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Using the MARCM system to positively mark mosaic clones in *Drosophila*.

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Introduction to the MARCM system: Mosaic analysis is a powerful tool to analyze gene functions in many biological processes. In *Drosophila*, the introduction of highly efficient FLP/FRT systems (Golic and Lindquist, 1989) and effective ways of marking clones (Xu and Rubin, 1993) allowed for both functional analysis of candidate genes and identification of new genes by mosaic screening. In the widely used marking system (Xu and Rubin, 1993), the marker transgene is placed distal to the FRT site in trans to the mutant of interest, such that homozygous mutant cells are the only cells that are not labeled. In many cases, it is desirable to visualize the mutant cells but not heterozygous parents or homozygous wild type siblings. The MARCM (for *M*osaic *A*nalysis with a *R*epressible *C*ell *M*arker) system we developed (Lee and Luo, 1999) serves this purpose.

An organism subjected to MARCM analysis must contain at least six transgenes: two transgenes for two homologous FRT sites, one for the FLP recombinase, one for a UAS-marker, one for a GAL4 driver, and lastly one for the *tubP-GAL80* transgene. The *tubP-GAL80* must be placed distal to the FRT site in trans to the mutant gene of interest. In heterozygous cells, GAL80 inhibits GAL4 induced UAS-marker expression. Only in homozygous mutant cells, in which the *tubP-GAL80* transgene is not present, will the marker be expressed (Lee and Luo, 1999).

Much interest has been generated in using the MARCM system since the publication of our original paper. In this Technique Note, we describe some of the recent additions to the MARCM system and discuss different parameters that affect the use of the system.

Completion of the MARCM chromosome arms: Since the publication of the original paper (Lee and Luo, 1999), we have been able to generate *tubP-GAL80* transgene insertions on 2L and 3L, using D2-3 mediated P-element transposition (Robertson *et al*., 1988). Thus a complete MARCM set is available for all major chromosome arms. New *tubP-GAL80* insertions were initially tested for their ability to suppress the phenotype created by *Drac1L89* expression in the eye using the UAS-GAL4 system (Brand and Perrimon, 1993; Lee and Luo, 1999). We also confirmed the ability of these *tubP-GAL80* transgenes to suppress GAL4 induced marker expression in larval brains and discs. We found and discarded many *tubP-GAL80* insertions that only partially suppressed GAL4 induced gene expression in at least one of the above assays, presumably due to positional effects that reduced the expression of *tubP-GAL80* in certain cells.

All *tubP-GAL80* insertions have been recombined with the most commonly used FRTs. These recombinant chromosomes, as well as other related strains, are available at the Bloomington Stock Center under the stock number 5128-5148 (for X, 2R, 3R), and 5190-5192 (for 2L, 3L). There is also a web page for strains containing the *tubP-GAL80* insertions (http://flystocks.bio.indiana.edu/gal80.htm).

FLP enhancer trapping: One of our original goals of establishing the MARCM system is to enable consistent labeling of identifiable neurons in different organisms. The best way to conduct tissue-specific mosaic analysis is to restrict FLP activity spatially. Unfortunately, one cannot use the combination of *UAS-FLP* (Duffy *et al*., 1998) and tissue specific GAL4 lines in the MARCM system because the expression of GAL80 inhibits the expression of *UAS-FLP* in the first place to prevent mitotic recombination. To achieve tissue-specific expression of FLP, we conducted an enhancer trap

screen. We inserted the open reading frame of the yeast FLP recombinase (Golic and Lindquist, 1989) into an enhancer trap vector (Giniger *et al*., 1993). Using third instar larval brain as the assay system, we screened through 750 independent insertions on the autosomes for FLP under the control of specific enhancer elements. We used two assays to test FLP activity (Figure 1). While almost 50% of the FLP enhancer trap lines gave patterns in the brain using the flip-out assay (Figure 1A) (Struhl and Basler, 1993; Pignoni and Zipursky, 1997), very few lines gave reproducible patterns using the trans-FRT assay (Figure 1B). For those few lines that yielded patterns in the CNS in the trans-FRT assay, each animal had a different subset of labeled neurons, suggesting that we trapped several pan-CNS enhancers, and each neuroblast lineage has a small frequency of productive mitotic recombination. These experiments suggest that either the frequency of FLP/FRT mediated mitotic recombination is much lower than that of intra-chromosomal flip-out, or that most of the lines that score positive for flip-out assay but negative for trans-FRT assay start their expression in post-mitotic neurons, or both.

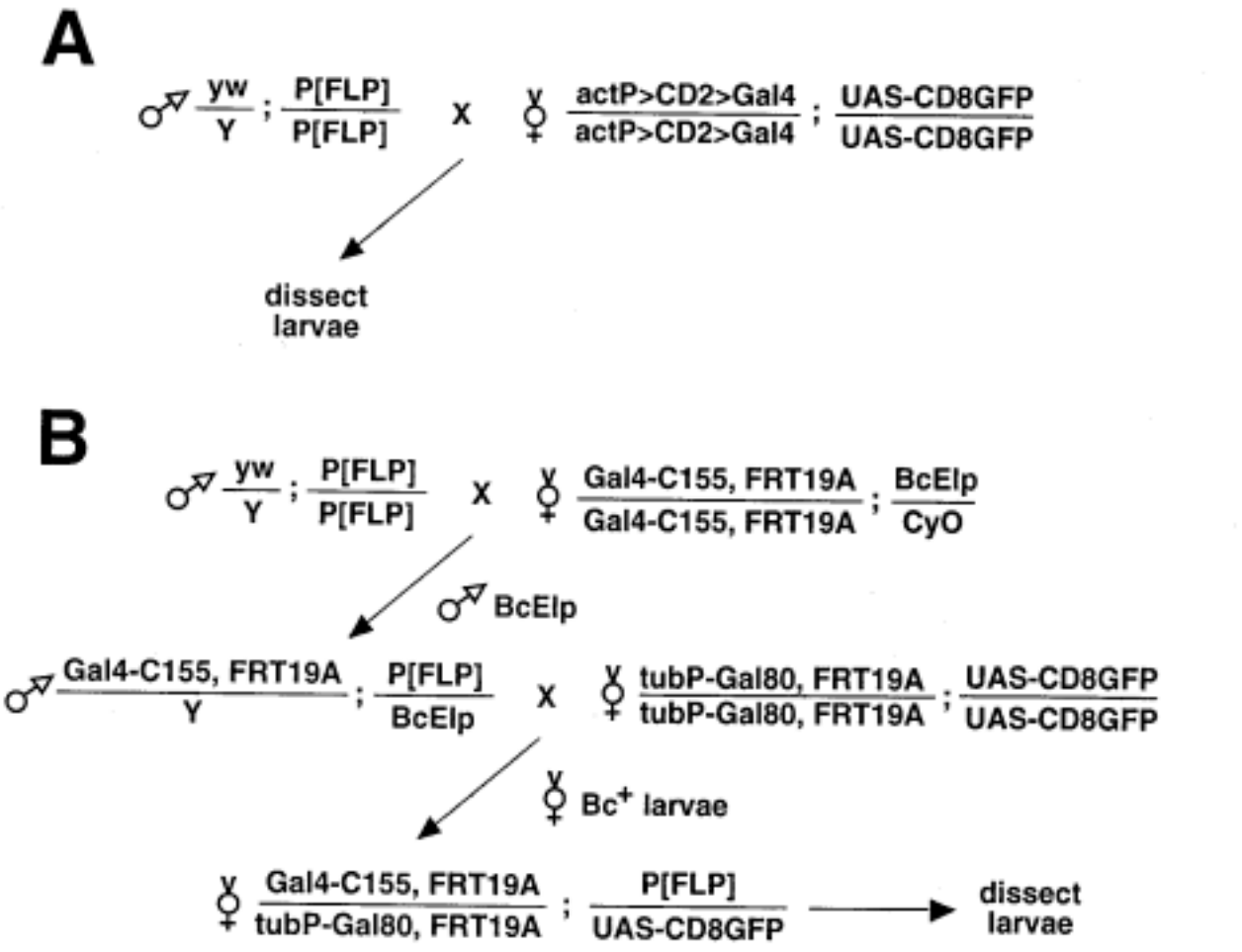

Figure 1. Genetic schemes to detect FLP enhancer trap activity. (a) Flip-out assay: the two FRT sites (>) are on the same chromosome. (b) Trans-FRT assay: the two FRT sites are on two homologous chromosomes. The scheme only illustrates the cases in which FLP is located on the second chromosome.

The low frequency of FLP-induced recombination was also confirmed by another experiment, in which we tested the efficiency of *GMR-FLP* using the trans-FRT assay. *GMR-FLP* is expressed posterior to the morphogenetic furrow, and is thus capable of generating clones in R1, R6 and R7 photoreceptor cells (Pignoni *et al*., 1997). In theory, there are potentially 3×800, or 2,400 mitosis that are targets of FLP-mediated mitotic recombination that gives rise to photoreceptor neurons. In each mitosis there is a 25-50% chance (Lee and Luo, 1999) that recombination would occur in a way such that one of the two daughter cells would lose *tubP-GAL80*, and a further 50% chance that the GAL80-negative daughter cell would be a photoreceptor neuron. Thus the estimated GAL80 deprived photoreceptor neuron is 300-600 if every *GMR-FLP* expressing precursor would undergo mitotic recombination. However, we observed an average of 30 neurons that were labeled in each eye when the MARCM clones were examined in the mid-pupal stage. This gave us an indication that even when FLP is under the control of a strong synthetic promoter like GMR, the frequency of generating mitotic recombination is between 5-10%.

We found that *hs-FLP* is the most efficient way to generate mitotic recombination at a reasonable frequency for MARCM analysis. Although *hs-FLP* lacks spatial control, one can sometimes choose heat shock window to favor the generation of clones in desired cell type, as only cells under going active proliferation are likely to be targets for mitotic recombination (Lee and Luo, 1999). For instance, we were able to generate mushroom body-specific clones by performing heat shock at the time when MB neuroblasts are preferentially active in proliferation (Lee and Luo, 1999).

Choice of GAL4 lines: In order to mark mosaic clones efficiently, strongest GAL4 drivers are always the best choice. This is especially true when one wants to visualize single-cell clones. There is no extra cell division to dilute the GAL80 protein inherited from the parental heterozygous cells. Consider the example of generating two-cell clones in MB lineage. We previously reported that the frequency of generating two-cell clones is much lower compared with Nb clones in the MB lineage when we used the *GAL4-C155* as a driver, whereas the theoretical prediction should be 1:1 (Lee and Luo, 1999). Subsequently we found that if we examined male progeny, in which *GAL4-C155* expression is at a higher level due to X-chromosome dosage compensation, the frequency of two-cell clones was higher. Recently, we have used *GAL4-201Y*, a MB GAL4 driver located on chromosome 2R, to visualize MB clones in combination with *FRT (2R-G13)*. We found the predicted 1:1 ratio of MB neuroblast clones to two-cell clones. In addition, all of the two-cell clones were much more strongly labeled (Lee *et al*., 1999). This intense labeling was presumably due to the strong expression of *GAL4-201Y* in MB neurons as well as the homozygosity of the *GAL4-201Y* transgene in mitotic clones.

Ideally, the GAL4 driver should be ubiquitous, such that all mutant cells devoid of *tubP-GAL80* can be visualized. This was the reason we developed the *tubP-GAL4* transgene (Lee and Luo, 1999). However, we found that a high level of ubiquitous GAL4 expression is toxic to the organism based on the following lines of evidence. First, the frequency of generating *tubP-GAL4* transgenic lines was 5-10 fold lower than other transgenes of comparable size. Second, it was very difficult to generate new insertions of *tubP-GAL4* using D2-3 transposase-induced P-element transposition. Third, of the all the insertions we obtained, none of them was homozygous viable. The homozygous lethality can be rescued by the presence of one copy of the *tubP-GAL80* transgene, thus identifying the high level of GAL4 expression as the cause of the lethality.

For certain applications, it may not be necessary to have GAL4 expressed in all GAL80-negative cells. In fact, sometimes it is advantageous to visualize only a subset of cells that have lost *tubP-GAL80*. For instance, selectively visualizing MB neurons using MB GAL4 lines in the MARCM system helped us to decipher the development of axon projection in wild-type animals (Lee *et al*., 1999). If

FLP is under the control of a specific promoter, there may only be a limited numbers of cells that can undergo mitotic recombination. The GAL80-negative and GAL4-negative "background" cells, when mutant, may not influence the development of cells of interest (GAL80-negative, GAL4-positive) due to, for instance, physical distance. In those cases using cell type-specific GAL4 lines to visualize only the cells of interest may facilitate mosaic analysis.

UAS-Marker: The membrane-targeted mCD8-GFP (Lee and Luo, 1999) has proven to be a good marker. We have generated insertions on X, 2L, 2R, and third chromosomes, which are all available at the Bloomington Stock Center.

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