

Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development

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We have modified an FLP/FRT-based genetic mosaic system to label either neurons derived from a common progenitor or isolated single neurons, in the *Drosophila* CNS. These uniquely labeled neurons can also be made homozygous for a mutation of interest within an otherwise phenotypically wild-type brain. Using this new mosaic system, not only can normal brain development be described with unprecedented single cell resolution, but also the underlying molecular mechanisms can be investigated by identifying genes that are required for these developmental processes.

Since Ramon y Cajal and his contemporaries first investigated the diverse and intricate neuronal morphologies in the brain¹, significant progress has been made in describing the formation of neural circuits based on the projection patterns of single neurons (reviewed in Ref. 2). However, such descriptive works only intensify our desire

for understanding how complex neural circuits are established. In order to visualize single neurons in the developing brain, and also to investigate the molecular mechanisms underlying the observed phenomena, we have developed a novel genetic system for performing mosaic analysis in the *Drosophila* CNS (Ref. 3).

The 'MARCM' system for performing mosaic analysis in the CNS Mosaic analysis involves the generation of homozygous mutant cells from heterozygous precursors via mitotic recombination (reviewed in Ref. 4). For most genes, one wild-type allele in a diploid cell is sufficient for normal function. Therefore, by creating homozygous mutant cells in heterozygous tissues, one can knock out gene function in a small subset of cells and then examine their phenotypes in an otherwise phenotypically wild-type organism. Such analysis is particularly

useful to determine the stage-specific, cell-autonomous roles of a gene that has pleiotropic functions in diverse tissues and at different developmental stages. In conventional mosaic analysis, homozygous mutant cells are identified as either unstained cells or cells that are stained with twice the intensity of heterozygous cells⁵. Because neurons are densely packed and neuronal processes are fasciculated and often extend over long distances, it is impossible to analyze such mutant cells in the CNS using conventional mosaic systems.

The system that we developed, called MARCM (mosaic analysis with a repressible cell marker)³, allows one to label homozygous mutant cells uniquely in mosaic tissues, which is essential for performing mosaic analysis in the complicated nervous system. To achieve this, the yeast GAL80 protein was introduced into the GAL4-UAS binary expression system in *Drosophila*⁶. The MARCM system initially contains cells that are heterozygous for a transgene encoding the GAL80 protein, which inhibits the activity of the transcription factor GAL4 (Ref. 7). Following FLP/FRT-mediated mitotic recombination⁸, the GAL80 transgene is removed from one of the daughter cells, thus allowing expression of a GAL4-driven reporter gene specifically in this daughter cell and its progeny (Fig. 1). If there is a mutation located on the chromosome arm *in trans* to the chromosome arm containing the GAL80 transgene, the uniquely labeled GAL80-negative (GAL80⁻) cells should be homozygous for this mutation. Therefore, one can specifically label the homozygous mutant cells in a mosaic tissue using the MARCM system. To examine the entire projection patterns of such labeled neurons, we have designed a membrane-targeted green fluorescent protein (GFP) (mCD8-GFP) as a marker in the MARCM system. This article describes three different examples that

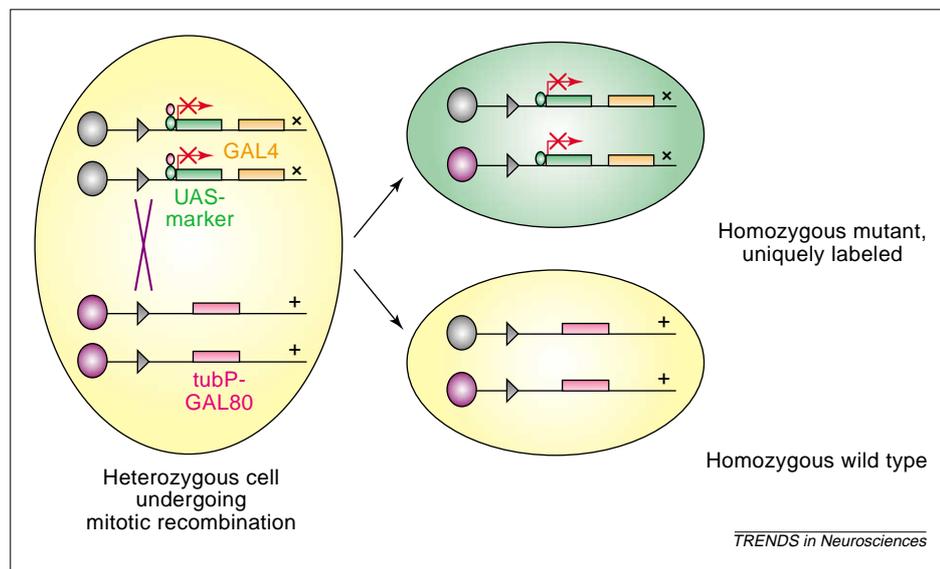


Fig. 1. Genetic basis of the mosaic analysis with a repressible cell marker (MARCM) system. After site-specific mitotic recombination, a heterozygous mother cell can give rise to two daughter cells in which the chromosome arms distal to the recombination site become homozygous. Driven by the *tubulin 1* α promoter, GAL80 is ubiquitously expressed and efficiently suppresses GAL4-dependent expression of a UAS-marker gene (i.e. the GAL80 pink rectangle binds to the GAL4 orange box at the UAS site, preventing transcription). If *tubP-GAL80*, but not *GAL4* or *UAS-marker*, is inserted on the chromosome arm carrying the wild-type (+) gene of interest, the daughter cell homozygous for the mutant gene (x) no longer contains *tubP-GAL80* (pink rectangle). Therefore, the marker gene can be specifically turned on by GAL4 (orange box) in homozygous mutant cells. Adapted from Ref. 3.

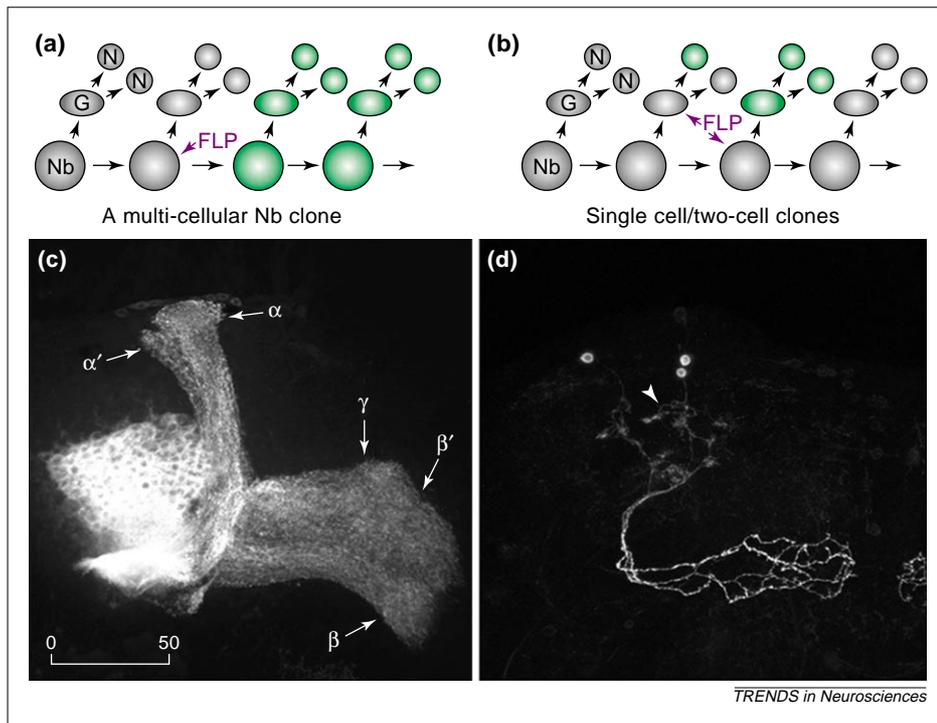


Fig. 2. Three distinct clone sizes of mushroom body (MB) neurons. (a) and (b) In the *Drosophila* CNS, a neuroblast (Nb) generates a series of ganglion mother cells (GMC or G in Fig.) via asymmetric divisions, and each GMC gives rise to two post-mitotic neurons (N). (a) In the MARCM (mosaic analysis with a repressible cell marker) system, if a Nb becomes GAL80-negative (GAL80⁻) after Flipase (FLP)-mediated mitotic recombination, all neurons subsequently derived from this GAL80⁻ Nb are specifically labeled and appear as a multicellular Nb clone. (b) If a GMC loses GAL80 after mitotic recombination, two neurons derived from the GAL80⁻ GMC are labeled and become a two-cell clone. By contrast, if mitotic recombination occurs in a dividing GMC, only one of the two post-mitotic neurons will be labeled. (c) and (d) Composite confocal images of MARCM clones of MB neurons. Using a membrane-targeted green fluorescent protein (GFP) (mCD8-GFP), the entire morphologies of uniquely labeled MARCM clones in intact brains could be observed. (c) A MB Nb clone generated by inducing mitotic recombination in a newly hatched larva (NHL) consists of hundreds of neurons at the adult stage. There are five axon bundles (lobes) in the adult MB: γ , β' and β projecting towards the midline and α' and α projecting dorsally. (d) Unique labeling of single-cell and two-cell clones of MB neurons generated in NHL reveals that each cell body extends a single process from which dendrites (arrowhead) branch out. The scale bar unit is in microns. Adapted from Ref. 10.

illustrate the utility of the MARCM system.

Example 1: cell lineage analysis

Formation of the *Drosophila* CNS involves a stereotypic set of neuroblasts (Nbs), each of which undergoes a series of asymmetric divisions to produce a characteristic clone of neurons. Each asymmetric division of a Nb generates another Nb and ganglion mother cell (GMC), which subsequently gives rise to two neurons after an additional cell division⁹. Consequently, three sizes of neuronal clones can be labeled using the MARCM system (Fig. 2)³. A multicellular clone is derived from a GAL80⁻ Nb, whereas a two-cell clone is the product of an isolated GAL80⁻ GMC. If mitotic recombination occurs in a dividing GMC, one of the two post-mitotic neurons might lose GAL80 and become a single-cell clone (Fig. 2).

The MARCM system can be used for cell lineage analysis by taking advantage of the following properties. First, one can use heat shock to induce FLP expression at a specific stage, and only precursors that divide actively at that stage are subject to mitotic recombination. Therefore, patterns of clones induced at various stages should reflect the spatial patterns of neurogenesis at the corresponding stages. Second, all neurons in a multicellular Nb clone are derived from a common progenitor, so examination of the compositions of Nb clones and their projection patterns reveals the roles of cell lineage in the construction of neural circuits. Third, in single-cell and two-cell clones, one can visualize morphological differentiation of single neurons. Induction of single-cell and two-cell clones at various time points allows the projection patterns of neurons that are generated at different stages to be determined.

MARCM analysis of the development of mushroom bodies (MB), the insect center for olfactory learning and memory, provides one example for such cell lineage analysis. Examining the Nb clones of MB neurons at different stages illustrates how adult MBs acquire their mature morphologies after production of post-mitotic neurons through larval and pupal stages¹⁰. The axon projection patterns of individual MB neurons are further revealed by examining single-cell and two-cell clones systematically induced at different stages¹⁰. There are three distinct types of neurons and their axons fasciculate into three different sets of MB lobes^{10,11}. Interestingly, they are sequentially derived from common progenitors, and the MB neurons that are generated before the mid-third instar stage remodel their projections after pruning larval-specific processes during early metamorphosis¹⁰. Similar strategies can be applied to study the development of other neural structures in the *Drosophila* CNS.

Example 2: functional analysis of candidate pleiotropic genes

Because one can selectively create clones of MB neurons that are homozygous for a mutation of interest within an otherwise phenotypically wild-type organism, the MARCM-labeled MB neurons have recently become model neurons for determining the cell-autonomous functions of pleiotropic genes in neuronal development. For example, the actin-binding protein encoded by *short stop* (*kakapo*) is important for extension and correct guidance of axons³ (Fig. 3b). The *Drosophila* small GTPase, RhoA, was found to be required for proliferation of neuroblasts and restriction of dendritic growth¹² (Fig. 3c,d). Trio, a guanine nucleotide exchange factor that activates the small GTPase, Rac, was shown to be autonomously required in MB neurons for extension and correct guidance of their axons¹³. Mutations in the *Drosophila* homolog of the mammalian *Lisencephaly 1* gene, *Lis1*, or a cytoplasmic dynein heavy chain, caused defects in neuroblast proliferation, dendritic elaboration, and axonal transport¹⁴. In addition, the Dachshund nuclear protein is autonomously required for proper differentiation of pupal-born MB neurons, as shown by the selective

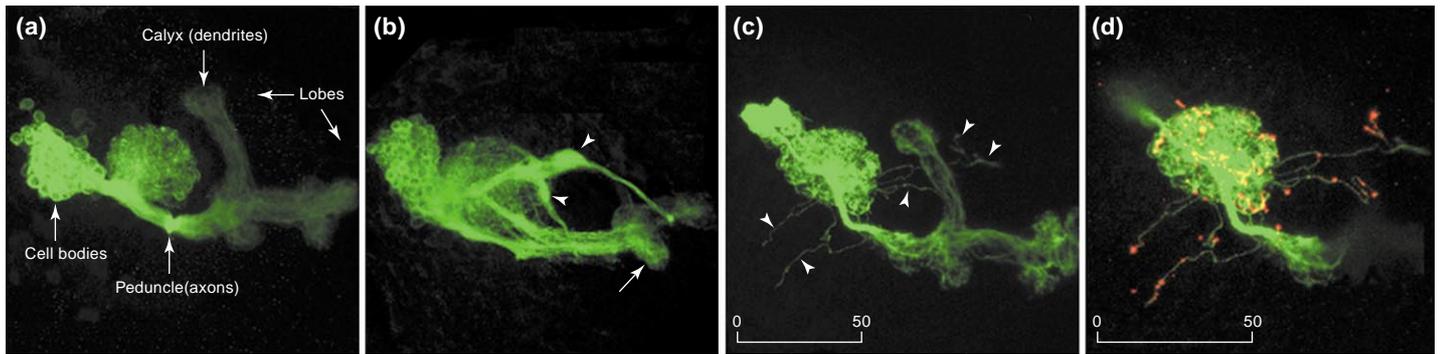


Fig. 3. Requirement of *short stop* and *RhoA* for different aspects of MB morphogenesis. Composite confocal images of neuroblast (Nb) clones of mushroom body (MB) neurons that were generated in newly hatched larva (NHL) and examined at the wandering third-instar stage. Compared with a wild-type clone (a), the *short stop* mutant clone (b) has ectopic axon bundles (arrowheads) and most axons in the peduncle stop at the bifurcation point (arrow). By contrast, the *RhoA* mutant clone (c) contains much fewer cell bodies with over-extended dendrites (arrowheads) but no detectable axon defect. A dendritic marker, the Nod- β -galactosidase chimeric protein [red in (d)], was used to confirm the dendritic nature of the overshooting processes. Scale bar is in microns. Adapted from Refs 3 and 12.

loss of a specific axonal lobe contributed by the pupal-born MB neurons in spite of normal numbers of neurons in mutant clones¹⁵.

If required, the non-cell autonomous effects of a mutant clone can be addressed by specifically labeling its immediate wild-type sibling cells using 'reverse MARCM'¹². If the mutation of interest is located on the same chromosome arm as the GAL80

transgene, the MARCM-labeled cells will be equivalent to the homozygous wild-type part of a 'twin spot' in conventional mosaic analysis⁵. With respect to cell lineage, they are closest to the mutant cells and should be the ideal subjects for examining any non-cell autonomous effects of a mutation. However, a clear-cut interpretation of the phenotypes might require visualizing both sides of the 'twin spot'.

One alternative for manipulating gene functions specifically in the MARCM-labeled cells involves ectopic expression of transgenes that encode gain-of-function proteins. In the absence of GAL80, GAL4 can activate expression of all the transgenes that are driven by the UAS promoter. Therefore, multiple marker genes can be visualized, and distinct aspects of neuronal morphologies can be visualized simultaneously^{12,14}, or mutant phenotypes could be rescued by selectively expressing various transgenes in the MARCM mutant clones^{12,14}. In combination with MARCM analysis of loss-of-function mutations, these other features greatly facilitate molecular genetic studies of complex developmental processes.

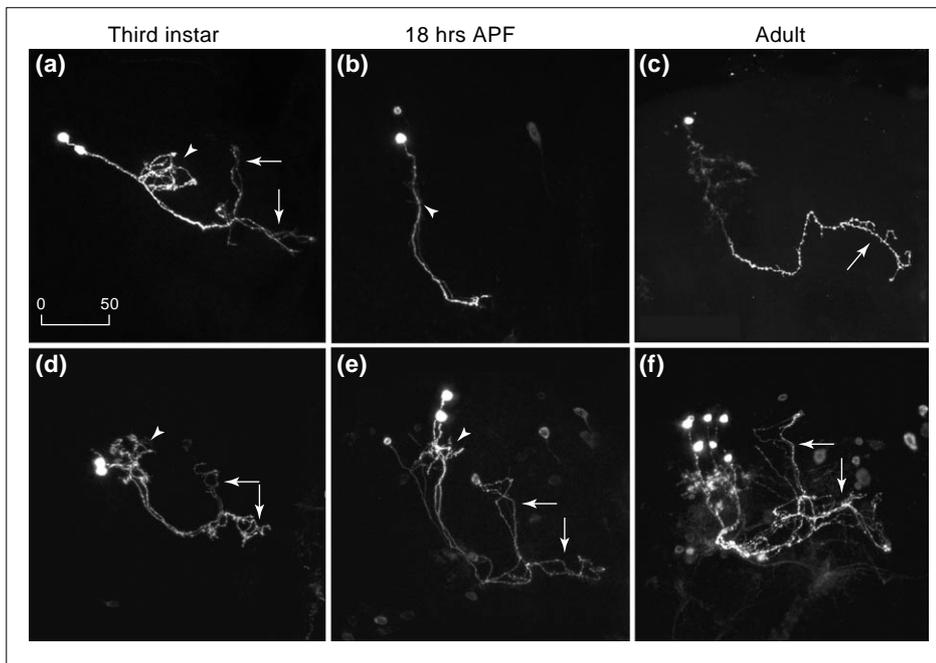


Fig. 4. Cell-autonomous requirement of *ultraspiracle* (*usp*) for remodeling of γ neurons. Composite confocal images of single-cell and two-cell clones of wild-type (a)–(c) or *usp* (d)–(f) γ neurons that were fixed at the late larval (a) and (d), early pupal (b) and (e), or adult stages (c) and (f). Wild-type γ neurons acquired different stage-specific axon projections [arrows, compare (c) with (a)] after pruning the larval-specific axonal branches [arrows in (a)] as well as most dendrites [arrowhead, compare (b) with (a)] during early metamorphosis. By contrast, no remodeling was observed in *usp* mutant γ neurons (d)–(f) such that the larval-specific bifurcation of axons (arrows) was retained through metamorphosis (e) and into the adult stage (f). Note that remodeling of dendrites (arrowheads) was also blocked. Adapted from Ref. 16.

Example 3: genetic screen to identify new genes

Following analysis of wild-type neuronal development using the MARCM system, one can in theory systematically investigate the underlying molecular mechanisms by screening for random mutations that disrupt the normal developmental processes. Indeed, a genetic mosaic screen has been used to identify genes required for remodeling of MB neurons¹⁶. Using single-cell mosaics, it was shown that all single-cell and two-cell clones of MB neurons generated in newly hatched larvae altered their axon projection patterns after pruning of the larval-specific branches followed by outgrowth of the adult-specific process during early metamorphosis (Fig. 4a–c). In order to elucidate the molecular mechanisms that control remodeling of neuronal processes, a genetic mosaic screen was conducted and mutations that block MB remodeling were identified (Fig. 4d–f). The first mutation was found to be in the *ultraspiracle* gene, encoding a co-receptor for the nuclear hormone ecdysone¹⁶. Identifying additional mutations should

shed more light on the molecular mechanisms of neuronal remodeling.

Future improvement and extension of the MARCM system

The MARCM system has noteworthy limitations. If the gene of interest is expressed in precursor cells, knocking out a gene in MARCM clones does not guarantee an immediate and complete loss of its encoded protein, the perdurance of which might complicate phenotypic analysis of mutant cells. Similarly, the homozygous mutant cells do not express the marker immediately because of the perdurance of the GAL80 protein, so the current MARCM system is not useful to examine events that happen shortly after the induction of mitotic recombination. Engineering a more labile GAL80 version might overcome this difficulty. Development of various means of controlling the patterns of mitotic recombination, which are required for better targeting of MARCM analysis to distinct types of neurons, will also extend the use of the MARCM system.

Although this review is focused on *Drosophila* brain development, MARCM-based mosaic analysis could in theory also be applied to study the function of neural genes in behavior. It could also be applied to study many developmental processes, in which positive marking of mutant cells is advantageous and sometimes essential. For example, MARCM has recently been used to studying *Drosophila* spermatogenesis¹⁷, asymmetric cell division in the adult sensory organ precursor¹⁸, and planar cell polarity in adult wing¹⁹. Finally, given that both the GAL4-UAS expression system²⁰ and the FLP/FRT (Ref. 21) or CRE/LOX (Ref. 22) mitotic recombination systems

function in mice, it might be possible to adapt the MARCM system to study neuronal development in the mouse brain.

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