principle, be overcome using a temperature-sensitive GAL80 protein[22]. In addition, if the protein product of the gene of interest is highly expressed in precursor cells and is stable, perdurance of the protein after generating clones might confound studies of its requirement in early morphogenesis.

MATERIALS

REAGENTS

- MARCM fly stocks: many MARCM stocks are readily available from the Bloomington Stock Center (**Table 1**; http://www.flybase.org)

EQUIPMENT

- Standard fly-culturing equipment and microscope (see ref. 23)
- 37 °C water bath for heat-shock (if using heat-shock promoter for FLP expression)
- 25 °C incubator to maintain fly crosses
- Imaging microscope and software (e.g., confocal microscope)

PROCEDURE

Generate MARCM-ready flies ● TIMING At least four generations depending upon the genetic elements used

1| Use standard genetic techniques to introduce the following transgenes (**Table 1**) into a single fly to create a MARCM-ready stock: (i) an *FRT* site and *tubP–GAL80* on the chromosome arm of interest; and on any other chromosome arm (ii) a tissue-specific or ubiquitous *GAL4*, (iii) *FLP recombinase* under the control of a tissue-specific or heat-shock promoter and (iv) a *UAS–marker*. An example of such a MARCM-ready fly is *yw, hs–FLP, UAS–mCD8::GFP; GH146–GAL4/CyO; FRT 82B, tubP–GAL80/TM6b, Tb*. This MARCM-ready stock features *GH146–GAL4*, which labels the majority of olfactory projection neurons. It also features *UAS–mCD8::GFP*, which is a membrane-associated GFP that effectively labels neurons and their processes. Thus, this stock can be used to analyze the effects of chromosome 3R genes on neurons by crossing to a fly of genotype *FRT82B, mutant* (**Fig. 2**).

▲ CRITICAL STEP These MARCM-ready flies might be weak due to the presence of multiple genetic elements. Special care should be taken to ensure that these stocks retain their transgenes. It is important to note that hs–FLP, especially a strongly expressing insertion, might make some genetic elements unstable. This could cause the gradual breakdown of the MARCM-ready stocks. The flies might therefore need to be re-established regularly.

■ PAUSE POINT Generated stocks can be maintained for an indefinite period of time, provided that they retain their transgenes.

? TROUBLESHOOTING

TABLE 1 | MARCM fly stocks available from the Bloomington Stock Center.

MARCM component	Bloomington Stock Center number	Important features
FRT and *tubP–GAL80* recombinant for X: *FRT19A*	5132	*FRT19A, tubP–GAL80* at 1C2
FRT and *tubP–GAL80* recombinant for 2L: *FRT40A*	5192	*FRT40A, tubP–GAL80* at 31E3
FRT and *tubP–GAL80* recombinant for 2R: *FRT42D* or *FRTG13*	5140	*FRTG13, tubP–GAL80* at 47A7
FRT and *tubP–GAL80* recombinant for 3L: *FRT80B* or *FRT2A*	5190	*FRT2A, tubP–GAL80* at 75E1
FRT and *tubP–GAL80* recombinant for 3R: *FRT82B*	5135	*FRT82B, tubP–GAL80* insertion unknown
Tissue-specific or ubiquitous *GAL4*	5138	*tubP–GAL4*
FLP driven by a tissue-specific or heat-shock promoter	5580	*ey-FLP*
FLP driven by a tissue-specific or heat-shock promoter	8862	*hs–FLP*
UAS–marker	5136	*UAS–mCD8::GFP* on X chromosome
UAS–marker	5137	*UAS–mCD8::GFP* on second chromosome
UAS–marker	5130	*UAS–mCD8::GFP* on third chromosome
UAS–marker	7118	*UAS–myr::mRFP* on second chromosome
UAS–marker	7119	*UAS–myr::mRFP* on third chromosome

See also Bloomington Stock Center numbers 5131, 5133, 5134 and 5139 for various combinations of the above components.

Generate mutant or UAS–transgene flies ● TIMING **At least three generations**

2| Choose appropriate genetic elements to introduce into a fly containing an *FRT* site, according to the purpose of the experiment. To study the effects of a particular mutation, follow option (A). To study the effects of overexpressing a specific gene using a *UAS–transgene*, follow option (B). Follow option (C) to analyze overexpression only in a mutant cell.

(A) Introduce a mutation in the gene of interest

(i) Use standard genetic techniques to introduce a mutation onto a chromosome arm with an *FRT* site. This can be done via EMS-induced random mutagenesis[24] of an *FRT*-containing stock or by recombining an existing mutation with an *FRT*-containing stock using standard meiotic recombination[23].

▲ CRITICAL STEP It is possible to add some of the MARCM components, such as the *GAL4*, *FLP* or *UAS–marker*, to the *FRT, mutant* fly. This becomes necessary if your gene of interest is on the same arm as the *GAL4*. In this case, make a double recombinant including the *FRT*, *GAL4* and *mutation*.

(B) Introduce a *UAS–transgene* for overexpression analysis

(ii) Use standard genetic techniques to introduce a *UAS–transgene* anywhere in the genome to overexpress a gene of interest.

▲ CRITICAL STEP The *UAS–transgene* can be introduced on any chromosome except the chromosome arm that contains the *tubP–GAL80*. However, if it is recombined distal to the *FRT* in *trans* to *tubP–GAL80*, the transgene will be doubled in MARCM clones yielding a higher level of expression.

(C) Introduce both a mutation and a *UAS–transgene*

(iii) Steps 2A(i) and 2B(ii) can be combined to express a UAS–transgene only in a mutant cell of interest. This is particularly useful to perform cell-autonomous rescue experiments.

▲ CRITICAL STEP The MARCM system ensures that all labeled cells, and no other cells, express the UAS–transgene. However, although the MARCM system ensures that all labeled cells are mutant, it does not ensure that all mutant cells are labeled unless a ubiquitous GAL4 line (such as tubP–GAL4) is used. There might be mutant cells left unlabeled because of restrictive expression of the GAL4 used.

■ PAUSE POINT Generated stocks can be maintained for an indefinite period of time, provided that they retain their transgenes.

? TROUBLESHOOTING

Cross MARCM-ready flies with *FRT, mutant* and/or *UAS–transgene* flies ● TIMING ~4 d

3| In a freshly yeasted vial, cross 10–20 MARCM-ready virgins (from the stock established in Step 1) to 1–10 males containing *FRT* alone (as a control or for studying morphology). In a separate vial, cross 10–20 MARCM-ready virgins to males carrying *FRT* with a mutant gene and/or a *UAS–transgene* (from the stock established in Step 2). Maintain these MARCM crosses in a 25 °C incubator for 2–3 d to allow fertilization of the females.

▲ CRITICAL STEP The crosses can be done with MARCM-ready males and *FRT* virgins; however, if the MARCM-ready males have important elements on the X chromosome, only female progeny will have MARCM clones.

? TROUBLESHOOTING

Generate MARCM clones

4| Generate clones for analysis according to whether the FLP is driven by a tissue-specific promoter (A) or by a heat-shock promoter (B).

(A) Use of a tissue-specific FLP to generate MARCM clones ● TIMING **Depends on the developmental stage when clones are examined; ~10 d until adults eclose**

(i) Continue transferring the adults into freshly yeasted vials to expand the cross that was set up in Step 3 to obtain sufficient material for analysis.

(ii) When the progeny of the cross reach the desired developmental stage, proceed to Step 5, and dissect and stain the tissue of interest. The promoter used to drive FLP expression will determine when and where the MARCM clones will be present.

(B) Use of hs–FLP to generate MARCM clones ● TIMING **Depends on the developmental stage when clones are examined; ~10 d until adults eclose including a 1 h heat-shock**

(iii) Transfer the MARCM cross into a freshly yeasted vial. Maintain this MARCM cross at 25 °C.

▲ CRITICAL STEP The timing of these steps will depend upon the tissue of interest. See **Figure 3** for an example. Time egg-laying appropriately to ensure that heat-shock is applied prior to when the cell of interest exits the cell cycle.

? TROUBLESHOOTING

(iv) Let the females lay eggs for a specific period of time. Then remove the adult flies by transferring them to a fresh vial.

▲ CRITICAL STEP The length of egg laying depends upon the tissue of interest and will determine the range of developmental times that will be heat-shocked at once. The shorter the egg-laying time, the more synchronized the heat-shock and, hence, the clone induction time. For example, to generate projection neuron neuroblast clones or single-cell clones in projection neurons targeting the DL1 glomerulus, transfer the MARCM cross into a freshly yeasted vial in the evening (e.g., at 18:00). Maintain this MARCM cross at 25 °C. Transfer the adults after 16 h (e.g., at 10:00 the next day).

As another example, for single-cell clones in projection neurons targeting the VA2 glomerulus, egg laying can also be allowed for 16 h. See **Figure 3a** for details.

(v) Allow the eggs to develop for the appropriate length of time at 25 °C. ▲ CRITICAL STEP The length of time before heat-shock induction depends upon the tissue of interest; heat-shock induction should be done when the cells of interest are being born. At 25 °C, embryogenesis lasts for ~21 h. For neuroblast clones or single-cell clones in projection neurons targeting the DL1 glomerulus, allow 24 h for larvae to complete hatching before heat-shock induction (e.g., at 10:00 the following day). For single-cell clones in projection neurons targeting the VA2 glomerulus, heat-shock immediately after the 16-h laying period. See **Figure 3a** for details.

(vi) At the desired time, when progenitors of the cell of interest are actively dividing (in embryos, larvae, pupae or even adults), heat-shock the developing progeny in a 37 °C water bath for 1 h. ▲ CRITICAL STEP Place the vial into a rack with a weight on top to prevent it from tipping or floating in the water bath. Make sure the water level is above the cotton plug level to ensure that the larvae or adults do not crawl above it to escape the heat. ? TROUBLESHOOTING

(vii) Return the developing progeny to 25 °C until the desired developmental stage for examining the clones.

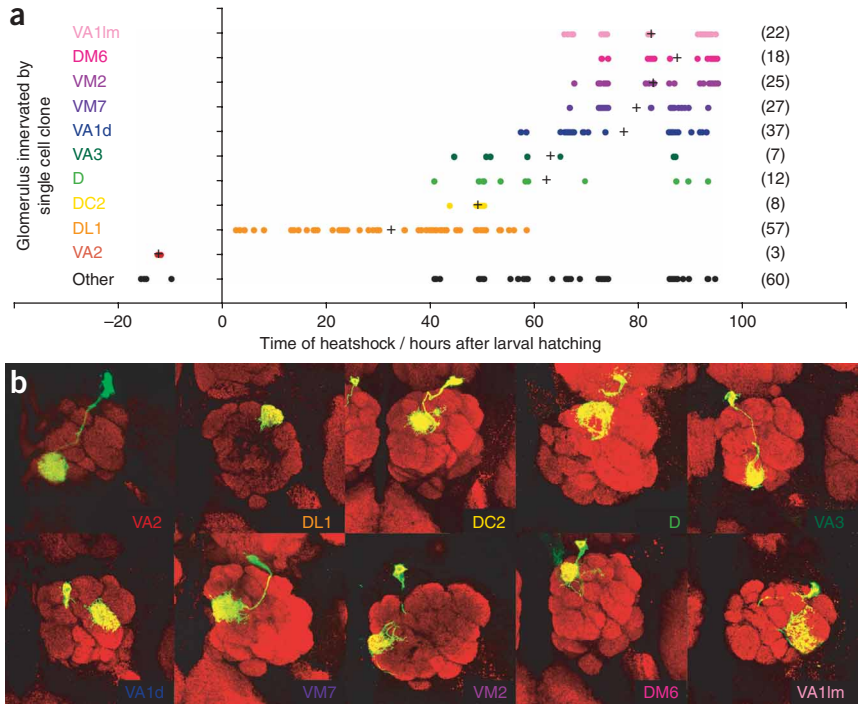

Figure 3 | MARCM example in which the birth order of projection neurons predicts their dendritic-projection pattern. (**a**) Guideline for heat-shock timing to obtain single-cell projection neuron clones in a particular glomerulus. The graph plots glomerular identity against heat-shock induction time for single-cell projection neuron clones from the anterodorsal neuroblast lineage. Each dot represents a single clone, and crosses represent mean heat-shock time for the glomerular class. Numbers in parenthesis represent total clones analyzed for the glomerular class. Heat-shock induction should be done when the cells of interest are being born. For example, to generate projection neuron neuroblast clones or single-cell clones in projection neurons targeting the DL1 glomerulus, heat-shock newly hatched larvae at 24 h after a 16 h laying period. At 25 °C, embryogenesis lasts for ~21 h. As another example, for single-cell clones in projection neurons targeting the VA2 glomerulus, allow egg laying for 16 h and heat-shock immediately. For other cell types, this style of analysis should be performed to determine proper heat-shock timing. (**b**) Representative images of the 10 landmark single-cell projection neuron clone classes. nc82 counterstaining is shown in red. Reprinted with permission from ref. 11.

Dissect and stain tissues ● TIMING **A few minutes (live examination) to several days (fix and stain)**

5| Clones can be analyzed in live or fixed tissues. Follow a dissection and staining protocol specific to the tissue of interest. A protocol for brain dissection and immunostaining is provided in ref. 25.

▲ CRITICAL STEP For MARCM clones using UAS–mCD8::GFP, use rat anti-mCD8a Ab (Invitrogen, CALTAG, cat. no. RM2200, 1:100) or anti-GFP Ab (Invitrogen, Molecular Probes rabbit anti-GFP, cat. no. A6455, 1:250). The pre-synaptic marker mouse anti-nc82 Ab can be used to label glomerular structure in the antennal lobe[26] (Developmental Studies Hybridoma Bank nc82, 1:40).

Image tissues ● TIMING **Variable, ~15 min per sample**

6| Follow instructions for imaging using a compound fluorescence or confocal microscope based on your laboratory's specific system.

▲ CRITICAL STEP Imaging should be done as soon as possible to get the best signal, and certainly within a few days of completing immunofluorescence staining. After imaging, store the samples in a dark slide holder at 4 °C for up to several months. Slides can be stored for longer (~3 yr) at –20 °C or for still longer at –80 °C.

? TROUBLESHOOTING

● TIMING

Steps 1,2: at least four generations to make MARCM-ready stocks; depending on the uniqueness of the particular stocks to be analyzed this might take several months.

Steps 3,4: up to 15 d to cross MARCM-ready stocks to *FRT* flies, heat-shock and allow to eclose.
Step 5: varies from a few minutes (to dissect live samples) to a few days (to dissect, fix and stain labeled tissues).
Step 6: ~15 min per sample; this might vary depending upon the images to be taken.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	Low-level marker expression	Weak GAL4	Use a stronger GAL4
		GAL80 perdurance from parental cells	Double the dose of GAL4 by (i) introducing it on the chromosome arm of interest such that marked clones will have two copies, or (ii) introducing GAL4 in both the MARCM-ready flies and the mutant flies
			Introduce more copies of the UAS–marker
			Increase experimental temperature to 29 °C where GAL4 activity is stronger
2C	Transgene expression cannot rescue mutant phenotype	GAL4 is not expressing where/when transgene function is needed	Use a different GAL4, keeping in mind where/when the UAS–transgene will be needed
		Non-cell-autonomous effects from unmarked clones	
3	Few flies develop in the MARCM cross	MARCM flies tend to be weak	Use more virgins per vial
			Use smaller vials
4B(iii)	Low efficiency of clone generation	Inherent bias of mitotic recombination	Extend G2 phase by allowing the flies to develop at lower temperatures; keep the vials at 18 °C after induction of FLP expression.
		MARCM-ready stocks have broken down	Use multiple copies or a stronger FLP
		Mutation causes cell death or inhibits GAL4 expression	Check MARCM-ready stocks to verify that all the components are present
			Verify that the heat-shock timing is correct and sufficient
			Perform multiple or longer heat-shocks
			Select a different GAL4 line that labels the same cells
4B(iii)	Too many clones	FLP is too strong	Reduce the length of heat-shock
			Reduce the number of copies of FLP
			Reduce the strength of the FLP by using a weaker insertion
4B(vi)	Few flies develop after heat-shock	Animals are dying in the heat	Weaker flies might require multiple heat-shocks for shorter periods; try two or three heat-shocks for 30 min each, with a 30 min recovery period between heat-shocks
6	Unexpected or no MARCM phenotype in a mutant known to have a phenotype otherwise	Time of clone generation is after the gene function is required	Induce clones at an earlier stage
		Non-cell-autonomous effects	Dissect and analyze tissues earlier, and over a time course
		Perdurance of mRNA or protein in clone	
6	MARCM phenotype has low penetrance or is variable	Gene is required at a specific time during development	Time embryo collections and heat-shock with more temporal precision
		Gene is required non-cell-autonomously	Use an alternative GAL4 that labels the cell in which the gene function is required

ANTICIPATED RESULTS

The efficiency of the MARCM system depends upon the genetic components used and the tissue being analyzed.
In flies that potentially contain clones, the observed clone frequency might vary between 5 and 100%, with one or more clones per fly.

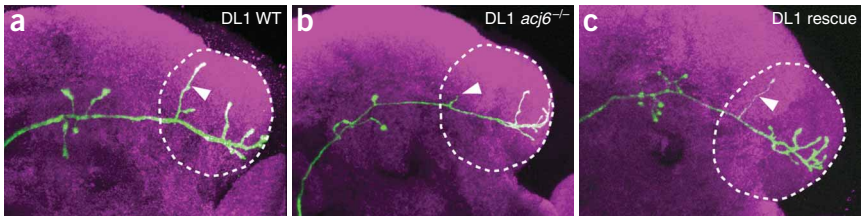

Figure 4 | MARCM example with high-resolution axon phenotypes in single-cell mutant and single-cell rescue experiments. The MARCM system can also be used to determine whether labeled mutant neurons can be rescued by cell-autonomous transgene expression. In addition to images of projection neuron dendrites in the antennal lobe shown in **Figure 3**, projection neuron axons can also be imaged. Each class of projection neurons exhibits a distinctive axonal branching pattern[12]. (**a**) Wild-type DL1 axonal branching pattern. (**b**) Mutations disrupting this branching pattern might cause phenotypes such as loss of the dorsal branch in *abnormal chemosensory jump 6* (*acj6*$^{-/-}$) projection neuron single-cell clones (white arrowhead). (**c**) Mutations can be also rescued in single labeled mutant cells using the MARCM system. *UAS–acj6* expression only in the labeled cell rescues the dorsal branch phenotype in *acj6*$^{-/-}$ projection neuron single-cell clones (white arrowhead). nc82 counterstaining is shown in violet. Reprinted with permission from ref. 15.

We give here two specific examples of MARCM analysis in the olfactory projection neurons. As shown in **Figure 3b**, confocal images of the antennal lobe show distinct glomeruli, which are innervated by specific classes of projection neurons that are born at specific times during larval development (**Fig. 3a**). Glomeruli can be identified by their stereotyped shape, size and position[27]. The neurons are labeled here in green, with counterstaining to show the glomerular structures in red.

Figure 4 shows the fine resolution of individual axon branches that can be achieved using the cell membrane-localized UAS–mCD8::GFP marker. The axonal branches of a single neuron can be visualized with fine resolution by confocal imaging. Specific results will depend upon the modifications to the protocol that have been made.

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