Video Article

Application of MultiColor FlpOut Technique to Study High Resolution Single Cell Morphologies and Cell Interactions of Glia in *Drosophila*

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Abstract

Cells display different morphologies and complex anatomical relationships. How do cells interact with their neighbors? Do the interactions differ between cell types or even within a given type? What kinds of spatial rules do they follow? The answers to such fundamental questions *in vivo* have been hampered so far by a lack of tools for high resolution single cell labeling. Here, a detailed protocol to target single cells with a MultiColor FlpOut (MCFO) technique is provided. This method relies on three differently tagged reporters (HA, FLAG and V5) under UAS control that are kept silent by a transcriptional terminator flanked by two FRT sites (FRT-stop-FRT). A heat shock pulse induces the expression of a heat shock-induced Flp recombinase, which randomly removes the FRT-stop-FRT cassettes in individual cells: expression occurs only in cells that also express a GAL4 driver. This leads to an array of differently colored cells of a given cell type that allows the visualization of individual cell morphologies at high resolution. As an example, the MCFO technique can be combined with specific glial GAL4 drivers to visualize the morphologies of the different glial subtypes in the adult *Drosophila* brain.

Video Link

The video component of this article can be found at https://www.jove.com/video/56177/

Introduction

Glia, the non-neuronal cell population of the nervous system (NS), were long believed to provide a static framework for neurons and therefore were not studied in detail. However, in humans, glia constitute the vast majority of cells in the NS (~90%) and fall into several different categories, including astrocytes, oligodendrocytes, microglia and Schwann cells. In *Drosophila*, glia constitute about 10% of the cells in the NS. Intriguingly, their morphologies and functions are remarkably similar to those found in vertebrates[1,2]. Their morphologies include blood-brain barrier (BBB) forming epithelia, ensheathing, and astrocyte-like cells.

The *Drosophila* central nervous system (CNS) consists of the following principal structures: cortex regions that contain the neuronal cell bodies; neuropils that harbor synaptic connections; small and large axon tracts that connect the different neuropiles; peripheral nerves that connect sensory organs and muscles with the CNS (**Figure 1**). Glia are found associated with all these anatomical structures: Cortex glia (CG) in the cortical regions, astrocyte-like glia (ALG) and ensheathing glia (EG) in the neuropile regions, ensheathing glia are also associated with central axon tracts and peripheral nerves (EGN), and finally, two sheet-like glia, perineurial glia (PG) and subperineurial (SPG), which together form a contiguous layer that covers the entire NS (**Figure 2**).

Previous studies have shown that glia play important roles in the development of the NS; they monitor neuronal cell numbers by reacting to systemically circulating insulin-like peptides, provide trophic support to neurons, such as the astrocyte-neuron lactate shuttle, and eliminate dying neurons by phagocytosis[3,4,5,6]. In the mature NS, glia maintain the BBB, take up neurotransmitters and maintain ionic homeostasis, act as the major immune cells in the NS, since macrophages cannot breach the BBB, and modulate synaptic activity as well as animal behavior[6,7,8,9,10,11].

Whether the different glial subtypes perform specialized functions remains an important open question. However, a systematic genome-wide analysis of glia, especially in the adult, has been hampered by a lack of appropriate genetic tools for their manipulation. Here, a method that allows the efficient and easy characterization of cell shapes to study complex cell-cell interactions is presented. This technique has been applied to characterize the morphology of the different glial subtypes in the adult *Drosophila* brain, but, depending on the specific GAL4 driver used, it could be adapted to study neurons[12,13], any kind of intermingling cells, and in principle any tissue in any developmental stages.

Protocol

1. Preparing Flies for MultiColor FlpOut (MCFO) Experiments

NOTE: The MCFO technique refers to a modified version of the so called Flp-mediated stop cassette excision (FlpOut). Transgenic MCFO flies carry a heat shock promoter (hsp)-Flp recombinase and different reporters under UAS control. Each reporter consists of a common backbone of a myristoylated (myr) super folder green fluorescent protein (sfGFP), in which 10 copies of an epitope tag (*e.g.*, HA, FLAG or V5) have been inserted. The resulting non-fluorescent proteins are named "spaghetti monster GFPs" (smGFPs)[14] and can be detected using specific antibodies against the different epitope tags.

1. **Cross flies with the MCFO cassette and the hsp-Flp (hsp-Flp; 10XUAS-FRT-stop-FRT-myr-smGFP-HA,10XUAS-FRT-stop-FRT-myr-smGFP-FLAG, 10XUAS-FRT-stop-FRT-myr-smGFP-V5) to the appropriate glial GAL4 driver lines (see Table of Materials).**
    1. As the hsp is already active at 25 °C and few cells may undergo recombination leading to an unspecific labeling, set up crosses at 18 °C in order to avoid leakiness of the system. Wait around 20 days at 18 °C to obtain the F1 generation. After the heat shock (see step 2), maintain flies at 25 °C (**Figure 3**).

2. Heat Shock

NOTE: Depending on the insertion site, the efficiency of the hsp-Flp may differ; therefore, the optimal heat shock time has to be optimized. For this protocol, a MCFO fly stock in which the hsp-Flp was inserted on the X chromosome has been used (see **Table of Materials**).

1. Transfer the progeny of the cross in step 1.1 (F1 generation flies, 3-4 days old) into new vials containing food and heat shock them at 37 °C in a water bath.
   NOTE: Young flies (1-2 days old) are very sensitive to heat shock. Wait 1 or 2 more days before performing the heat shock in order to reduce the mortality rate caused by the treatment. Use some of the F1 generation flies as a control, without heat shock.
2. During the heat shock, maintain flies in normal vials with food in order to decrease the rate of stress-induced mortality. Put females and males in separate vials.
3. Make sure to immerse the entire vial in the water to ensure a homogeneous heat shock. Use a 5-8 min shock as a suitable starting point for sparse labeling of glia cells with many GAL4 driver lines; the shock duration must be optimized for each driver and experiment (shorter or longer heat shock times may be necessary).
4. After the heat shock, lay the vials horizontally on the bench for a few minutes in order to allow the flies to recover from the heat shock.
   NOTE: It is not necessary to change the vials.
5. Maintain the flies at 25 °C and dissect (see step 3 and 4) them 2 or more days after the heat shock for optimal reporter expression.

3. Dissection Preparation and Solutions

1. The day of the dissection, prepare fresh fixative solution in 200 µL PCR tubes by mixing 180 µL of S2 cell culture medium with 20 µL of 20% paraformaldehyde (PFA) to obtain a 2% PFA solution. Maintain the fixative solution on ice before and during the dissection.
   Caution: PFA is toxic and must be handled with care.
   NOTE: Mix 160 µL of S2 cell culture medium with 40 µL of 20% paraformaldehyde (PFA) in a 200 µL PCR tube to prepare a 4% PFA solution instead of a 2% solution.
2. Use one PCR tube per genotype with a maximum of 8-10 brains per tube for optimal staining results.
3. Prepare the adult brain washing solution: 0.5% bovine serum albumin, 0.5% Triton X-100 in PBS.
   NOTE: Depending on the staining, a solution containing 1% Triton X-100 may be needed. A higher concentration of Triton X-100 increases the penetration of the antibody into the tissue and it is necessary to stain regions that lie deep inside the tissue (*e.g.,* synaptic regions in the adult *Drosophila* brain).
4. Prepare the blocking solution: 3% normal goat serum, 3% normal donkey serum, and 0.5% Triton X-100 in PBS.
5. The adult brain washing solution and the blocking solution can be stored at 4 °C for a few weeks.
6. Put deep depression wells glass plate on ice and fill each well with the following solutions: one with 70% ethanol, one with PBS, and one with S2 cell culture medium.

4. Adult Brain Dissection

1. Position a 3 cm dissecting dish on the stage of a stereomicroscope and fill it with cold S2 cell culture medium. Conduct all the dissections in this medium to ensure that the tissue remains healthy during the dissection procedure.
   NOTE: Use a dissecting dish lined with several millimeters of black silicone which provides a good background contrast (in the images), and preserve the forceps during the dissection.
2. Anesthetize the flies of the desired genotype with CO_2.
3. With forceps, grab the fly by the wings and wash it in cold 70 % ethanol for 30 s, then with cold PBS for additional 30 s.
4. Transfer the fly into the deep depression well that is filled with S2 cell culture medium.
5. Repeat the same procedure for all flies of the same genotype and maintain them in cold S2 cell culture medium until dissection, which will preserve the tissue.
   NOTE: Anesthetize only the number of flies that can be dissected in a time period of about 30 min. Long incubation times in S2 cell culture medium may damage the tissue.
6. Grab the fly with forceps and, under a stereomicroscope, submerge the fly in S2 cell culture medium.

7. Detach the head from the rest of the body. Discard the body and keep the head submerged in S2 cell culture medium. It is extremely important to hold the head with forceps for the entire duration of the dissection. If the head starts to float, it will be difficult to retrieve it without damaging the tissue.
8. Begin the dissection by pulling out one eye, while holding the head with at least one forceps at all times. Make sure that the tip of the forceps is just below the retina to avoid damaging the tissue underneath. Pull out the other eye and begin removing the cuticle until the brain appears.
9. Remove all tracheal tissue and cuticle around the brain until the tissue appears clean. Make sure to remove all tracheal tissue around the brain for optimal staining results; the tissues are now ready to be fixed and stained.
10. Maintain all the dissected brains in cold S2 cell culture medium before transferring them into the PFA solution in order to time the fixation step.

5. Adult Brain Staining

1. Transfer the isolated brain with a P10 pipette into the PFA solution at room temperature (RT). To avoid tissue damage, never use forceps for transferring the brains after the dissection. Perform all steps in 200 µL PCR tubes on a nutator.
2. For all steps, before discarding the old solution with a P200 pipette, allow the brains to settle on the bottom of the tube. Try to remove the supernatant without aspirating the tissue. Protect the tissue from light exposure.
3. Fix the brains in 200 µL of 2% PFA in S2 cell culture medium for 1 h or in 4% PFA for 30 minutes. Keep fixation times identical between samples in order to increase reproducibility.
4. After 3 (or more) washes of 15 minutes each in 200 µL of adult brain washing solution, block the tissues with 200 µL of blocking solution for 30 minutes. Longer incubation times in blocking solution are possible.
5. **Incubate the brains overnight at 4 °C with primary antibodies diluted in adult brain washing solution in a total volume of 200 µL.**
   NOTE: The incubation times may vary depending on the type of antibody used. Longer incubations may be necessary.
   1. For labeling of the three MCFO markers, incubate the brains with anti-HA (1:500), anti-FLAG (DYKDDDDK epitope) (1:100), and anti-V5 primary antibodies.
   2. Primary directly-conjugated antibodies could be used instead of the normal primary + secondary combination (*e.g.*, anti-V5:DyLight 549 (1:200)). In this case, treat the directly-conjugated antibodies as secondary antibodies.
      NOTE: For each genotype, keep some brains as controls to verify the specificity of the staining. Skip the primary antibody incubation and go directly to the next step.
6. Wash the tissues 3 times for 1 h in 200 µL of adult brain washing solution (step 3.3) and incubate them with secondary fluorophore-conjugates/primary directly-conjugated antibodies diluted in adult brain washing solution overnight at 4 °C or for 4 h at RT, in a total volume of 200 µL
   NOTE: Examples of appropriate antibodies: AlexaFluor 488 (1:250), anti-V5:DyLight 549 (1:200) and DyLight 647 (1:100)-conjugated antibodies.
7. Wash the brains 3 times for 1 h in 200 µL of adult brain washing solution, followed by a final wash in 200 µL of PBS overnight at 4 °C or for 1-2 h at RT; the final washing step in PBS removes any traces of Triton X-100 and is necessary for optimal staining results. Mount the brains in mounting medium with an anti-fade agent on glass coverslips.

6. Mounting of Brains for Imaging

1. Trim an imaging spacer to the appropriate size using scissors. For high-resolution microscopy, sandwich the specimen and imaging spacer between two glass coverslips.
   NOTE: Imaging spacers are thin (0.12 mm thick) adhesive spacers that peel and stick to glass coverslips or microscope slides, allowing the mounting of specimens without compression.
2. Using forceps, remove the adhesive liner from one surface and apply the spacer, adhesive side down, on the surface of a glass coverslip (22 mm x 60 mm). Apply 10 µL of mounting medium to a new glass coverslip.
3. With a P10 pipette, transfer the brains from the 200 µL tube to the coverslip, next to the drop of mounting medium. Together with the tissues, transfer some PBS in order to maintain the brains in solution.
4. With a P10 pipette, move the brains from the PBS to the drop of mounting medium trying to limit the amount of PBS. Transfer the specimens in mounting medium on the coverslip with the imaging spacer. Use enough mounting media to cover the specimen.
5. Remove the other adhesive liner from the upper side of the spacer and add a glass coverslip on top of the specimens. With the tip of a forceps, apply a gentle pressure over the adhesive area to seal. Image the mounted tissues immediately or store the slides at -20 °C for later analysis.

7. Image Acquisition

1. Acquire stacks of confocal fluorescence images using a confocal microscope with a 40X (NA = 1.2, water immersion) objective or 63X (NA =1.4, oil immersion) objective (pixel sizes = 1,024 x 1,024 in the xy plane; Distance between two confocal sections = 0.5 µm). Adjust detector gain and offset in each channel in such a way that no saturated pixels and zero pixel values are present in the images; this avoids information loss and ensures use of the full dynamic range of the detector.
   NOTE: Since 3D imaging is time consuming, the measurements can be automated by scanning multiple samples in parallel at different locations. To avoid evaporation with the water immersion objective, use special oil immersion medium with refractive index n = 1.33.
2. Process the confocal stacks for maximum density projections, changes in channel hue, and adjustment of brightness and contrast using any standard image analysis software (see **Table of Materials**).

Representative Results

This section illustrates examples of results that can be obtained by using the MCFO technique in the adult *Drosophila* brain. **Figure 3** shows a schematic of the method. Three differently membrane-tagged reporters (myr-smGFP-HA, myr-smGFP-FLAG and myr-smGFP-V5) under UAS control are kept silent by a transcriptional terminator flanked by two FRT sites (FRT-stop-FRT). A heat shock pulse induces the expression of Flp recombinase which randomly removes the FRT-stop-FRT cassettes in individual cells. This leads to an array of differently colored cells of a given cell type, specified by the GAL4 driver used.

Overall, seven colors are possible. **Figure 4** and **Figure 5** show the single morphologies of EG and ALG cells in the *Drosophila* antennal lobe (AL), respectively. Increasing heat shock time induces an increased amount of labeled cells, depending on the type of GAL4 driver, therefore an initial optimization of the heat shock protocol is necessary. **Figure 6** shows the labeling of EG in the *Drosophila* optic lobe (OL) with single MCFO reporters.

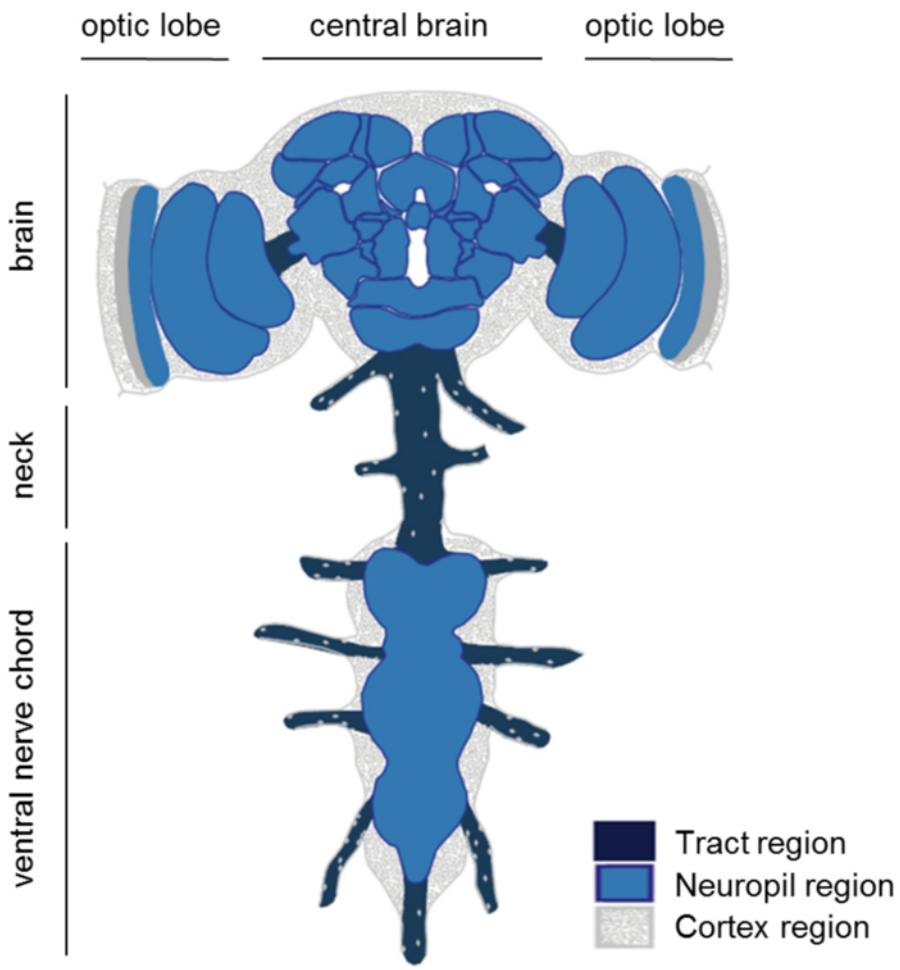

Figure 1: Anatomy of the adult *Drosophila* NS. The cortical regions (dotted gray areas) contain all of the neuronal and most of the glial cell bodies, while the neuropile regions (blue areas) contain the synaptic connections. Tract regions (dark blue)connect the different neuropiles. This figure has been modified from Kremer *et al.* (2017)[18]. Please click here to view a larger version of this figure.

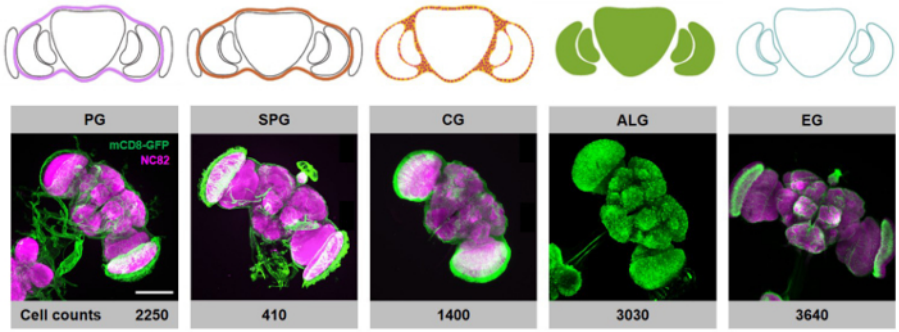

Figure 2: Generic glial subtypes in the *Drosophila* CNS. A membrane-tagged GFP (UAS-mCD8-GFP) reporter driven by specific GAL4 drivers allows the visualization of the five generic glial subtypes (PG: perineurial glia, SPG: subperineurial glia, CG: cortex glia, ALG: astrocyte-like glia and EG: ensheathing glia). The neuropil regions (magenta areas) are detected with the presynaptic marker NC82 (BRUCHPILOT). At the bottom, cell counts for the different glial subtypes are also shown. Scale bar = 100 µm. Please click here to view a larger version of this figure.

Figure 3: Crossing scheme for the MCFO technique. Schematic of the MCFO technique. Homozygousvirgins carrying the MCFO cassette and the hsp-Flp (hsp-Flp; 10XUAS-FRT-stop-FRT-myr-smGFP-HA,10XUAS-FRT-stop-FRT-myr-smGFP-FLAG, 10XUAS-FRT-stop-FRT-myr-smGFP-V5) are crossed with specific homozygous GAL4 driver males. The F1 generation is then heat shocked. According to the number of FRT-stop-FRT cassettes randomly removed from the reporters, seven different colors are possible. Please click here to view a larger version of this figure.

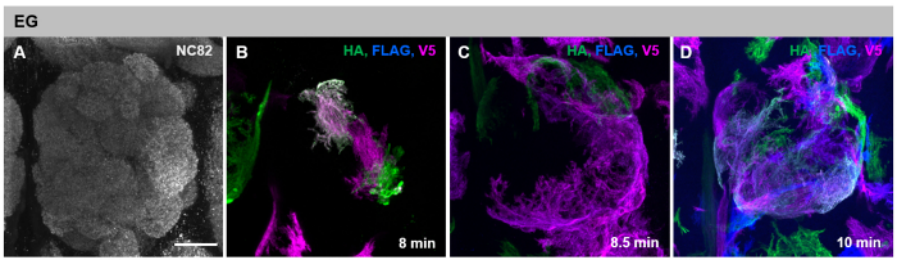

Figure 4: Morphology of ensheathing glia (EG) in the antennal lobe (AL). Confocal z-stacks of *Drosophila* AL after wholemount immunostaining. (**A**) Morphology of AL detected with the presynaptic marker NC82 (BRUCHPILOT). (**B-D**) Stochastic labeling of EGcells induced by increasing heat shock times: 8 min (**B**), 8.5 min (**C**) and 10 min (**D**). The EG take on many different shapes and sizes, as they ensheath the surface of the AL. Neighboring EG cells cover distinct areas but partially interdigitate in regions of contact. Scale bar = 20 µm. HA: Human influenza hemagglutinin. Please click here to view a larger version of this figure.

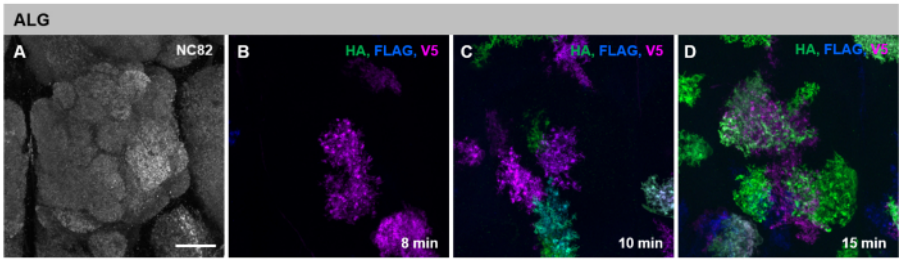

Figure 5: Morphology of astrocyte-like glia (ALG) in the antennal lobe (AL). Confocal z-stacks of *Drosophila* AL after wholemount immunostaining. (**A**) Morphology of the AL detected with the presynaptic marker NC82 (BRUCHPILOT). (**B-D**) Stochastic labeling of ALG cells induced by increasing heat shock times: 8 min (**B**), 10 min (**C**) and 15 min (**D**). ALG show variable size and morphologies but cover largely non-overlapping areas. Scale bar = 20 µm. HA: Human influenza hemagglutinin. Please click here to view a larger version of this figure.

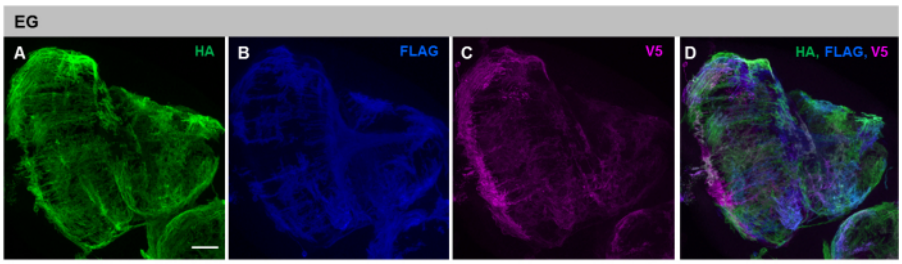

Figure 6: Morphology of ensheathing glia (EG) in the optic lobe (OL). Confocal z-stacks of *Drosophila* OL after wholemount immunostaining. (**A-C**) MCFO labeling with three stop-cassette reporters with HA, FLAG and V5 myr-smGFPs. Flp recombinase was induced by a 10 min heat shock at 37 °C. (**A-C**) Individual MCFO reporters and merge (**D**) are shown. Scale bar = 20 µm. HA: Human influenza hemagglutinin. Please click here to view a larger version of this figure.

Discussion

This protocol describes an easy and efficient method to study the morphology of different cell types within a tissue of interest at high resolution. With the MCFO technique, multiple reporters with different epitope tags are used in combination for multicolor stochastic labeling (**Figure 2**). Similar to other methods such as Brainbow/Flybow[15,16,17], MCFO increases the label diversity through marker coexpression, allowing the visualization of cell boundaries for cell-cell interaction studies. For example, with three reporters, seven potential marker combinations are possible. Compared to previous labeling methods, the MCFO technique shows a more precise and easy control of the cell labeling density[17]: Flybow 1.0 flies express a default marker that makes single cell labeling more difficult; Flybow 2.0 flies require the expression of two different Flp recombinases making the system more complicated.

Depending on the specific GAL4 driver used, the MCFO technique may be adapted to study any tissue at any developmental stage. Here, the technique has been applied to characterize the morphology of the generic glial subtypes in the adult *Drosophila* brain at high resolution. The multicolor labeling of single cells allows the visualization of the different boundaries and helps in understanding, for example, the spatial interaction between two adjacent cells; apart from the PNG and SPG cells, which are meant to form epithelia, all other glial subtypes show tiling, that is they minimize contact with their glial neighbors, while maximizing contact with the enveloped neuronal compartment (cell body, axons, dendrites, and synapses). They all send fine lamellipodial or filopodial processes not only into their local neighborhood but also into distant surroundings[18].

For the success of this protocol, it is crucial to rear flies at 18 °C in order to avoid leakiness in the system. When dissecting and throughout the entire staining procedure, it is important to avoid any damage to the tissue for optimal staining results. Finally, two technical aspects have to be considered: the heat shock time defines the amount of cells in which the tags are expressed. A short heat shock will lead to labeling of a few cells, while a long heat shock will induce the expression of tags in many cells up to the extreme case in which all FRT-stop-FRT cassettes are removed from the reporters, in all cells. In this case, only one color (the merge of the three epitope tags) will be present, preventing the visualization of single cell morphologies. The heat shock time varies depending on the insertion site of the hsp-Flp and the GAL4 driver, therefore an initial optimization step may be necessary. The marker expression is random and cannot be targeted to a specific subpopulation of cells driven by the GAL4 driver. Within one cell, different tags can be expressed enabling the antibody staining of neighboring cells with different colors. However, occasionally, more than one tag is expressed in one cell leading to the overlap of the two colors.

Disclosures

None of the authors have competing or conflicting interests.

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