A protocol for dissecting *Drosophila melanogaster* brains for live imaging or immunostaining

Joy S Wu & Liqun Luo

Howard Hughes Medical Institute, Department of Biological Sciences, Neurosciences Program, Stanford University, Stanford, California 94305-5020, USA. Correspondence should be addressed to L.L. (lluo@stanford.edu).

Published online 7 December 2006; doi:10.1038/nprot.2006.336

This protocol describes a basic method for dissection and immunofluorescence staining of the *Drosophila* brain at various developmental stages. The *Drosophila* brain has become increasingly useful for studies of neuronal wiring and morphogenesis in combination with techniques such as the 'mosaic analysis with a repressible cell marker' (MARCM) system, where single neurons can be followed in live and fixed tissues for high-resolution analysis of wild-type or genetically manipulated cells. Such high-resolution anatomical study of the brain is also important in characterizing the organization of neural circuits using genetic tools such as *GAL4* enhancer trap lines, as *Drosophila* has been intensively used for studying the neural basis of behavior. Advantages of fluorescence immunostaining include compatibility with multicolor labeling and confocal or multiphoton imaging. This brain dissection and immunofluorescence staining protocol requires approximately 2 to 6 d to complete.

INTRODUCTION

In recent years, the *Drosophila* brain has been intensively used for many neurobiological studies. For developmental neurobiologists, neurons in the *Drosophila* brain have become increasingly accessible to functional analysis of genes involved in processes including neuronal polarity, axonal and dendritic growth, guidance, branching and pruning, and establishment of wiring specificity. Single-neuron labeling in the *Drosophila* brain with techniques such as the MARCM system allow for high-resolution analysis of wild-type or genetically manipulated cells in live or fixed tissues[1–3]. For those who wish to use *Drosophila* as a model system to investigate the neural basis of behavior, understanding the anatomical organization of neural circuits is essential. Here, the availability of thousands of *GAL4* enhancer trap lines, in combination with additional genetic tools such as the MARCM system and FLP-out[4], have allowed high-resolution mapping of neuronal circuits.

The *Drosophila* brain is most accessible to immunofluorescence staining techniques. Such techniques require primary antibodies specific to the protein or structure of interest as well as fluorescently labeled secondary antibodies to immunolabel the primary antibodies. The advantages of immunofluorescence staining, as compared to other techniques such as immunoperoxidase staining, include the ability to simultaneously use multifluorescent labeling for double, triple or quadruple staining. Fluorescence staining also allows high-resolution confocal or multiphoton imaging of deep structures.

In this protocol, we describe a basic method for dissecting adult, pupal and larval brains. Dissection is then followed by either live imaging or fixing and immunostaining for confocal imaging. We give specific examples of olfactory system studies, including the antennal lobes and the mushroom bodies.

MATERIALS

REAGENTS

- 1× PB (100 mM Na_2HPO_4/NaH_2PO_4, pH 7.2)
- Triton X-100 (Sigma-Aldrich, cat. no. 9002-93-1)
- SlowFade Gold antifade reagent (Invitrogen Molecular Probes, cat. no. 36936)
- One or more appropriate primary antibodies: for staining MARCM[3] clones using UAS-mCD8::GFP, use rat anti-mCD8α (Invitrogen Caltag, cat. no. RM2200, 1:100) or anti-GFP (Invitrogen Molecular Probes rabbit anti-GFP, cat. no. A6455, 1:250); to label relevant architectural features, the presynaptic marker mouse anti-nc82 can be used to label general neuropil, including the glomerular organization in the antennal lobe[5] (Developmental Studies Hybridoma Bank nc82, 1:40); mouse anti–Fas II can be used to label axon tracts of specific neuron classes in the brain, notably the mushroom body neurons, where, in the adult, the α/β axons are strongly labeled and the γ neurons are weakly labeled[6] (Developmental Studies Hybridoma Bank 1D4 anti-Fasciclin II, 1:50)
- One or more appropriate secondary antibodies: these may include goat anti-rat Alexa 488 (Invitrogen Molecular Probes, cat. no. A11006, 1:200), goat anti-rabbit Alexa 488 (Invitrogen Molecular Probes, cat. no. A11034, 1:200), goat anti-mouse Alexa 568 (Invitrogen Molecular Probes, cat. no. A11031, 1:200) or goat anti-mouse Cy3 (Jackson ImmunoResearch Laboratories, cat. no. 115-165-166, 1:200)
- 4,6-diamidino-2-phenylindole (DAPI; Invitrogen Molecular Probes, cat. no. D1306; 1:20,000)
- Self-hardening mounting medium (for example, Biomeda no. M01)

EQUIPMENT

- Dissecting microscope
- Nutator (VWR, cat. no. 15172-203)
- 0.5-ml microcentrifuge tubes
- Two pairs of sharp forceps (Dumont, no. 5)
- Three-well glass dissection dishes (Fisher Scientific, cat. no. 21-379)
- Petri dish
- Grape plate
- Paintbrush
- Kimwipes
- Microscope slides
- No. 1 coverslips (VWR, cat. no. 48366-067)
- No. 2 coverslips (VWR, cat. no. 48368-062)
- Clear nail polish
- Imaging microscope and software (for example, confocal microscope)

REAGENT SETUP

0.3% (vol/vol) PBT Add 1.5 ml Triton-X 100 to 498.5 ml PB. Store this nonhazardous buffer at room temperature (20–25 °C). Different staining procedures or antibodies may require different concentrations of Triton-X 100, often 0.1%–0.5%, or a different detergent such as Tween-20.

4% (vol/vol) paraformaldehyde In a 0.5-ml tube, add 100 µl 20% w/v paraformaldehyde (Electron Microscopy Sciences, cat. no. 15713-S) to 400 µl PBT. Prepare fresh and place on ice. Different staining procedures or antibodies may require different concentrations of paraformaldehyde or a different fixative. ! CAUTION Toxic, handle as a possible carcinogen. After one use, properly dispose of this waste according to appropriate guidelines.

5% (vol/vol) normal goat serum (NGS) Add 50 µl normal goat serum (Lampire Biological Laboratories, cat. no. S2-0609) to 950 µl PBT. Store this block solution for short periods at 4 °C (overnight at the very most).

Primary antibody Dilute the primary antibody in freshly prepared 5% NGS. A 0.5-ml tube requires 400 µl of diluted antibody. Diluted primary antibodies can be reused up to three times. Some antibodies may last even longer. Store antibodies between uses at 4 °C for no more than 1 month. ▲ CRITICAL As little as 150 µl of diluted antibody can be used in a 0.5-ml tube when quantities of antibodies are very limited. For even smaller volumes, less than 100 µl, use 0.2-ml PCR tubes.

Secondary antibody Dilute the secondary antibody in freshly prepared 5% NGS. A 0.5-ml tube requires 400 µl of diluted antibody. Prepare fresh and discard secondary antibodies after use. ▲ CRITICAL As little as 150 µl of diluted antibody can be used in a 0.5-ml tube when quantities of antibodies are very limited. For even smaller volumes, less than 100 µl, use 0.2-ml PCR tubes. ▲ CRITICAL Antibodies can be diluted 1:500 or 1:1,000 if background signal is too high.

PROCEDURE

Dissect brains

1| Brains can be analyzed as fixed or live tissues. The protocols below describe how to dissect brains from adults (A), pupae (B) and larvae (C).

(A) Adult brains ● TIMING **~5 min per brain for a novice, ~30 s per brain for an expert**

(i) Anaesthetize and place adults of the appropriate genotype and age into a three-well dish on ice.

(ii) Fill another three-well dish with PBT (see REAGENT SETUP) and place it under a dissecting microscope.
▲ CRITICAL STEP It is also possible to dissect in PB (see REAGENT SETUP) or in different concentrations of PBT.

(iii) Place a 0.5-ml tube containing 500 µl of freshly prepared 4% paraformaldehyde (see REAGENT SETUP) on ice. For live imaging, use PB instead of paraformaldehyde.

(iv) With gentle forceps manipulation, remove the brain from the head cuticle and place it into the 4% paraformaldehyde on ice (or directly into a drop of PB on a microscope slide, for live imaging). One method of performing this dissection is to hold the fly with forceps in the PBT, belly up. With another pair of forceps, gently insert one side into the cavity just below the eye to obtain a grip of the eye. Be careful to avoid internal head structures such as the brain. Gently pull the head off of the fly and discard the body. With the free forceps, obtain a grip of the other eye from the underside. Gently pull the two pairs of forceps away from each other to open the head cuticle. After removing the brain from the head cuticle, carefully remove the surrounding trachea, as these air-filled sacs can cause the brains to float in staining solutions or interfere with live imaging.
? TROUBLESHOOTING

(v) Repeat Step 1A(iv) for the remaining flies. To stain brains, proceed to Step 2. For live imaging, proceed directly to Step 16A, omitting the staining procedure (Steps 2–15).
? TROUBLESHOOTING

(B) Pupal brains ● TIMING **~10 min per brain for a novice, ~1 min per brain for an expert**

(i) Collect white prepupa with a PB-soaked paintbrush and place these into a small Petri dish in a humidifying chamber at 25 °C. This is taken to be 0 h after puparium formation. To study a specific pupal development stage, wait the appropriate number of hours before proceeding.
▲ CRITICAL STEP Pupae will not develop properly when wet. Be sure to remove excess PB from the dish.

(ii) Place the Petri dish on ice to stop development.

(iii) Fill a three-well dish with PB and place it under a dissecting microscope.
▲ CRITICAL STEP Staining is often better when pupae are dissected in PB instead of PBT.

(iv) Place a 0.5-ml tube containing 500 µl of freshly prepared 4% paraformaldehyde on ice. For live imaging, use PB instead of paraformaldehyde.

(v) With gentle forceps manipulation, remove the brain from the pupa and place it into the 4% paraformaldehyde on ice (or directly into a drop of PB on a microscope slide for live imaging). One method of performing this dissection is to hold the pupal case with forceps in the PB, dorsal side up. With the other pair of sharp forceps held closed, puncture a hole into the abdominal end of the pupa. Open the forceps to tear the pupal case apart and pull the pupa out. Use forceps to gently remove the abdominal tissue, up to the level of the thorax. Gently hold the pupa at the very front of the head case. Use the side of the second pair of forceps to gently push the brain from the head and out through the thorax. Alternatively, try tearing apart the head case from the opening in the thorax. Gently remove excess fat particles.
? TROUBLESHOOTING

(vi) Repeat Step 1B(v) for the remaining pupa. To stain brains, proceed to Step 2. For live imaging, proceed directly to Step 16A, omitting the staining procedure (Steps 2–15).
? TROUBLESHOOTING

(C) Larval brains ● TIMING ~5 min per brain for a novice, ~15 s per brain for an expert

(i) Synchronize a vial of hatching larvae to ensure that a substantial number are at the developmental stage of interest.
▲ **CRITICAL STEP** For more carefully staged larvae, collect embryos on a grape plate. Wait until embryos start to hatch (~21 h at 25 °C) and remove all hatched larvae from the plate. After 2 h, collect all newly hatched larvae. These are taken to be 0–2 h after larval hatching. Allow the larvae to develop in a vial until the proper stage.

(ii) Collect larvae of the stage of interest with a PBT-soaked paintbrush.

(iii) Place the larvae into a three-well dish containing cold PBT.

(iv) Place a 0.5-ml tube containing 500 µl of freshly prepared 4% paraformaldehyde on ice. For live imaging, use PB instead of paraformaldehyde.

(v) With gentle forceps manipulation, remove the brain, keeping it attached to the mouth hook, and place it into the 4% paraformaldehyde on ice (or directly into a drop of PB on a microscope slide for live imaging). One method of performing this dissection is to gently hold the larval body with one pair of forceps, and with a second pair of forceps, hold the larval mouth hook. Pull the two pairs of forceps apart gently so the mouth hook detaches from the body, pulling the brain out with it. Alternatively, try peeling the larval cuticle apart like a banana, starting at the mouth hook. Discard the larval body. While maintaining a grip on the mouth hook, gently remove excess tissue surrounding the brain.
▲ **CRITICAL STEP** Remove the mouth hook and eye discs only when mounting larval brains on a microscope slide. Larval brains without the mouth hook are easily damaged and their small size makes them easy to lose during staining.
? TROUBLESHOOTING

(vi) Repeat Step 1C(v) for the remaining larvae. To stain brains, proceed to Step 2. For live imaging, proceed directly to Step 16A, omitting the staining procedure (Steps 2–15).
? TROUBLESHOOTING

Stain brains: day 1 ● TIMING ~3 h for 1–20 tubes handled simultaneously, followed by two overnight incubations

2| Place the 0.5-ml tube containing brains in 4% paraformaldehyde onto a nutator. Allow the brains to fix for 20 min at room temperature.
? TROUBLESHOOTING

3| Remove the tube from the nutator and place it into a tube rack at room temperature. Allow the brains to settle to the bottom of the tube. Use a P-200 pipet to remove the paraformaldehyde.
! CAUTION Properly dispose of this hazardous waste according to the appropriate guidelines.

4| Add 0.5 ml PBT to the tube. Close and invert the tube. Allow the brains to settle to the bottom. Remove the PBT. Repeat once more for a total of two quick washes at room temperature.

5| Add 0.5 ml PBT to the tube. Place on nutator to wash for 20 min. Repeat twice more for a total of three 20-min washes at room temperature.
? TROUBLESHOOTING

6| Remove the PBT from the brains and add 0.5 ml block solution (5% NGS; see REAGENT SETUP). Place brains on nutator to block for at least 30 min at room temperature.

7| Remove block solution from the brains and add primary antibody solution. Place on nutator at 4 °C for 2 nights.
■ **PAUSE POINT** This step can be reduced to one night at 4 °C, but antibody penetration is not as thorough, depending on the cells of interest. This can also be increased to 1 week at 4 °C, but the increase in penetration is not much greater.
? TROUBLESHOOTING

Stain brains: day 3 ● TIMING ~1.5 h, followed by two overnight incubations

8| Remove the primary antibody and store it at 4 °C. The antibody can be reused three times or more.
? TROUBLESHOOTING

9| Add PBT for two quick washes, as in Step 4.

10| Perform three 20-min washes in PBT, as in Step 5.

11| Remove PBT. Add secondary antibody. Place on nutator at 4 °C for 2 nights. Blocking before adding secondary antibody is unnecessary.
■ **PAUSE POINT** This step can be reduced to 2 h at room temperature, but antibody penetration is not as thorough, depending on the cells of interest. This can also be increased to 1 week at 4 °C, but the increase in penetration is not much greater.
? TROUBLESHOOTING

Stain brains: day 5 ● TIMING ~1.5 h

12| Remove the secondary antibody and discard.

13| Add PBT for two quick washes, as in Step 4.

14| Add PBT for three 20-min washes, as in Step 5. DAPI can be added to one of the 20-min washes to stain nuclei for analyses such as counting cell numbers.

15| Remove PBT and add 200 µl SlowFade. Allow brains to settle in SlowFade at 4 °C.

■ PAUSE POINT Brains can be left at this stage for up to 1 week at 4 °C.

Mount brains

16| To mount brains for live imaging, follow option (A). For mounting fixed and stained brains, follow option (B).

(A) Mount brains for live imaging ● TIMING ~30 min per 50 brains

(i) Place the dissected brains into a drop of PB on a microscope slide.

(ii) Using forceps, carefully align the brains for ease of imaging. Pay careful attention that the anterior side of the antennal lobes is facing upward, as it is easier to image these structures in this orientation.

(iii) Arrange two broken coverslips on the microscope slide to form a bridge around the brains. This prevents the brains from becoming too compressed under the top coverslip. For larval and early pupal brains, use broken no. 1 coverslips. For late pupal and adult brains, use broken no. 2 coverslips.

? TROUBLESHOOTING

(iv) Gently place a no. 1 coverslip on top of the bridge to cover the brains.

(v) Proceed directly to imaging (Step 17). This is useful for a quick examination or a forward genetic screen[7]. If samples are prevented from drying out, they last for 20–30 min depending on the tissue. In a self-hardening mounting medium, live samples last ~2 h.

(B) Mount fixed and stained brains ● TIMING ~30 min per 50 brains

(i) Cut the end off a P-200 pipet tip. Use this to pipet the brains from the tube and transfer them onto a mounting slide. Avoid adding excess SlowFade. Excess SlowFade can be removed with a P-200 pipet or Kimwipe.

(ii) Carefully align the brains, as in Step 16A(ii). Remove excess SlowFade with a P-200 pipet or Kimwipe.

(iii) Arrange two broken coverslips on the microscope slide to form a bridge around the brains, as in Step 16A(iii).

? TROUBLESHOOTING

(iv) Gently place a no. 1 coverslip on top of the bridge to cover the brains.

(v) Slowly pipet SlowFade, starting from one side of the coverslip, until the entire sample is covered.

(vi) Seal the edges of the coverslip with nail polish. Store at 4 °C in a dark slide holder.

■ PAUSE POINT Mounted slides can be stored for several months at 4 °C, but the fluorescence will gradually fade. It is best to proceed to imaging within a few days of staining. Slides can be stored for longer at –20 °C (~3 years) or even longer at –80 °C. It is good practice to periodically check the slides to make sure that the nail-polish seal is maintained. If the slide appears to be drying out, additional mounting media can be pipetted under the coverslip and the nail-polish seal reapplied.

Image brains ● TIMING ~15 min per brain

17| Follow instructions for imaging using a compound fluorescence or confocal microscope, according to your laboratory's specific system.

▲ CRITICAL STEP Imaging should be done as soon as possible to get the best signal, certainly within a few days of completing immunofluorescence staining. For confocal imaging of the antennal lobe, we recommend the use of a ×40 oil objective and a *z*-step of 1 µm to allow for identification of the individual glomeruli. After imaging, return the samples to a dark slide holder.

? TROUBLESHOOTING

● TIMING

Step 1: ~2 h, dissect brains.

Steps 2–15: ~5 d, fix and stain brains.

Steps 16–17: ~5 h, mount and image brains and analyze data. This may take substantially longer depending on the complexity of the experiment.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1A(iv)	Brains float at the surface instead of sinking	Air-filled trachea can cause adult brains to float	Remove trachea during adult brain dissection
1B(v)		Fat particles can cause pupal brains to float	Remove fat particles during pupal dissection
1C(v)		Larval brains without the mouth hook can float	Do not remove the mouth hook during larval dissection
		The solution does not contain enough Triton-X detergent	Add a small amount of Triton-X or a higher concentration of PBT
1A(v) 1B(vi) 1C(vi)	Degradation of tissue	Dissection time too long	Dissect and keep fixed on ice for no more than 1 h
2	Fuzzy images	Fixed for too long	Take care to fix only for 20 min
5	Brains stick to tube walls	Pupal brains in particular can stick if washed for long periods of time	Take care to wash only twice quickly and three times for 20 min each
			Add a small amount of Triton-X or a higher concentration of PBT
7	Nonspecific staining	Primary antibody not diluted enough	Make a more dilute solution of primary antibody
		Primary antibody needs to be preadsorbed	Preadsorb primary antibody before use
8	Mold growth in primary antibodies	Antibody stored for too long	Check for growth and discard moldy dilutions
			Make fresh dilutions
			Add sodium azide (Sigma-Aldrich cat. no. 26628-22-8; 0.1% wt/vol) to the primary antibody stock; ! **CAUTION**: highly toxic
11	Background signal is too high	Secondary antibody not diluted enough	Make a more dilute solution of secondary antibody
11	Bright, aberrant puncta covering images	Secondary antibody precipitated before use	Briefly centrifuge the secondary antibody before making the dilution; take only the supernatant
11	Unexpected dual-labeling	Cross-reactivity of secondary antibodies	Ensure that minimally cross-reactive secondary antibodies are purchased
			Use primary antibodies generated in a different animal
16A(iii)	Brains float under top coverslip	Bridge is thicker than the brain	Use thinner broken coverslips for a bridge
16B(iii)			Instead of broken coverslips, use a dot of thick silicon vacuum grease on the four corners around the brains; place coverslip on top, press down until the brains touch the coverslip
17	Dim fluorescence signal	Fluorescence bleached by exposure to light	Ensure that samples are not left under room or fluorescent lights for long periods of time
		Primary and/or secondary antibodies too dilute	Use a higher concentration of antibodies
		Primary and/or secondary antibody penetration is poor	Incubate in primary and/or secondary antibodies for longer periods of time

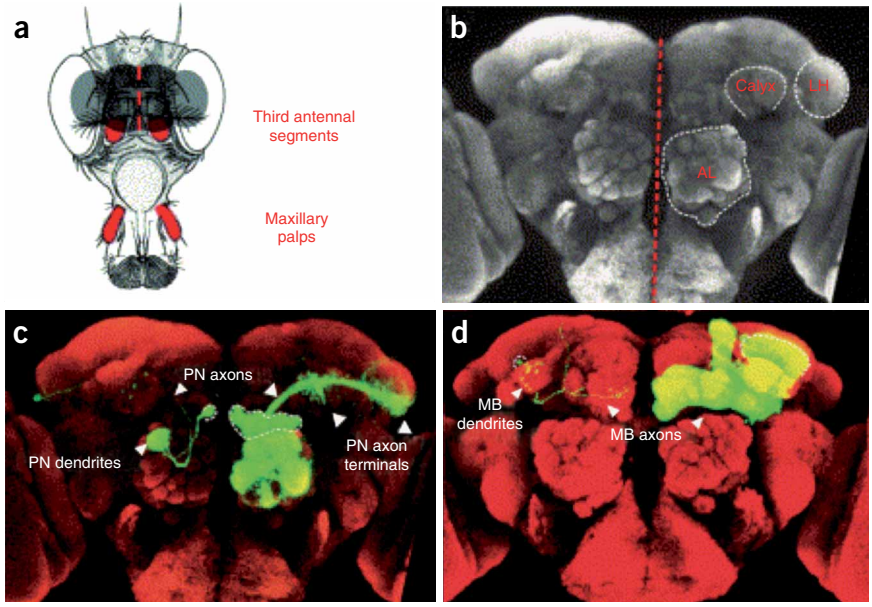

Figure 1 | Visualizing neurons in the *Drosophila* brain. (**a**) Schematic of the *Drosophila* head (anterior view), with the brain in dark gray. Olfactory appendages are shown in red. (**b**) A brain in the same orientation, stained with the monoclonal antibody nc82, a presynaptic marker recognizing neuropil. Major brain structures are labeled: AL, antennal lobe; calyx, mushroom body calyx; LH, lateral horn. Dotted red line represents midline. (**c**) A brain showing MARCM clones. Shown in green on the left is a projection neuron (PN) single-cell clone; on the right is a PN neuroblast clone. nc82 staining is shown in red. White dotted lines outline cell bodies. (**d**) A brain showing MARCM clones. Shown in green on the left is an MB single-cell clone of the α′/β′ type; on the right is an MB neuroblast clone. nc82 staining is show in red. White dotted lines outline cell bodies. Reprinted with permission from refs. 8,9.

ANTICIPATED RESULTS

Figure 1 illustrates the location of the brain in the *Drosophila* head and gives examples of immunostaining results. This protocol can yield staining with high resolution of single neurons within the brain.

ACKNOWLEDGMENTS We thank members of our laboratory for their helpful comments on this protocol. Research in our lab has been generously supported by grants from the US National Institutes of Health, and more recently from the Howard Hughes Medical Institute, for which L.L. is an investigator.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

Published online at http://www.natureprotocols.com
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Lee, T. & Luo, L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451–461 (1999).
2. Lee, T. & Luo, L. Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. *Trends Neurosci.* **24**, 251–254 (2001).
3. Wu, J.S. & Luo, L. A protocol for mosaic analysis with a repressible cell marker (MARCM) in *Drosophila*. *Nat. Prot.* (doi:10.1038/nprot.2006.320).
4. Wong, A.M., Wang, J.W. & Axel, R. Spatial representation of the glomerular map in the *Drosophila* protocerebrum. *Cell* **109**, 229–241 (2002).
5. Wagh, D.A. *et al.* Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. *Neuron* **49**, 833–844 (2006).
6. Crittenden, J.R., Sloulakis, E.M.C., Han, K.-A., Kalderon, D. & Davis, R.L. Tripartite mushroom body architecture revealed by antigenic markers. *Learn. Mem.* **5**, 38–51 (1998).
7. Reuter, J.E. *et al.* A mosaic genetic screen for genes necessary for *Drosophila* mushroom body neuronal morphogenesis. *Development* **130**, 1203–1213 (2003).
8. Jefferis, G.S.X.E., Marin, E.C., Stocker, R.F. & Luo, L. Target neuron prespecification in the olfactory map of *Drosophila*. *Nature* **414**, 204–208 (2001).
9. Jefferis, G.S.X.E., Marin, E.C., Watts, R.J. & Luo, L. Development of neuronal connectivity in *Drosophila* antennal lobes and mushroom bodies. *Curr. Opin. Neurobiol.* **12**, 80–86 (2002).