Video Article

Dissection and Immunofluorescent Staining of Mushroom Body and Photoreceptor Neurons in Adult *Drosophila melanogaster* Brains

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Abstract

Nervous system development involves a sequential series of events that are coordinated by several signaling pathways and regulatory networks. Many of the proteins involved in these pathways are evolutionarily conserved between mammals and other eukaryotes, such as the fruit fly *Drosophila melanogaster*, suggesting that similar organizing principles exist during the development of these organisms. Importantly, *Drosophila* has been used extensively to identify cellular and molecular mechanisms regulating processes that are required in mammals including neurogenesis, differentiation, axonal guidance, and synaptogenesis. Flies have also been used successfully to model a variety of human neurodevelopmental diseases. Here we describe a protocol for the step-by-step microdissection, fixation, and immunofluorescent localization of proteins within the adult *Drosophila* brain. This protocol focuses on two example neuronal populations, mushroom body neurons and retinal photoreceptors, and includes optional steps to trace individual mushroom body neurons using Mosaic Analysis with a Repressible Cell Marker (MARCM) technique. Example data from both wild-type and mutant brains are shown along with a brief description of a scoring criteria for axonal guidance defects. While this protocol highlights two well-established antibodies for investigating the morphology of mushroom body and photoreceptor neurons, other *Drosophila* brain regions and the localization of proteins within other brain regions can also be investigated using this protocol.

Video Link

The video component of this article can be found at https://www.jove.com/video/56174/

Introduction

Although the *Drosophila* nervous system is smaller than that of humans and rodents, its complexity provides a powerful, approachable model to better understand its vertebrate counterparts. In many cases, the genomes of vertebrates and flies encode very similar proteins that dictate the mechanisms of nervous system development. In fact, many of the genes required for neuronal development in vertebrates have orthologs in flies, including those involved in signaling pathways that control patterning, neurogenesis, and axonal guidance[1,2,3,4,5]. For example, netrin is a ligand required for axonal guidance in mammals and *D. melanogaster*[6,7,8]. While netrin was originally isolated from embryonic chick brain tissue[6], subsequent studies have revealed that netrin plays a conserved role during development of the embryonic central nervous system (CNS) in *Drosophila*[8]. Other studies have used genetic screens in the embryonic *Drosophila* CNS to identify conserved ligands and receptors required for pathfinding in both *Drosophila* and vertebrates[9].

While the embryonic fly CNS has been used extensively in the past to identify ligands, receptors, and intracellular signaling proteins required for axonal guidance[8,9], recent work has investigated the ways in which many of these proteins also control pathfinding decisions during later stages of development. Specifically, investigation of mushroom body (MB) and retinal photoreceptor neuron development (**Figure 1**) has provided insight into the mechanisms that control pathfinding, synapse formation, axon pruning, and several other aspects of neuronal development[10,11,12,13,14,15,16,17]. Photoreceptor neurons connect the fly retina to regions of the adult brain called the lamina and medulla and are critical for relaying visual information to the brain (reviewed by[18,19]), while mushroom body neurons are centrally located in the fly brain and are required for learning and memory[20,21]. Both photoreceptor neurons and the intrinsic neurons of the mushroom bodies, called Kenyon cells, utilize evolutionarily conserved diffusible and contact-dependent axonal guidance mechanisms to find their post-synaptic targets. In addition to being visible in adult flies, photoreceptor and MB neurons can also be directly visualized in larvae and pupae with antibodies or reporter genes[22,23,24,25]. The ability to easily visualize these two sets of neurons at different developmental time points has promoted their use as superb models for many aspects of neuronal development.

In addition to being used as a model to understand the mechanisms of normal nervous system development, recent studies have demonstrated that flies can also serve as accurate models of a wide variety of human diseases, including Fragile X Syndrome (FXS)[26], Intellectual Disability (ID)[27,28,29,30,31], and others[32]. For example, to study the molecular function of ZC3H14, a gene recently linked to human intellectual disability, we

created a fly model of ID using a null allele of the fly ZC3H14 ortholog, called Nab2[30]. Flies lacking Nab2 have severe memory impairments and extended poly(A) tails, recapitulating what is observed in human patients or patient derived cell lines[33,34]. Importantly, flies lacking Nab2 also display severe brain morphology defects in their adult mushroom bodies[34], similar to what is observed in flies lacking the FXS syndrome gene, FMR1[35]. Thus, flies can serve as an important model organism to study both normal brain development and diseases that disrupt it.

Finally, the accessibility of high throughput methods to monitor behavior, combined with the vast array of available genetic tools, make *Drosophila* a model organism of choice for identifying the brain regions that control complex behaviors, such as learning and memory, sleep, courtship, thirst, and others[36,37,38,39]. One particularly useful tool that is at the center of a fly geneticist's "toolbox" is the GAL4/UAS system (**Figure 2**). This system[40,41] uses tissue specific expression of the Gal4 transcriptional activator to increase the expression of genes or transgenes downstream of an Upstream Activating Sequence (UAS). Modifications of this system have allowed researchers to, for example, precisely control the excitability of specific neurons[42,43], overexpress or knock-down specific genes of interest[44,45], analyze real-time calcium dynamics *in vivo*[46], and express reporter genes to mark neuronal lineages[41]. The combination of the GAL4/UAS system with mitotic recombination also allowed for the creation of the Mosaic Analysis with a Repressible Cell Marker (MARCM) system[12,47]. MARCM has been used extensively in single neuron tracing to identify the cellular signaling components required for axonal guidance[12,47]. Although these and other techniques have provided a number of valuable insights on the cellular mechanisms required for nervous system function, most require that the *Drosophila* brain first be dissected; careful removal of the brain is required to retain correct brain morphology and connection patterns between brain regions. The following protocol uses mushroom body and photoreceptor neurons as example neuronal populations as it guides you through the dissection and immunofluorescent staining of adult *Drosophila* brains.

Protocol

1. *Drosophila melanogaster* Genetics and Optional Heat Shock Procedures

1. Once flies have been crossed and F1 progeny have hatched, obtain females and/or males of the appropriate genotype. Depending on the brain region being investigated, flies should be collected daily and separated by sex so that age-dependent and/or sexually dimorphic patterns of brain connectivity can be more easily distinguished.
   NOTE: Optional: If flies are being used for Mosaic Analysis with a Repressible Cell Marker (MARCM) analysis[12,47], embryos, larvae, or pupae should be heat shocked at 37 °C for 30 - 45 min to induce mitotic recombination. In order to target specific regions of the mushroom bodies for MARCM analysis, heat shock should be timed according to the schedule determined by[12] and outlined in **Figure 5B**. If using MARCM to investigate brain regions other than the mushroom bodies, pilot experiments should be performed to determine the optimal stage to be heat-shocked. While the steps below are written in regard to eclosed flies, pharate adults can also be dissected using these steps once the pupal case has been removed.

2. Dissection Station Preparation

1. Position the stereomicroscope and light source with attached fiber optic goosenecks on a large benchtop. To promote steady hand movements and reduce hand "shake" while dissecting, it is essential that adequate hand and arm rest space is available around the microscope. Ensure that there is approximately 8 - 10 inches on either side of the microscope and 4 - 6 inches between the base of microscope and edge of the bench.
2. **Fill 2 or 3 wells of a glass 9-well or 3-well dish with 1.0 mL of PTN buffer (0.1 M Sodium Phosphate Buffer pH 7.2, 0.1% nonionic surfactant, see Materials Table for complete buffer components) and place next to the dissecting station on ice. Newly dissected brains will be transferred to this dish and stored until the fixation step.**
   1. Note: If live imaging is required, dissection buffer should be 1x Phosphate Buffered Saline (PBS) or HL3 buffer[48]. If intracellular protein localization is required, PBS can also be used as an alternative buffer for dissection and fixation. Following fixation, permeabilization of cell membranes should then be performed using PTN washes containing 0.1% or 0.3% nonionic detergent.
   2. If PBS is used for dissections and fixation, pipette tips should be rinsed at least one time with a detergent-containing buffer (such as PTN) to prevent brains from sticking to the plastic pipette tips during transfer to microcentrifuge tubes.
3. Using an empty 35 mm glass or plastic petri dish, construct a dissection dish containing a silicone elastomer. Briefly, mix the elastomer components according to the manufacturer's directions, pour into 35 mm dishes, and let it polymerize overnight on a flat surface. Elastomer containing dissection dishes should be used to protect the fine tips of dissecting forceps, which can easily be damaged if contact is made between the forceps and a harder surface, such as a glass dish. We also regularly purchase commercially available silicone coated dishes from online retailers. To increase contrast during dissections, silicone elastomer dissection dishes containing inactivated charcoal (and thus colored black) are particularly useful.

3. Adult Brain Dissection Procedure

1. Anesthetize 3 - 5 day old adult *D. melanogaster* with CO_2 or by using ice. If using ice, place the vial containing flies upside down (plug end down) into an ice bucket for ~5 min. Placing the vial into ice upside down prevents flies from becoming lodged in the food. Once flies have been anesthetized, place the flies on a cold metal pad or petri dish sitting in ice or on a CO_2 emitting fly pad. If dissecting brains to analyze neurodegeneration, older flies may also be used.
2. Place a small amount (150 - 200 µL) of PTN in the center of the dissection dish using a transfer pipette or a p200 pipette to create a "bubble" of PTN. Place the dissection dish under the stereomicroscope and adjust the lighting and focus so that the bubble of PTN fills the field of view and is uniformly illuminated.
3. Manipulate flies so that they are "belly up" (*i.e.*, ventral side up) while lying on the metal or CO_2 pad.
4. Using one pair of #5 forceps, grasp the abdomen of a fly to be dissected and, keeping hold of the fly, completely submerge it in the PTN on the dissection dish.

NOTE: For the remainder of the protocol, all steps should be performed while the head is submerged in PTN.

5. Using a second pair of #5 forceps, grasp the base of the fly proboscis and pull the two pairs of forceps apart to detach the fly head from the body. Discard the abdomen and thorax. During this step, it is critical that the head is not released and allowed to float on the surface of the PTN. Once the head is floating, it can be very difficult to grasp again without crushing the brain.
   NOTE: Using this method, connections between the brain and ventral nerve cord are severed. If intact connections between these regions of the CNS are required, an alternative dissection protocol, such as[49,50], should be followed. If the proboscis detaches from the fly head before the head is removed, there will be a hole where the proboscis was. In this case, grasp the fly head at the edge of the hole near one eye. Then remove the head using a moderate amount of force while pulling the two pairs of forceps apart from one another. Occasionally, when the head is removed from the body, the gut and/or ventral nerve cord remains attached to the head and should be removed before continuing the dissection.
6. While one pair of forceps grasps the proboscis, the second pair should grasp the medial edge of the right fly eye. Slowly, pull the forceps apart from one another. This step should be performed with a small amount of steady lateral force. As the forceps slowly move apart from one another, the proboscis should pull away from the head and create a central hole in the head cuticle. Discard the proboscis with the first pair of forceps without releasing the medial portion of the right eye from the second pair.
   Note: The adult *D. melanogaster* brain is in the caudal (*i.e.*, posterior/rear) region of the fly head. Thus, grasping that region of the head should be avoided. Ideally, only the rostral (*i.e.*, front) portion of the head near the medial retina should be directly grasped by the forceps. The brain and associated trachea should now be visible through the central hole in the cuticle. At this time, any white stringy threads of trachea protruding from the hole can be removed and discarded.
7. With the second pair of forceps, grasp the medial edge of the left retina (at the edge of the central hole in the head cuticle). To remove the retinas and associated cuticle, slowly pull the forceps away from one another at a 180° angle. As the retina dissociates from the underlying optic lobe, you should feel a slight decrease in tension. Proceed slowly to prevent tearing the optic lobe.
   NOTE: Separating the forceps too quickly during this step may result in the tearing of the optic lobe or disruption of mushroom body structures. Occasionally, the cuticle will be removed but pieces of the retina will remain attached to the optic lobe. If imaging the mushroom bodies, it is not completely necessary to remove the entire retina. However, analysis of other brain regions (such as retinal neuron innervation of the optic brain lobes) may require the retina to be completely removed as described by[48,51].
   NOTE: As the retina is slowly separated from the underlying optic lobe of the brain, the optic lobe should be observable as an opaque white structure covered with white, stringy trachea. Once one retina has been removed, it can be discarded. When analyzing pathfinding of retinal neurons, particular care should be taken during this step to prevent damage to the optic lobe. An additional protocol focused on dissection and live imaging of the photoreceptor neurons is also available[48].
8. Now, carefully remove as much of the visible trachea as possible. The trachea may already contain or later fill with air, causing brains to float and, potentially, be lost during later immunostaining steps. To remove trachea, pick it off the brain using a very sharp pair of #5 forceps.
9. Remove the remaining retina and surrounding cuticle by using both pairs of forceps to grasp the medial region of the left fly retina. Carefully tear the retina in half to remove pieces of the retina and cuticle. In some cases, removing the remaining cuticle without crushing the brain proves especially challenging. In these cases, we have found that the remaining strands of the ventral nerve cord can instead be grasped by one pair of forceps while the other pair of forceps is used to carefully remove the last of the cuticle.
10. Using a p200 pipette, move dissected brains to one well of the 9- or 3-well dish containing PTN. Brains of the same genotype should be pooled together into the same well and kept on ice. Brain tissue should be fixed within one hour of dissection. Brains can be fixed in small batches and pooled if a larger number of brains is required. In most circumstances, an experienced researcher can usually dissect a brain and transfer it to the glass collection dish in approximately 3 - 5 min.

4. Fixation and Immunofluorescent Staining Procedure

1. Using a p200 pipette, transfer dissected brains from the 9-well dish to a 0.5 mL microcentrifuge tube filled with 0.5 mL of 4% paraformaldehyde diluted in PTN. At least 10 - 15 brains of the same genotype can be combined into one microcentrifuge tube. All remaining steps (until mounting of brains onto slides) are completed in 0.5 ml microcentrifuge tubes.
   CAUTION: Paraformaldehyde (PFA) should be handled in a fume hood. PFA waste should be saved and disposed of properly. 20% paraformaldehyde purchased in glass ampules can be aliquoted into microcentrifuge tubes and stored at -20 °C until needed.
2. Inside a fume hood, incubate brains in 4% paraformaldehyde for 20 min with slow speed rocking at room temperature.
3. Following fixation, allow brains to settle to the bottom of the microcentrifuge tube by gravity.
   Note: Occasionally, the brains may stick to the side of the microcentrifuge tube. If this occurs, it is usually helpful to laterally rotate the tube between the index finger and thumb or to very gently tap the tube on the bench to promote sinking of the brains.
4. Remove fixative using a p1000 pipette and perform two "quick" washes with 500 µL of PTN, allowing the brains to settle to the bottom of the microcentrifuge tube between washes. During these quick washes, once all the brains have settled by gravity to the bottom of the microcentrifuge tube, the PTN can be immediately exchanged for fresh buffer; no additional wash time is required.
   NOTE: Typically, leaving extra buffer in the tube is preferable to risking brain removal. Careful examination of the pipette tip is often required to ensure that no brains have been mistakenly removed from the tube. If brains have been accidently pipetted into the tip, dispense them back into the microcentrifuge tube, wait for the brains to settle, and then continue removing any extra PTN that remains.
5. After the last quick wash, use a p1000 pipette to perform three "long" washes: add 500 µL of PTN and wash for 20 min at room temperature on a rocker/nutator. All future "long" washes should be for 20 mins.
   NOTE: Following these washes, fixed brains may be stored overnight at 4 °C in PTN.
6. **Remove the last wash using a p1000 pipette and incubate brains on a rocker or nutator at room temperature in 0.5 mL of blocking solution [PTN + 5% normal goat serum (NGS)] for at least 30 min at room temperature.**
   1. Goat secondary antibodies will be used in subsequent protocol steps. If secondary antibodies from another species will be used, normal serum from that species (rather than NGS) should be used in the blocking and antibody solutions.
7. Using a p1000 pipette, remove blocking solution and add the primary antibody diluted in PTN (PTN + 5% NGS + diluted primary antibody). When using a primary antibody for the first time, the optimal dilution of that antibody should be determined empirically.
   Note: For visualizing mushroom body neurons, antibodies recognizing Fas2 are typically used. These antibodies are available from the Developmental Studies Hybridoma Bank (DSHB) as antibody 1D4 and should be diluted 1:20 in PTN + 5% NGS.

NOTE: For visualizing photoreceptor neurons, antibodies recognizing chaoptin are typically used. Chaoptin antibodies are available from the DSHB as antibody 24B10 and should be diluted 1:20 in PTN + 5% NGS.
NOTE: The fixation process typically eliminates fluorescence from fluorescent protein such as green fluorescent protein (GFP). Therefore, when using MARCM to analyze axonal guidance of individual MB neurons, use an antibody recognizing GFP.

8. Incubate brains in primary antibody solution on a rocker/nutator for 2 - 3 nights at 4 °C.
9. Following incubation with primary antibodies, allow brains to settle to the bottom of the microcentrifuge tube and then remove the primary antibody solution.
10. Using a p1000 pipette, perform 2 "quick" washes and 3 "long" 20 min washes with 0.5 mL of PTN as described above in Steps 4.4 and 4.5, carefully allowing brains to settle by gravity to the bottom of the microcentrifuge tube between each wash.
11. Incubate brains for 3 h at room temperature with appropriate fluorescently-labeled secondary antibodies. Secondary antibodies are usually diluted in 0.5 mL of PTN + 5% NGS at a concentration of 1:200.
NOTE: Once fluorescent secondary antibodies have been added, brains should be kept in the dark for the remainder of the experiment.
NOTE: In case residual GFP fluorescence remains, when performing MARCM analysis it is advisable to use a secondary antibody labeled with a fluorophore having similar excitation/emission wavelengths as GFP (*e.g.*, Fluoroscein isothyocyanate (FITC) or Alexa488).
12. Following secondary antibody incubation, allow brains to settle to the bottom of the microcentrifuge tube and remove the secondary antibody solution.
13. Perform 2 "quick" washes and 3 "long" 20 min washes with 0.5 mL of PTN as described above in Steps 4.4 and 4.5, carefully allowing brains to settle to the bottom of the microcentrifuge tube between each wash.
14. Following the third "long" 20 min wash, use a p200 pipette to remove as much buffer as possible.
15. Add 75 µL of fluorescent anti-fade mounting medium to the brains. Pipette brains and mounting medium into the pipette tip once to mix. Do not invert the tube since brains may become stuck on the cap or sides of the microcentrifuge tube.
NOTE: Following suspension of brains in mounting medium, tubes may be wrapped in aluminum foil to slow fluorophore quenching and stored at 4 °C overnight. If necessary, brains can be stored for several days at 4 °C, but should ideally be mounted onto slides as soon as possible.

5. Mounting Adult *D. melanogaster* Brains onto Microscope Slides and Imaging

1. Build a "bridge" slide. Position two "base" coverslips roughly 1 cm apart on a positively charged slide. Ensure that the positively charged side of the slide is facing up. Adhere the coverslips to the slide with fingernail polish as shown in **Figure 3A**. It is usually helpful to seal the three outer edges of each base cover slip with fingernail polish to ensure mounting media does not wick under these base cover slips. Let fingernail polish dry completely (10 - 15 min) before proceeding.
2. Place the slide under the stereomicroscope and pipette the mounting media solution containing the dissected brains into the space between the two coverslips. To provide more contrast, it is useful to maneuver the gooseneck lights so that they are parallel with the bench top.
3. Remove extra mounting media from the slide using a pipette, being careful not to pipette the brains off the slide.
4. Wick away extra mounting media. This will allow the brains to be positioned more precisely during the next step.
5. Using a pair of forceps and the stereomicroscope, position the brains on the slide in a grid pattern with antennal lobes facing up.
6. Place a cover slip (the "bridge") over the brains (**Figure 3A**). Use fingernail polish to seal the sides of the bridge cover slip where they contact the "base" cover slips.
7. Using a p200 pipette, slowly fill the center cavity under the bridge with fresh mounting media (**Figure 3B**). Place one drop at a time on the open edge of the center coverslip and allow the mounting media to wick under the center bridge coverslip. Continue until the entire cavity is filled with mounting media, then seal the top and bottom with clear fingernail polish.
8. Once the fingernail polish is dry, image the slides immediately or store in a lightproof tight slide box at -20 °C.
9. Within one week, image brains using a laser-scanning confocal microscope with excitation lasers and filter cubes appropriate to the chosen fluorescent secondary antibodies. Z-stack images of mushroom body neurons are typically obtained using 20X or 40X objectives. Imaging of retinal photoreceptor neurons may require higher magnification.

Representative Results

The method described above allows for the reliable and reproducible visualization of virtually any region of the adult *Drosophila* brain. Here we have focused on the mushroom bodies and photoreceptor neurons, but other studies have used similar methods to visualize brain regions such as the *pars intercerebralis*[52], clock neurons[53,54], and antennal lobe projection neurons[55], among many others. Importantly, this technique can be used to visualize both entire brain structures as well as individual neurons within those structures using techniques such as MARCM[12,47]. **Figure 4**, **Figure 5**, and **Figure 6** show several different types of data from mushroom bodies and photoreceptor neurons in adult brains that can be generated using this dissection and immunostaining technique.

First, the method described above can be used to directly visualize mushroom body morphology using either antibodies that recognize Fasciculin 2 (Fas2) or reporter genes expressed in mushroom body neurons[34]. As shown in **Figure 4A** and **Figure 4B**, Fas2 antibodies can be used to visualize the α, β, and (to a lesser extent) γ lobes of the mushroom bodies in adult *Drosophila* brains. Fas2 is a cell-cell adhesion protein required for neuron fasciculation and is expressed at high levels in the α and β lobes[23,56,57], making it a reliable and well-established marker of these mushroom body neurons. Notably, the circular-shaped ellipsoid body located in the central region of the adult brain can also be visualized using this antibody (**Figure 4A**).

Defects in axonal guidance proteins often cause incompletely penetrant mushroom body mutant phenotypes[15,34,35]. Therefore, in most circumstances, several dozen brains should be imaged and analyzed. For example, the mushroom body β lobe axons of Nab2 null flies inappropriately cross the brain midline. This "crossing over" or β lobe "fusion" phenotype is typically observed in ~80% of adult Nab2 null flies but is mainly absent from wild-type controls and can be classified as slight, moderate, or complete fusion[34]. The "fusion" of β lobes across the midline results from the incorrect contralateral projection of axons into the opposite brain hemisphere. As shown in **Figure 4C** and detailed in[34,35], slight fusion refers to β lobes connected by a "thin strand of Fas2-positive fibers," while moderate fusion refers to more substantially connected β lobes that show a slightly decreased lobe thickness at the midline. Complete (or "extreme") fusion refers to β lobes that are

completely connected and show no decrease in lobe thickness or Fas2 staining at the midline. The extent of mushroom body β lobe fusion can be quantified and displayed as demonstrated in [34,35] or as a table showing the percentage of brains showing each type of morphology defect.

In addition to staining with Fas2 antibodies, the MARCM technique[12,47,48] can also be used to visualize axon guidance decisions of individual GFP^+ neurons within the mushroom body lobes. MARCM utilizes mitotic recombination during development to create individual neurons, or clonally related groups of neurons, marked with GFP (**Figure 5A**). MARCM provides a way to generate a small number of neurons that completely lack a protein within an otherwise heterozygous fly. As such, this technique has been especially useful in analyzing the axonal guidance functions of proteins that are also essential for overall organismal viability[12,47]. Homozygous null neurons marked with GFP can be directly compared to control neurons marked with GFP in a wild-type genetic background. Furthermore, depending on when developing larvae or pupae are heat-shocked, different classes of mushroom body neurons can be targeted (**Figure 5B**).

An example of the type of data generated using this technique is shown in **Figure 5C**, where wild-type (*i.e.*, control) MARCM clones are generated as an example. To generate the individual GFP^+ mushroom body neurons shown in **Figure 5C**, developing F1 larvae were initially housed at 25 °C. Approximately 5 - 6 days after larval hatching (ALH), pupae were heat shocked for 30 min at 37 °C and then returned to 25 °C until eclosion. Following adult hatching, brains were dissected in PTN, fixed, and simultaneously incubated with antibodies recognizing Fas2 (1D4, diluted 1:20 in PTN) and GFP (diluted 1:500 in PTN). Brains were then incubated with secondary antibodies and mounted on slides as described above. GFP^+ cells were identified and imaged using a laser-scanning confocal microscope and maximum intensity projections were created using ImageJ. As demonstrated in **Figure 5C**, control GFP^+ neurons generated in the α and β lobes colocalize with Fas2 and terminate prior to contacting the midline that separates the two *Drosophila* brain hemispheres. A single axon projects anteriorly, bifurcates, and then projects both dorsally and medially to form the α and β lobes. Several previous studies have used this technique to investigate whether certain proteins are required to study cell division autonomously for many aspects of axonogenesis, including extension, pathfinding, branching, and/or pruning[10,11,12,13,14,15,16,17,34].

In addition to the mushroom bodies, the axonal pathfinding decisions of retinal photoreceptor neurons (R-cells) can also be visualized using the dissection method described above (as well as by the dissection method described in reference[48]). Each ommatidia within the fly eye contains 8 photoreceptor neurons that can be classified into three groups (**Figure 1**): R1-R6 cells, which project axons to the superficial lamina of the brain optic lobe; R7 cells, which project axons to the deeper M6 layer of the medulla; and R8 cells, which project axons to the intermediate M3 layer of the medulla[19,58]. The projection pattern of each class of photoreceptor has been studied extensively and together these neurons have been successfully used as a model to understand the signaling pathways involved in axon guidance[19,59]. Importantly, all photoreceptor axons can be easily visualized in dissected *Drosophila* brains by immunostaining of the adult, pupal, or larval tissues using an antibody that recognizes chaoptin, a cell surface glycoprotein[24,60,61]. Reporter genes expressing GFP or β-galactosidase in each R-cell type (R1-R6, R7, and R8 cells) have also been constructed and can be used to visualize each class of photoreceptor[62]. Since each type of R-cell terminates in a different layer of the developing optic lobe, these reporters have allowed for detailed comparisons of the signaling pathways required for each cell type to correctly find its target. An example of the expression patterns produced by two of these reporter genes in combination with chaoptin localization (which can be used as a marker of all photoreceptor neurons) is shown in **Figure 6**. To visualize R7 photoreceptors, brains containing the R7-specific *Rhodopsin4-LacZ* reporter gene were dissected and stained with antibodies to chaoptin and β-galactosidase. *Rhodopsin 4* (*Rh4*) is expressed specifically in a subset of R7 cells[63]; the *Rh4* promoter can therefore be used to drive reporter gene expression in only these cells. As expected, R7 cells terminate in the deeper M6 layer of the medulla (denoted with a yellow dotted line **Figure 6**). Similarly, brains expressing β-galactosidase from the *Rhodopsin 6* (*Rh6*) promoter, which is expressed specifically in R8 photoreceptors[63], were dissected and immunostained using antibodies recognizing chaoptin and β-galactosidase. As shown in **Figure 6**, R8 photoreceptors terminated at the R3 layer of the*medulla.* Although not shown here, *Rh1-LacZ* can also be used to visualize the termination of R1-R6 photoreceptors in the lamina.

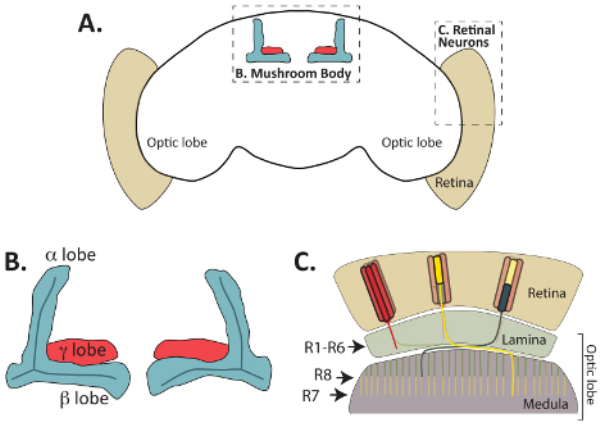

Figure 1: The adult *Drosophila melanogaster* brain consists of functionally distinct but interconnected regions. (**A**) An outline of the adult *D. melanogaster* brain is shown, highlighting the centrally located mushroom bodies and peripheral retinal photoreceptor neurons. (**B**) Enlarged diagram of the adult mushroom body axon bundles (also called lobes) that are recognized by Fas2 antibodies. The intrinsic neurons of the mushroom bodies, called Kenyon cells, project axons anteriorly from dorsally located cell bodies (cell bodies omitted from this diagram) and form distinct lobe structures. In the adult brain, the γ lobes (shown in red) form from a single medial bundle of axons, while the dorsal α and medial β lobes (shown in blue) form from a single axon that bifurcates during the pathfinding process. α'/β' neurons develop axonal lobes that partially overlap with those of α/β neurons, but are not recognized by Fas2 antibodies and are omitted from this diagram. (**C**) Retinal neurons are critical in relaying visual information from the retina to the optic lobe. Axons project from cell bodies located in the retina (beige) and form connections with post-synaptic target cells in the lamina or medulla. R1-R6 photoreceptor cells (shown in red) form specific connections with cells in the outer layer of the optic lobe, called the lamina. R7 photoreceptor cells (yellow) synapse with targets in the M6 layer of the medulla, while R8 cells (shown in green) project axons to the slightly more superficial M3 layer of the medulla. Part C adapted by permission from Macmillan Publishers Ltd: Nature Neuroscience (reference[64], copyright (2011)). Please click here to view a larger version of this figure.

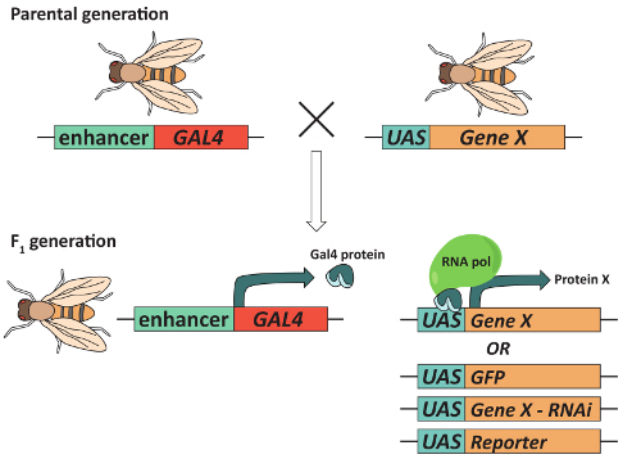

Figure 2: The GAL4/UAS system can be used for targeted gene expression. To obtain flies expressing a gene of interest ("Gene X") in a tissue specific pattern, flies must contain both a transgene expressing the Gal4 transcriptional activator protein under the control of a tissue specific enhancer and a transgene containing the Gal4 DNA binding sequence (called the Upstream Activating Sequence or UAS) adjacent to Gene X. Typically, this combination is achieved by mating parental flies that each contain one transgene and selecting for F_1 progeny that contain both. In the resulting F_1 generation, Gal4 will be expressed and will bind to the UAS to activate transcription of Gene X in a tissue specific manner. Importantly, different UAS-containing transgenes can be used in combination with the same *GAL4* "driver". For example, a transgene expressing Gal4 in the mushroom bodies (MBs) can be combined with a UAS-containing transgene to express GFP, a transgene that knocks down expression of a gene of interest using RNA interference, or a UAS-transgene that produces a reporter. Please click here to view a larger version of this figure.

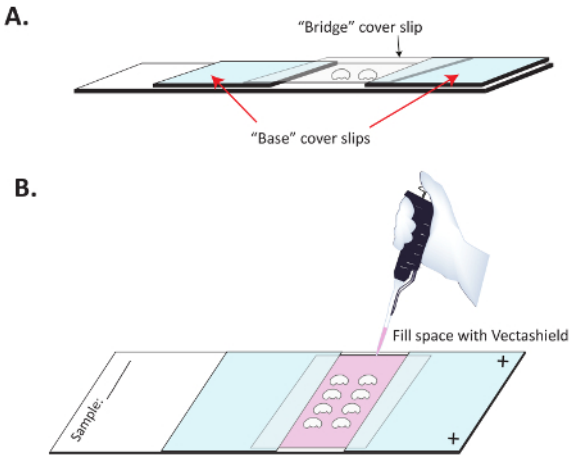

Figure 3: Mounting brains in preparation for immunofluorescence imaging. (**A**) Brains are mounted on SuperFrost Plus slides with a bridge cover to prevent flattening of the brains. Two "base" cover slips are adhered to a Superfrost Plus positively-charged slide with clear fingernail polish and dissected brains are then placed on the slide between them. A clear "bridge" cover slip is placed above the brains and adhered to the base cover slips. (**B**) Once the fingernail polish has dried, Vectashield is slowly pipetted under the bridge to preserve the fluorescence of the secondary antibody. Clear fingernail polish is then used to seal the top and bottom of the "bridge" cover slip. Please click here to view a larger version of this figure.

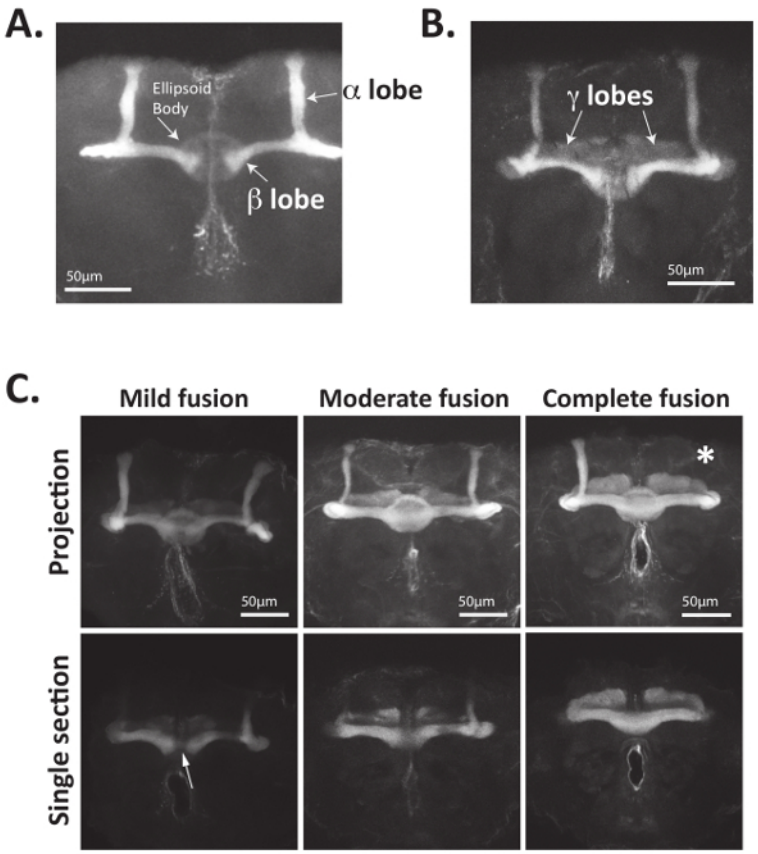

Figure 4: Mushroom body axons can be clearly visualized using antibodies that recognize Fas2. (**A**) Adult *Drosophila* brains were dissected, fixed, and incubated with antibodies recognizing Fas2. Primary antibodies were recognized using Alexa488-coupled goat-anti-mouse secondary antibodies and brains were mounted onto positively-charged slides and imaged using a laser scanning confocal microscope. Bisymmetrically located mushroom body cell bodies extend axons anteriorly, bifurcate, and form axon bundles (also called lobes). Axons that form the dorsally-projecting α lobes and medially-projecting β lobes express Fas2. Critically, β lobes of wild-type flies terminate prior to the brain midline. The centrally located ellipsoid body can also be visualized using the 1D4 antibody. (**B**) The medially-projecting γ lobe axons of the adult *Drosophila* mushroom body also express Fas2 and can be visualized using the 1D4 antibody. Typically, the expression of Fas2 in γ lobe axons is less than that of α and β lobes. (**C**) The mushroom body axons of Nab2-null brains (genotype: $Nab2^{ex3}/Nab2^{ex3}$) mis-project contralaterally and often lack lobes. Nab2-null brains were dissected, fixed, and stained with the 1D4 antibody to visualize mushroom body α, β, and γ lobes. Maximum intensity Z-stack projections as well as individual optical sections focused on the midline region are shown. While wild-type mushroom body β lobe axons rarely cross the brain midline, Nab2-null brains have varying amounts of mis-projection across the midline into the contralateral brain hemisphere, resulting in "fused" β lobes. As defined in references[34,35], mild fusion refers to <β lobes with only several "strands" of axons crossing the midline, moderate fusion refers to situations where Fas2 positive β lobe neurons cross the brain midline but β lobe width at the midline is decreased, and complete fusion refers to situations where there is no reduction of β lobe thickness as the lobes cross the brain midline. Since the ellipsoid body also expresses Fas2, optical sections showing the midline are often more useful in visualizing β lobe fusion. Notably, missing lobes are also frequently observed (denoted here with the white asterisk). Data in part C is used in quantification of mushroom body phenotypes from reference[34], with permission. Please click here to view a larger version of this figure.

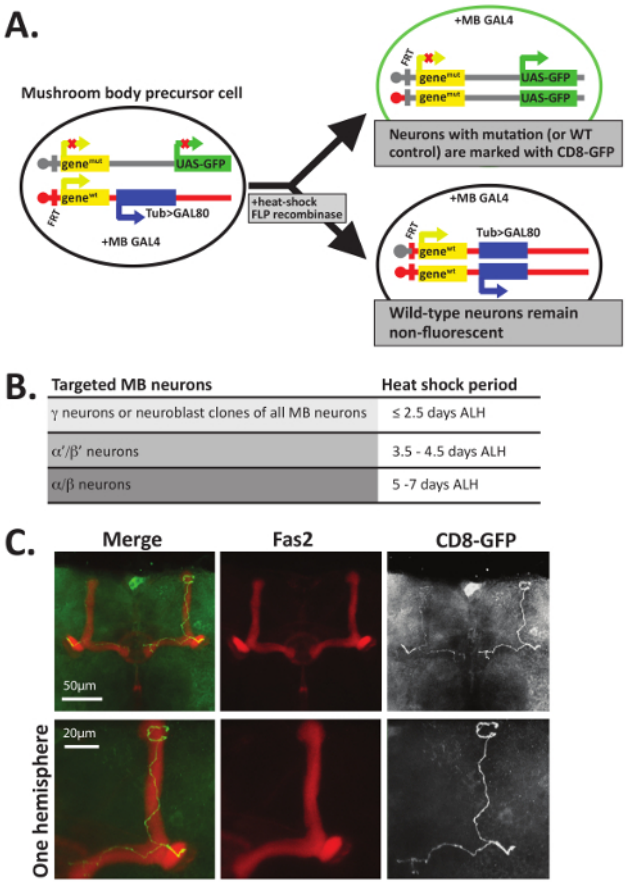

Figure 5: MARCM can be used to visualize the axons of single neurons. (**A**) Mosaic Analysis with a Repressible Cell Marker (MARCM) uses FLP recombinase-mediated mitotic recombination at FRT sites to create two distinct daughter cells. In this example, all cells contain a mushroom body-specific GAL4 protein on a separate chromosome. Following mitotic recombination during cell division, one daughter cell (top) expresses GFP (or the membrane bound CD8-GFP) and contains two mutant alleles of a gene of interest, while the other daughter cell (bottom) inherits two wild-type (WT) alleles and a transgene expressing the Gal80 protein using a tubulin promoter. Gal80 inhibits Gal4, so any cells producing Gal80 will be non-fluorescent and should be either heterozygous or homozygous wild-type. Only those cells that are GFP^+ will contain two copies of the mutant allele. Note that only one of several methods for generating GFP^+ cells that also contain two mutant alleles of a gene of interest is shown; please see[12,45,56] for other examples. (**B**) Since development of mushroom body neurons begins with γ neurons, then α'/β' neurons, and ends with α/β neurons, each class of neuron can selectively be targeted and visualized by heat shocking at different developmental time points. As described in [12], a 40 min heat shock at 37 °C that occurs ≤2.5 days after larval hatching (ALH) will specifically target γ ;neurons, while heat shock that occurs 3.5 - 4.5 days ALH (in the late L3 larval stage) will target α'/β' neurons, and a heat shock that occurs between 5-7 days ALH (during pupal development) will target α/β neurons. (**C**) Individual wild-type axons were visualized using MARCM. Wild-type ~5 - 6 day old pupae (genotype: hsFLP, UAS-CD8-GFP;FRT82B, UAS-CD8-GFP/FRT82B, tub>Gal80; OK107-GAL4/+) were heat shocked at 37 °C for 40 min to induce mitotic recombination. Brains were dissected, fixed, and stained with antibodies recognizing GFP (1:500) and Fas2 (1:20). Brains were then incubated with fluorescently labeled secondary antibodies and visualized by confocal microscopy. In this example data from a "control" genotype, GFP positive cells generated using MARCM are wild-type and instead of containing two $gene^{mut}$ alleles, contain two $gene^{WT}$ alleles. Mushroom body β lobes (visualized using antibodies recognizing Fas2) terminate before reaching the brain midline; α lobe neurons are also present. To show greater detail, a zoomed in view of a single brain hemisphere is shown in the bottom row of images. Part C was adapted with permission from reference[34]. Please click here to view a larger version of this figure.

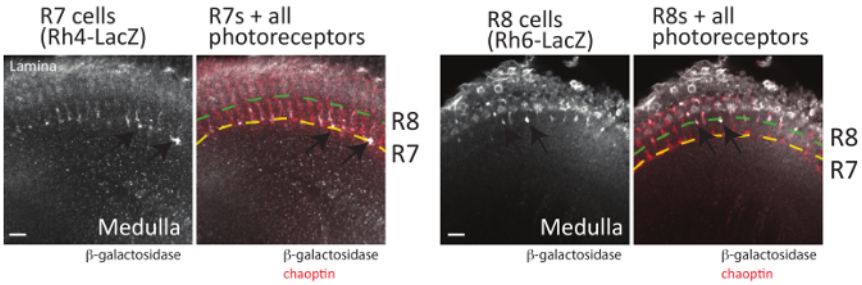

Figure 6: Photoreceptor axons can also be visualized. Adult brains expressing β-galactosidase in R7 photoreceptors (left, using *Rh4-LacZ*) or R8 photoreceptors (right, using *Rh6-LacZ*) were dissected, fixed, and incubated with antibodies recognizing β-galactosidase or chaoptin. Brains were then incubated with fluorescently labeled secondary antibodies and visualized by laser scanning confocal microscopy. Single optical sections are shown from each brain. On the left, several R7 photoreceptor axons (arrows) can be seen terminating in the deeper M6 layer of the medulla (shown by the yellow dotted line). On the right, R8 photoreceptor axons (arrows) terminate in the outer M3 layer of the medulla (shown by the green dotted line). Since R7 photoreceptors express either Rh3 or Rh4 and R8 photoreceptors express either Rh5 or Rh6[63], all R7 or R8 photoreceptors cannot be visualized using a single *LacZ* reporter gene. Scale bars = 10 μm. Please click here to view a larger version of this figure.

Discussion

The dissection and visualization method described above can be used in a wide variety of immunostaining and live imaging applications. We have outlined a general immunostaining protocol and have highlighted one way in which MARCM can be used to visualize axonal morphology of individual mushroom body neurons. In addition, these general procedures can also be used for imaging other brain regions in fixed or freshly dissected adult brains[48,65]. Live imaging may provide a more efficient approach when analyzing expression patterns of enhancer traps, reporter genes, or MiMIC lines[66], for example. To prevent capturing artifacts from cell death during live imaging of GFP in unfixed tissue, brains should be dissected in 1x Phosphate Buffered Saline (PBS) or HL3 media[48], mounted on bridge slides in 1x PBS (or HL3), and imaged in less than 15 - 20 min. Care should also be taken to remove as much trachea as possible from the dissected brain prior to imaging, since it can interfere with visualization of GFP fluorescence.

While staining conditions for assessing the morphology of mushroom body and photoreceptor neurons are relatively well-established[24,61,67], the localization of a specific protein of interest in these cell types may require extensive troubleshooting and optimization. In particular, several critical steps, including antibody dilution, blocking buffer component concentration, and fixation, should be optimized to generate the most reproducible and reliable results. First, the optimal concentration of primary antibody should be determined. Although commercially available antibodies often have a suggested dilution (and we often initially start by using that concentration), these values are rarely determined empirically on *Drosophila* tissues. Therefore, testing a series of primary antibody dilutions that range above and below the manufacturer's starting suggestion often results in more specific staining. While primary antibody dilution can have a significant effect on staining accuracy and should be precisely determined, the time brains are incubated in primary antibody containing solutions can vary considerably with little effect on the overall result. For example, we have observed similar results when incubating dissected brains with the Fas2 antibody primary antibody for two, three, or even four days at 4 °C. Although we recommend at least two nights, we have also incubated brains in primary antibody solution for a single night and obtained reproducible staining. Importantly, limiting the amount of time brains are incubated in the secondary antibody to 3 h at room temperature (or one night at 4 °C) helps limit non-specific binding of secondary antibodies to brain tissue.

Second, it may be necessary to use additional "blocking" reagents to eliminate unwanted background signal. Options include increasing the concentration of NGS to ~10%, adding 1 - 10% Bovine Serum Albumin (BSA) to the blocking and primary/secondary antibody solutions, or pre-adsorption of primary antibodies overnight at 4 °C with *Drosophila* embryos (see reference[68], section 2.9, step 3).

While we have written the above protocol using PTN as the suggested buffer for all fixation, wash, and antibody incubation steps, tissue fixation in other buffers (such as PLP and PEM, listed in the **Materials Table**) may result in profound differences in signal strength. For example, previous studies have demonstrated that Cyclin E is undetectable in larval imaginal discs when tissues are fixed in traditional 4% paraformaldehyde diluted in PBS, but is clearly visible when tissues are fixed in PLP buffer (Ken Moberg, personal communication, and reference[69]). In addition to changes in buffer components, altering the time and temperature for tissue fixation can also significantly affect immunofluorescent staining and can be empirically determined if needed. Generally, fixation time should be long enough to allow for sufficient crosslinking of cellular components and long-term maintenance of overall cellular morphology while being limited enough to prevent over-crosslinking and "burying" of protein epitopes. Therefore, when initially optimizing staining conditions for a newly acquired primary antibody, we usually limit fixation time to approximately 20 min and will often fix tissues at colder temperatures.

Several controls should be included in any immunofluorescence experiment to investigate the specificity of primary and secondary antibodies as well as the effect of transgenes/genetic background on the observed phenotype. To ascertain whether the primary antibody being used specifically recognizes the protein of interest, tissue from flies lacking this protein and/or tissue overexpressing the protein should be included as controls. The addition of excess purified antigen protein can also be included to determine whether the primary antibody recognizes other epitopes in the fly brain. Finally, any fluorescent signal present when primary antibodies are omitted represents the level of non-specific binding by the selected secondary antibodies.

Several important controls should also be included to assess the contribution of genetic background or the presence of transgenes to staining intensity or neuronal morphology. For example, the flies used in the MARCM experiment in **Figure 5** contain multiple transgenes (hsFLP, UAS-CD8-GFP, FRT82B, Tub>GAL80, and OK107-GAL4), each of which should be analyzed separately for effects on mushroom body morphology.

At a very minimum, mushroom body morphology of flies containing both OK107-GAL4 and UAS-CD8-GFP should be analyzed. It may also be necessary to assess the effect of heat shock and Flp recombinase production on mushroom body development by analyzing flies containing both hsFLP and FRT82B. Although MARCM can provide significant insight into whether a given protein autonomously controls axonal guidance, the number of controls required to accurately make this conclusion can be a minor limitation of this technique. In less complicated experiments, similar controls are also applicable. For example, take an experiment where the *GAL4/UAS* system is being used in combination with an RNAi transgene to knockdown expression of a protein of interest in all neurons. In this experiment, at least two transgenes will be present: a pan-neuronal GAL4 driver, such as *elav-GAL4,* and the *UAS-RNAi* transgene. The morphology of mushroom body neurons in flies containing each of these transgenes alone should be investigated in addition to the experimental condition where flies harbor both transgenes in the same fly.

Finally, to determine whether the protein of interest is expressed in mushroom body neurons it is usually necessary to co-stain brains with antibodies to Fas2. Alternatively, fluorescent proteins such as GFP, RFP, or the membrane bound CD8-GFP can also be expressed using the *GAL4/UAS* system and used in combination with antibodies recognizing the protein of interest. Since Fas2 only recognizes the fasciculated axons that form the mushroom body α and β lobes, the use of GAL4-driven, membrane-bound CD8-GFP has been particularly useful for marking mushroom body neurons.

Defects in axonal guidance are often not 100% penetrant and even brains of the same genotype may show some variability. Therefore, when analyzing newly generated mutant alleles for defects in mushroom body or photoreceptor pathfinding, a combination of different alleles and approaches should be utilized. Most studies in the literature use several different approaches to investigate whether a protein plays a cell-autonomous role in controlling axonal guidance: i) analysis of pathfinding defects in homozygous null flies (preferably using different null alleles), ii) analysis of pathfinding defects in flies lacking the protein of interest only in neurons (usually via RNAi), iii) MARCM analysis of pathfinding defects, and iv) rescue experiments where the gene is re-expressed in the neurons of homozygous null flies. Ideally, several dozen brains per genotype should be analyzed for defects in neuronal morphology.

Although the protocol described here primarily focuses on immunofluorescent localization of proteins within fixed tissue, several future applications of this technique are being developed to image live brain tissue. Once brains are dissected (usually in cell culture media), they can then be cultured for several days at 25 °C. These *ex vivo* culturing methods are being developed in order to investigate a wide variety of biological processes, such as protein activity that promotes axon regeneration following injury[70,71], intracellular signaling dynamics[72], and neuronal development[73].

Disclosures

The authors declare that they have no competing financial interests.

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