Immunofluorescent antibody staining of intact *Drosophila* larvae

Laurina Manning & Chris Q Doe

Institute of Molecular Biology, Institute of Neuroscience, Howard Hughes Medical Institute, University of Oregon, Eugene, Oregon, USA. Correspondence should be addressed to L.M. (rina@uoregon.edu) or C.Q.D. (cdoe@uoregon.edu).

Published online 1 December 2016; doi:10.1038/nprot.2016.162

Antibody staining is a vital technique for studying the development of many model organisms, including *Drosophila*. Reliable protocols have long been available for antibody staining of *Drosophila* whole-mount embryos and dissected larvae. By contrast, methods for staining whole larvae have rarely been reported, are unreliable, and fail to work on large third-instar larvae. This has become a major limitation to understanding the role of multitissue interactions such as neural circuit formation and cell metastasis. We have modified existing embryo protocols to develop a reliable method for antibody staining of whole *Drosophila* larvae of any developmental stage. The procedure consists of a bleach wash, enzymatic digestion, first fixation, 'cracking', second fixation, (optional) Proteinase K (Pro-K) or sonication treatment, antibody staining, clearing, and mounting. The method takes longer than typical antibody stains of dissected larval tissues—12 or 16 d, depending on the size of the larvae, compared with 2–3 d for embryos or dissected tissue stains—but time is saved by eliminating the need for larval dissections and by allowing hundreds of larvae to be batch-processed. The method also works well for staining embryos, even late-stage embryos with cuticles, allowing characterization from early embryogenesis to the end of larval development.

INTRODUCTION

Immunofluorescent antibody staining is an important research tool in many fields, including developmental biology and neuroscience. In particular, the characterization of neural circuit formation and function requires methods for imaging the distant connections between sensory neurons and the CNS, or between motor neurons and muscles, in intact organisms. The development of novel clearing protocols such as those that use dibutyl phthalate in xylene (DPX) for *Drosophila*[1] or Clarity for mammals[2], has allowed unprecedented resolution of larger tissues and organisms.

Drosophila is a premier system for studying developmental biology and neuroscience. Immunostaining of intact embryos has been possible for more than three decades[3], and it has been instrumental in furthering our understanding of the development and neurobiology of the embryo. However, the focus of *Drosophila* neuroscience has recently shifted to the larva and adult. Although expression of GFP or other genetically encoded fluorophores can be detected within intact larvae, imaging of live larvae yields poor optical resolution, which limits the detection of sparsely labeled populations or fine processes. Dissection of the larval CNS, followed by immunofluorescent staining, has been useful for studying neural stem cell self-renewal[4], stem cell proliferation control[5], and the generation of neuronal diversity[6,7]. So-called 'fillet preparations' have been used to preserve these connections[8], but they do not preserve neural connections to internal organs, nor are they amenable to processing more than a few larvae per day.

Here we present a detailed protocol for immunofluorescent antibody staining of intact *Drosophila* larvae of all stages—from newly hatched larvae to late third-instar wandering larvae—that is based on our published methods[9]. The protocol has been optimized for nuclear antigens and for membrane antigens that decorate sensory and central neurons following multicolor flip-out (MCFO)[1], although we expect it to work equally well with most antigens.

Development and overview of the procedure

The protocol is based on the standard *Drosophila* embryo staining protocol (**Fig. 1**, top)[3,10], but the final protocol (**Fig. 1**, bottom) has a number of modifications taken from staining techniques for a variety of organisms including *Caenorhabditis elegans*, *Euprymna scolopes*, and *Artemia franciscana* (brine shrimp)[11–13]. Furthermore, our protocol allows visualization of fine neuronal processes, unlike a previous method[14]. Each modification is essential to an improved outcome, but none work as stand-alone additions to the original embryo protocol.

In sequence, the procedure consists of the following: bleach wash; enzymatic digestion; first fixation; 'cracking' by exposure to a methanol/heptane interface; second fixation step; optional Pro-K or sonication treatment (most useful for the large third-instar larvae); antibody staining; clearing; and mounting. The specific modifications that we have incorporated at key steps are discussed below.

Bleach wash. The bleach wash has been modified from the embryo procedure to be more concentrated and of longer duration so that it begins the process of permeabilization.

Enzyme digestion. We have incorporated an initial enzyme digestion step before fixing; larvae are incubated at 37 °C for 1 h in 'CCD' buffer, which is a mix of chitinase and chymotrypsin in a HEPES buffer with 1% (vol/vol) DMSO. Chitinase–chymotrypsin digestion steps were found in several protocols and varied widely in terms of enzyme concentration, incubation duration, and incubation temperature—ranging from 24 h at 37 °C for *E. scolopes*[14] to 3 min at room temperature (22–24 °C) for *C. elegans*[11]. We tried several concentrations, from 0.4 to 5.0 units of chitinase per ml and from 0.4 to 100.0 units of chymotrypsin per ml. Concentrations of either enzyme lower than 2 units per ml did not yield satisfactory results. We settled on 5 units of chitinase per ml and 100 units of chymotrypsin per ml, but the concentrations, as well as the length of the incubation time and the incubation temperature, are points in the protocol that could be optimized

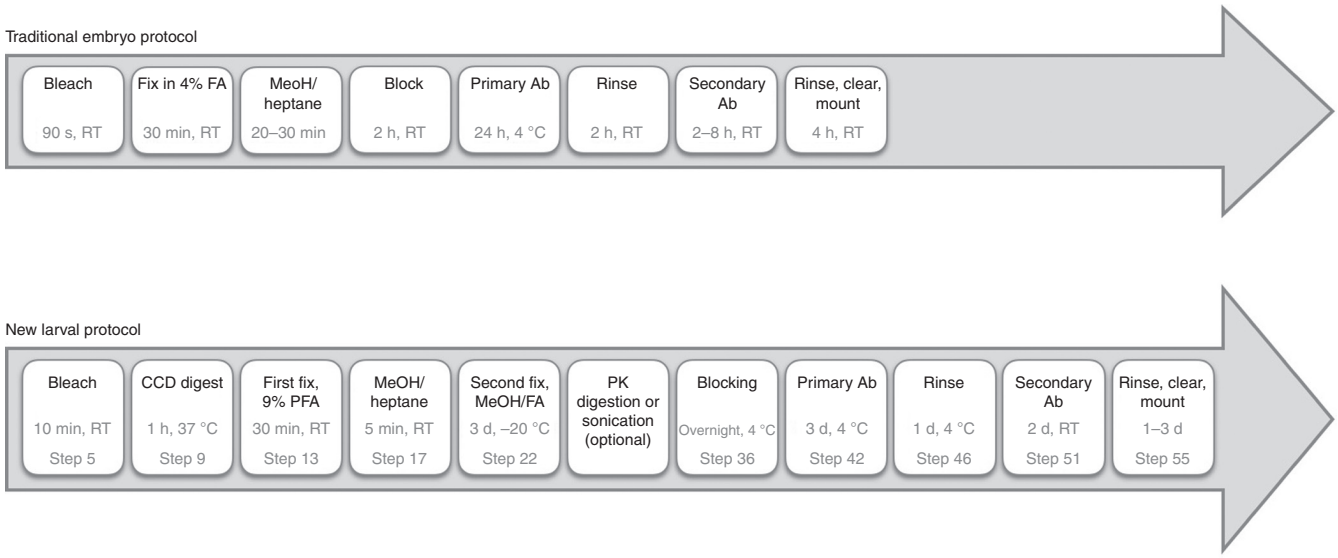

Figure 1 | Comparison of old and new staining pipelines. Top: traditional *Drosophila* embryo staining protocol pipeline. Bottom: new *Drosophila* larval staining protocol pipeline. Ab, antibody; CCD, chitinase–chymotrypsin DMSO digestion; FA, formaldhyde; MeOH, methanol; PFA, paraformaldehyde; PK, proteinase K; RT, room temperature.

for specific applications. We found that the addition of 1–2% (vol/vol) DMSO to the chitinase–chymotrypsin buffer improves the permeability and intensity of the staining results.

First fixation and cracking. The first fixation is performed after the enzyme digest, and we found that 9% (vol/vol) paraformaldehyde, as compared with 4% (vol/vol) formaldehyde, provided better antibody penetration in all animals tested. The duration of the first fixation incubation (20 min) is unchanged from that of the original protocol. However, the concentration of formaldehyde, incubation time, and incubation temperature could be adjusted to further optimize performance. For reasons that remain unclear, the heptane/methanol 'cracking' procedure from the original embryo fixation protocol—included to remove the inner vitelline membrane after the fixation step—is also critical to the success of the larval procedure.

TABLE 1 | Typical second fixation and primary antibody incubation durations for different developmental stages.

Developmental stage	Description	Duration of second (MOF) fixation[a]	Duration of primary antibody incubation[a]	Comments
L0 larvae[b] (**Fig. 2**)	No older than 4 h post hatching	3 d	From 48 h	L0 larvae are more easily stained because of the softened state of the chitin at this age
L1 larvae (**Fig. 3**)	4 h post hatching to the point of the first molting	5 d	3–5 d	Percentage of usable animals is typically >90% at this stage. Modify incubation times as needed
L2 larvae (**Fig. 4**)	Defined by mouth hooks	5–7 d	3–7 d	Penetration is more difficult. Percentage of usable animals is typically >70% at this stage. Modify incubation times as needed
L3 larvae (**Fig. 5**)	Defined by mouth hooks	7 d	7 d	Penetration is very difficult. Consider doing a sample set with Pro-K treatment to see deep proteins (see limitations). Percentage of usable animals is typically 10–30% at this stage. Modify incubation times as needed
Embryos (**Fig. 6**)	Stages 0–17	2–3 d		No CCD incubation needed for embryos. Percentage of usable animals is typically >90% at this stage

[a]For each antibody, incubation length will need to be user-optimized. [b]L0 larvae are 0–4 h post hatching from embryo.

TABLE 2 | Primary and secondary antibodies.

	Source	Concentration	Dilution
Primary antibody			
Guinea pig α-Deadpan	Abcam, cat. no. ab195172	1 mg/ml	1:100
Rat α-Elav	DHSB, cat. no. 7E8A10	333 µg/ml[a]	1:100
Mouse α-Repo	DHSB, cat. no. 8D12	172 µg/ml[a]	1:50
Mouse α-Fas2	DHSB, cat. no. 1D4	212 µg/ml[a]	1:100
Rabbit α-GFP	Thermo Fisher, cat. no. A11122	2 mg/ml	1:500
Chicken α-mCherry	Novus, cat. no. NBP2-25158	3 mg/ml[a]	1:1,000
Rabbit α-V5-549 tag	Rockland, cat. no. 600-442-378	1 mg/ml	1:400
Mouse α-HA-488 tag	Cell Signaling, cat. no. 2350	100 µg/ml[a]	1:200
Rabbit α-Flag-680 tag[b]	Rockland, cat. no. 600-444-383	1 mg/ml	1:200
Rat α-OLLAS-650 tag[b]	Novus, cat. no. NBP 1-06713C	0.9 mg/ml[a]	1:100
Secondary antibody			
Donkey α-guineapig-alexa647	Jackson, cat. no. 706-605-148	1 mg/ml	1:400
Donkey α-rat-alexa488	Jackson, cat. no. 712-545-153	1 mg/ml	1:400
Donkey α-mouse-alexa647	Jackson, cat. no. 715-605-151	1 mg/ml	1:400
Goat α-mouse-alexa555	Thermo Fisher, cat. no. A21424	2 mg/ml	1:400
Goat α-rabbit-alexa488	Thermo Fisher, cat. no. A11034	2 mg/ml	1:400
Goat α-chicken-alexa555	Thermo Fisher, cat. no. A21437	2 mg/ml	1:400

[a]Concentration may vary by lot—optimal working concentration is typically around 3–4 µg/ml. [b]The anti-Flag 680 and anti-OLLAS 650 were used simultaneously because of the low number of MCFO neurons occurring with Flag and OLLAS tags.

Second fixation. We added a second fixation step to the procedure to increase permeability, based on the WormBook protocol 2.7.7 (ref. 15). We call our modification of their protocol the 'Meth-O-Fix' (MOF) step. The MOF step takes place at −20 °C for a minimum of 3 d for younger samples and for up to 7 d for larger larvae. Inclusion of heptane during this step greatly improves permeability, but it may reduce intensity in staining for some epitopes or antibodies. This step can also be optimized according to experimental requirements.

Optional Pro-K treatment or sonication. Once the two fixation steps are complete, the optional Pro-K digestion step or sonication steps may be performed. Pro-K increased the level of internal staining deep in the larval structures, but at the cost of peripheral structure and protein integrity. Sonication increased the penetration of antibodies into the body cavity, producing strong staining of internal cell types in L1–L3 animals, but had two drawbacks: the cuticle and epidermis were visibly damaged, and fine axonal and dendritic projections were disrupted (data not shown). At this time, we recommend using Pro-K only for nonepidermal antigens, and sonication only for nuclear proteins.

Mounting. DPX (Fluka, Milwaukee, WI) is the preferred method of mounting the stained whole larvae because of its outstanding clarity. We have modified the original DPX procedure[1] to include longer washes during the dehydration steps and to incorporate the use of a silicone-covered slide. This latter modification allows small 22-mm diameter round coverslips to be used in order to minimize drying time while maximizing drying efficiency. The silicone also helps keep larvae in place during drying; in the absence of the silicone coat, the larvae have a tendency to slide out from under the coverslip.

Advantages, limitations, and applications

Our protocol can be used for all stages of *Drosophila* larvae and embryos, even late embryonic stages that were previously inaccessible to antibody staining; typical conditions for obtaining optimal staining at different developmental stages are outlined in **Table 1**. The major advantage of this protocol is that it preserves cell and tissue relationships that would be lost during tissue dissection, which was the only previously known method of staining internal organs in *Drosophila* larvae. In addition, we do not see 'hot spots' of staining, which would be expected if permeabilization treatments created a few large holes in the cuticle; rather, we observe even staining in all regions of the larva, suggesting uniform penetration of the cuticle by antibodies.

Our protocol has some limitations, the first being that it is lengthy, requiring 12–16 d to complete. However, most steps are

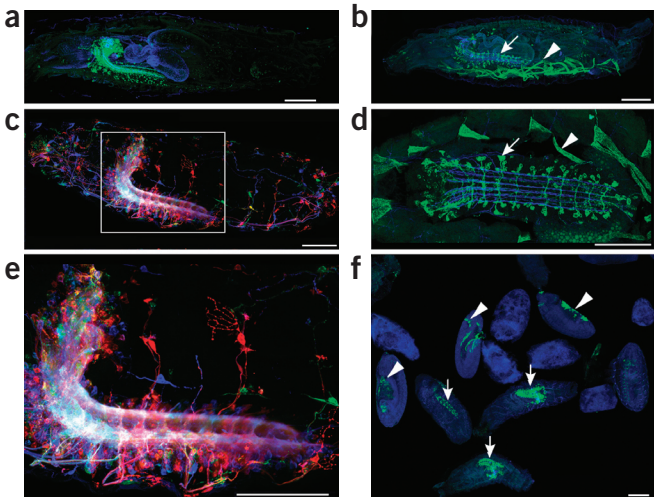

Figure 2 | Staining of newly hatched larvae (L0). (**a**,**b**) L0 larvae stained for R15D07-gal4 UAS-myr:GFP (green) and FasII (blue). Neuronal staining (arrow) and muscle staining (arrowhead) are visible. For additional details, see Clark *et al.*[9]. (**c**) L0 larvae stained for the multicolor flip-out antigens that decorate random neuronal membranes of a subset of Elav-gal4-57C10-MCFO-line-6-positive neurons. (**d**) L0 larvae stained for R31G06-gal4 UAS-myr:GFP (green) and FasII (blue). Neuronal staining (arrow) and muscle staining (arrowhead) are visible. For more details, see Clark *et al.*[9]. (**e**) Enlargement of boxed portion of panel **c**. (**f**) Field of view showing L0 larvae and various embryo stages stained for R76F05-gal4 UAS-myr:GFP (green) and FasII (blue). CNS staining (arrow) and amnioserosa staining (arrowhead) are visible. For more details, see Clark *et al.*[9]. Scale bars, 50 µm. Additional details regarding antibodies used can be found in **Table 2**.

short-interval solution changes, and thus once a staining workflow is established, it is easy to generate the same quantity of data per week as with the faster standard protocol. Second, the percentage of usable stained samples varies based on the antibodies used, and decreases as the larvae get larger. Using the MCFO, Deadpan, or Elav antibodies (**Table 2**), the number of well-stained larvae, similar to those shown in **Figures 2–5**, is ~95% for young larvae (L0–L1), ~50% for L2 larvae, and ~20% for the largest L3 larvae—but drops to as low as 10% for other antibodies that we used, such as rabbit anti-GFP. Modifications of the protocol will be needed to optimize the staining of each new antibody.

Although the procedure is substantially longer than existing protocols for staining dissected larval tissue, it is the only way to image entire larvae, preserving cell and tissue relationships that are lost during tissue dissections. Furthermore, it saves time by avoiding delicate larval dissections and allows batch processing of many larvae simultaneously. We recommend that our protocol be used when the number of larvae to be stained is large (thus avoiding tedious dissections) or when the cells or cell type of interest is widely distributed within the larva. We recommend that the shorter standard protocol be used only if embryos are to be stained or if dissected larval tissues are needed.

It is possible that this procedure may also be useful for a variety of arthropod embryos and larvae. As the protocol came from combining elements of crustacean, worm, and fly antibody staining protocols, it is possible that our protocol may be widely adaptable to many organisms such as non-Drosophilid insects, nematodes, waterbearers (*Tardigrades*), beach hoppers (*Parhyale hawaiensis*), and brine shrimp (*A. franciscana*).

Experimental design

Optimizing fixation. Optimal fixation is critical to success and is dependent on the antibody used. Both the first and second fixation steps can be adapted to ensure maximal penetration of different antibodies: variables include the percentage (vol/vol) of paraformaldehyde used, as well as the duration and temperature of the incubations. In addition, we have had promising results using Ott's ZnFA fixation protocol (zinc chloride–paraformaldehyde in HEPES buffer and 1% (vol/vol) DMSO)[16] for the first fixation step (used on large third-instar larvae (L.M., data not shown)).

Solution-to-sample ratio. The solution-to-sample ratio is critically important at all stages of the protocol, but particularly during the chitinase–chymotrypsin digestion, the fixation/cracking procedure, and the MOF second fixation. There must be a sufficiently large solution-to-sample ratio to ensure complete digestion or fixing. A rough estimate is that the sample should comprise no more than 10–15% of the total volume. In addition, we recommend

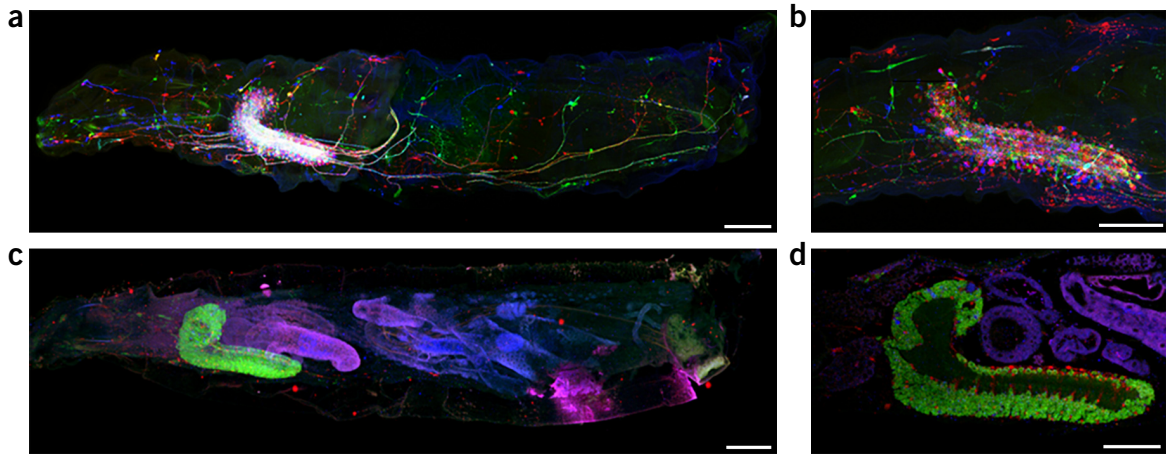

Figure 3 | Staining of first-instar larvae (L1). (**a**,**b**) First-instar larvae stained for the multicolor flip-out antigens that decorate random neuronal membranes of a subset of Elav-gal4-57C10-MCFO-line-6-positive neurons. (**a**) Whole larva and (**b**) CNS only. (**c**,**d**) First-instar larvae stained for the nuclear proteins Dpn (blue), Elav (green), and Repo (red). (**c**) Whole larva and (**d**) CNS only. Scale bars, 50 µm.

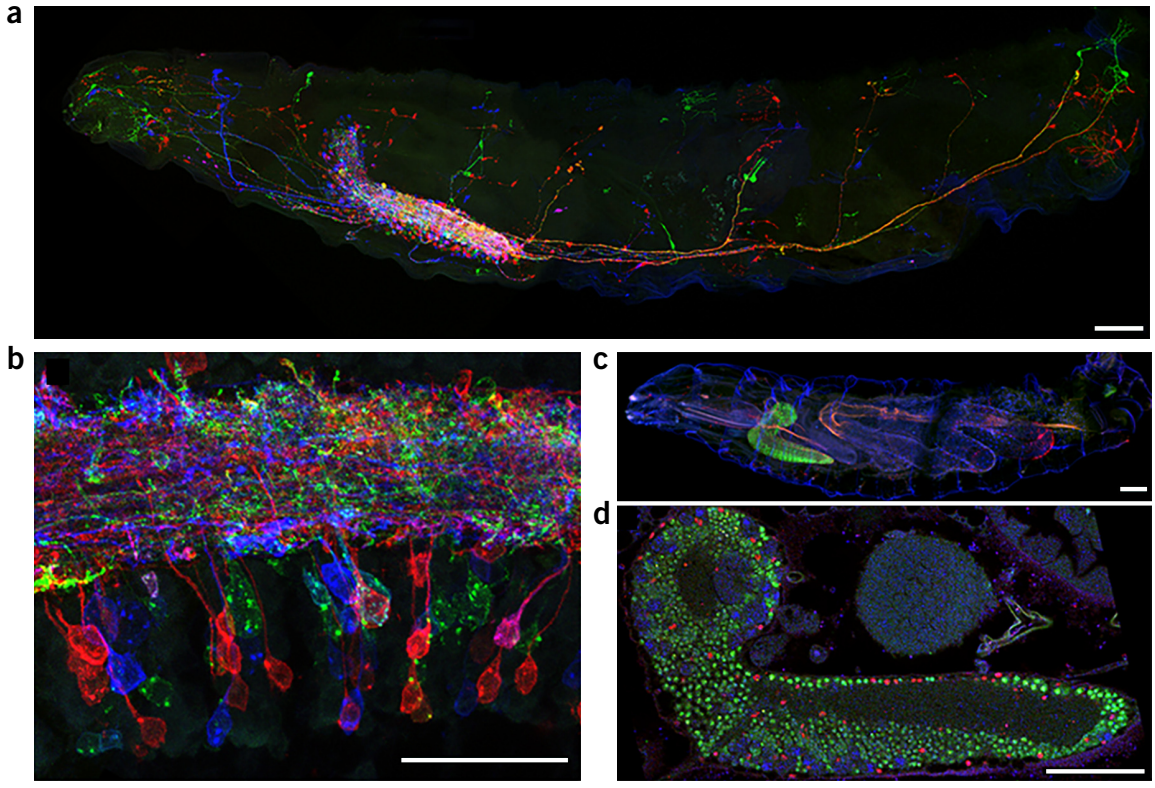

Figure 4 | Staining of second-instar larvae (L2). (**a**,**b**) Second-instar larvae stained for the multicolor flip-out antigens that decorate random neuronal membranes of a subset of Elav-gal4-57C10-MCFO-line-6-positive neurons. (**c**,**d**) Second-instar larvae stained for the nuclear proteins Dpn (blue), Elav (green), and Repo (red), using the optional Pro-K treatment (Steps 31–35) to increase detection of deep-tissue nuclear antigens. Scale bars, 50 µm. Additional details regarding antibodies used can be found in **Table 2**.

laying the tubes on their side to spread out the larvae and maximize contact with the solutions during incubations.

Post-MOF washes and incubations. After the two fixation steps and optional Pro-K or sonication treatments are complete, we do not recommend rocking or shaking at any point from the overnight block step (Step 35) onward. The absence of rocking or shaking is particularly critical during, and in all steps following, the primary antibody incubation (Step 43). We found that aggressive or excessive changing of washes beyond what is indicated in the procedure led to reduced signal. Wash duration between incubation in primary and secondary antibody could be increased without harm, and in fact it reduced nonspecific background in the staining. However, extensive rinsing after the secondary antibody incubation beyond what is recommend in the procedure is not advised. We find that as the larval size increases the 'MOF' second fixation, antibody incubation length, wash duration between primary and secondary antibodies, and DPX clearing times need to be increased correspondingly.

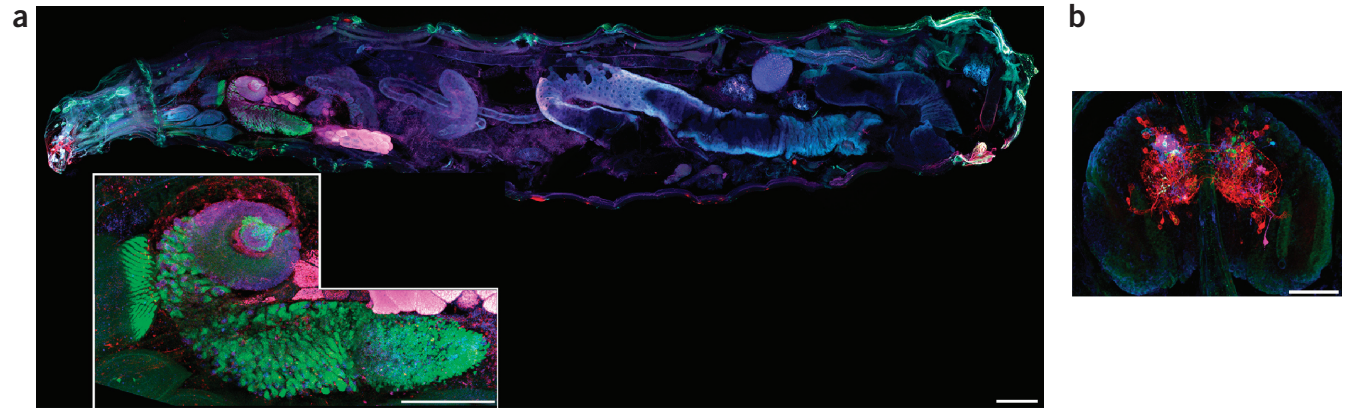

Figure 5 | Staining of third-instar larvae (L3). (**a**) Third-instar larvae stained for Dpn (blue), Elav (green), Repo (red), and processed using the optional Pro-K treatment (Steps 31–35). Inset: High-magnification view of larval CNS. (**b**) Third-instar larvae stained for the multicolor flip-out antigens that decorate neuronal membranes of a subset of Elav-gal4-57C10-MCFO neurons. Scale bars, 50 µm. Additional details regarding antibodies used can be found in **Table 2**.

MATERIALS

REAGENTS

- Bleach (Clorox). It is harmful to eyes and skin, and it is corrosive.
- Chitinase (Sigma-Aldrich, cat. no. C6137) ! CAUTION Wear gloves when handling the compound; chitinase is not hazardous, but avoid inhalation.
- Chymotrypsin (Sigma-Aldrich, cat. no. CHY5S) ! CAUTION Wear gloves when handling the compound. It is harmful to the skin, eyes, and respiratory system; avoid inhalation, wear gloves, and work in a well-ventilated fume hood.
- DMSO (EMD Millipore, cat. no. 102950) ! CAUTION Wear gloves when handling the compound; DMSO is flammable, and it is an irritant to the skin and eyes.
- Ethanol, 200 proof (Decon Labs, cat. no. 2716) ! CAUTION Wear gloves when you are handling the compound; the compound is flammable, and it is harmful to the skin and eyes.
- Methanol (Sigma-Aldrich, cat. no. 322415) ! CAUTION Wear gloves when handling the compound; methanol is flammable and harmful; it is a respiratory and skin irritant, and it is toxic.
- Sodium azide (Sigma-Aldrich, cat. no. S2002) ! CAUTION Use in a laminar flow hood while wearing gloves and a lab coat. It is harmful on inhalation, and it is an irritant to the skin and eyes. It is toxic, harmful, and an irritant; it can cause brain damage, and exposure can be fatal. It can also cause an explosion if used near lead or copper.
- Paraformaldehyde (32% (vol/vol)) (Electron Microscope Sciences, cat. no. 15714) ! CAUTION Use in a laminar flow hood while wearing gloves and a lab coat; paraformldehyde is toxic, a carcinogen, corrosive, harmful, and an irritant to the skin, eyes, and respiratory tract.
- Heptane (Sigma-Aldrich, cat. no. 246654) ! CAUTION Use in a laminar flow hood while wearing gloves and a lab coat; heptane is flammable and toxic, and exposure can be fatal. It is harmful to the eyes, skin, and respiratory tract.
- Xylene (Alfa Aesar, cat. no. 16371) ! CAUTION Use xylene in a laminar flow hood while wearing gloves and a lab coat; it is flammable, harmful, and an irritant to the eyes, skin, and respiratory tract. Dibutyl phthalate in xylene (DPX; Sigma-Aldrich, cat. no. 06522) ! CAUTION Use DPX in a laminar flow hood while wearing gloves and a lab coat; DPX is flammable, fatal, harmful to the reproductive system, and an irritant to the skin, eyes, organs, and the respiratory tract. Silicone rubber sealant (DAP; Dow Corning, cat. no. 00688). It is a mild irritant to the eyes, skin, and respiratory tract.
- Proteinase K (Sigma-Aldrich, cat. no. P6556) (optional reagent)
- Falcon 60 × 15 mm Petri dish (Corning, cat. no. 351007)
- Saf-instant yeast (Walmart, cat. no. 009214590)
- NaCl (Sigma-Aldrich, cat. no. S9888) (5 M stock solution in ddH_2O)
- KCl (Sigma-Aldrich, cat. no. P9541) (1 M stock solution in ddH_2O)
- CaCl2 (Sigma-Aldrich, cat. no. 793639) (0.5 M stock solution in ddH_2O)
- MgCl2 (Sigma-Aldrich, cat. no. M8266) (1 M stock solution in ddH_2O)
- HEPES (Sigma-Aldrich, cat. no. H9136) (1 M stock solution in ddH_2O)
- Sodium phosphate dibasic (Na2HPO4·2H2O; Sigma-Aldrich, cat. no. 71643)
- Sodium phosphate monobasic (NaH2PO4·2H2O; Sigma-Aldrich, cat. no. 71505)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- BSA (Sigma-Aldrich, cat. no. A7906)
- Glycine (Sigma-Aldrich, cat. no. G7126)
- Normal goat serum (Sigma-Aldrich, cat. no. G9023)
- Normal donkey serum (Sigma-Aldrich, cat. no. D9663)
- Primary and secondary antibodies of choice (see **Table 2** for details of antibodies we have used successfully)

Fly stocks

- *yellow white* (as wild type; Bloomington Drosophila Stock Center, cat. no. 1495)
- MCFO line no. 6: *R57C10-flpL* in su(Hw)attP8; HA_V5_FLAG_OLLAS[1]
- *w*; *elav-gal4*, *w+* on third chromosome[17](Bloomington Drosophila Stock Center, cat. no. 458)
- R15D07, R31G06, R76F05—Bloomington Stock nos. 47467, 47345, and 41305, respectively (ref. 18)

EQUIPMENT

- Rotator or rocker (Fisher Scientific, cat. no. 1404120Q)
- Water bath (Thermo Scientific, cat. no. TSGP05)
- Eppendorf Snap-Cap Microcentrifuge Safe-Lock Tubes: 1.5-ml, 2.0-ml, and 5.0-ml (Fisher Scientific, cat. nos. 05-402-25, 05-402-7, and 14282302)
- Collection baskets (Genesee Scientific, cat. no. 46-101; see Equipment Setup)
- Embryo mesh (Sefar Nytex Nylon, cat. no. 03-100/32)
- Forceps (Fisher Scientific, cat. no. 16-100-113)
- Pyrex spot plates (Fisher Scientific, cat. no. 13-748B)
- Micro-spatula, 20 cm (Fisher Scientific, cat. no. S50821)
- Wash bottles (Fisher, cat. no. 02-897-10)
- Small paintbrush, size 0–1 (local art supply store)
- Aluminum foil (Fisher Scientific, cat. no. S05356A)
- Parafilm (Fisher Scientific, cat. no. S37441)
- Sterile filter units (500-ml; Fisher Scientific, cat. no. 166-0045)
- Plastic transfer pipette (Fisher Scientific, cat. no. 13-711-5AM)
- Poly-L-lysine coverslips, 12-mm round (Fisher Scientific, cat. no. 08774383)
- Fisher premium microscope slides 3 inches × 1 inch (Fisher Scientific, cat. no. 22-178-277)
- Silicone-coated slides (see Equipment Setup)
- Glass spot plates (Fisher Scientific, cat. no. 13-748B)
- Kimwipes (Fisher Scientific, cat. no. 06-666)
- 15-ml conical tubes (Fisher Scientific, cat. no. 12-565-268)
- 50-ml conical tubes (Fisher Scientific, cat. no. 12-565-270)
- 4.0-ml Pyrex disposable glass culture tubes, rimless (Sigma-Aldrich, cat. no. CLS9944510-250EA)
- Sonicator (Branson SFX250, cat. no. 101-063-965R)
- Confocal microscope (Zeiss 710 with 405 nm, 488 nm, 561 nm, and 633 laser lines using 10×, 20×, 40×, or 63× objectives)

REAGENT SETUP

HEPES larval buffer (pH 7.0) HEPES larval buffer (HLB) is 118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 25 mM HEPES. Adjust the pH to 7.0 (with NaOH–HCl) at 95% volume, and then bring it to the final volume with ddH_2O. Filter-sterilize the buffer and store it for up to 6–12 months at 4 °C.

Chitinase–chymotrypsin–DMSO buffer (pH 6.8) Chitinase–chymotrypsin–DMSO buffer (CCD buffer) is 5 U/ml chitinase, 100 U/ml chymotrypsin, and 1–2% (vol/vol) DMSO in HLB. Adjust the pH to 6.8 (with NaOH–HCl) at 95% volume, and then bring it to a final volume with HLB. Prepare aliquots in small tubes (500–1,000 µl) and store them at −20 °C for up to 6–12 months. We have had success refreezing and reusing this solution. ▲ CRITICAL Use units per milliliter (% solution is not accurate—units will vary in each 'lot' of enzyme). ▲ CRITICAL Calcium is required to induce chitinase and chymotrypsin activity, so do not add PBS to this solution at any point during or before the digestion; PBS causes the calcium to salt out. ▲ CRITICAL Temperatures higher than 37 °C can inactivate and/or destroy the enzyme activity.

Fixative 1 solution Fixative 1 solution is 9% (vol/vol) paraformaldehyde in PBS. ▲ CRITICAL: Freshly make the solution for each use

Fixative 2 (MOF) solution Fixative 2 (MOF) solution is 2% (vol/vol) paraformaldehyde in methanol. ▲ CRITICAL: Freshly make the solution for each use

Pro-K 2 mg/ml concentrated stock (pH 7.5) Pro-K 2 mg/ml concentrated stock is 2 mg/ml Pro-K, 20 mM Tris HCl, and 1 mM CaCl2. Adjust the pH to 7.5 (with NaOH–HCl) at 95% volume, and then bring it to the final volume in 50% (vol/vol) glycerol (in ddH2O). This stock can be stored for several years at −20 °C in 1-ml aliquots; avoid freeze–thaw cycles.

Proteinase K 15 µg/ml working solution Dilute 2 mg/ml Pro-K stock in HLB to a working solution of 15 µg/ml. ▲ CRITICAL: Freshly make the solution with each use. ▲ CRITICAL Calcium is required to induce Pro-K activity, so do not add PBS to this solution at any point during or before the digest; PBS causes the calcium to salt out.

PBS (pH 7.2) PBS is 30 mM NaCl, 7 mM Na2HPO4·2H2O, and 3 mM NaH2PO4·2H2O. Adjust the pH to 7.2 (with NaOH–HCl) at 95% volume, and then bring it to the final volume with ddH2O. Filter-sterilize the solution and store it for up to 6 months at room temperature.

Wash buffer (pH 7–7.2) Wash buffer is 1× PBS, 0.1% (vol/vol) Triton X-100, 3% (wt/vol) BSA, and 10mM glycine. Adjust the pH to 7.2 (with NaOH–HCl) at 95% volume, and then bring it to the final volume with 1× PBS. Filter-sterilize the wash buffer and store it for up to 6–12 months at 4 °C.

Blocking buffer (pH 7–7.2) Blocking buffer is 1× PBS, 1.1% (vol/vol) Triton X-100, 3% (wt/vol) BSA, 10 mM glycine, 1% (vol/vol) normal goat serum, 1% (vol/vol) normal donkey serum, 0.3% (vol/vol) Triton X-100, and 2% (vol/vol) DMSO. Adjust the pH to 7.2 (with NaOH–HCl) at 95% volume, and then bring it to a final volume with 1× PBS. Filter-sterilize the buffer and store it for up to 6–12 months at 4 °C.

Primary antibodies Dilute the antibodies to the appropriate concentration (**Table 2**) in sterile PBS and add 0.02% (wt/vol) sodium azide for reuse. Antibodies can be stored for several weeks at 4 °C.

Secondary antibodies Secondary antibodies from Jackson and Invitrogen are all highly cross-absorbed and are used at a 1:300 ratio diluted in wash buffer.

EQUIPMENT SETUP

Collection basket assembly Open a collection basket and place a square of embryo mesh (cut to appropriate size) over the thread end of the tube. Place the ring over the mesh and screw onto the mesh and the tube. Be sure that the mesh is tight and that there are no gaps.

Silicone-coated slides Using a micro-spatula, place a small bead of silicone on a slide. Press hard with the flat edge of the 20-cm micro-spatula and smooth the silicone into a thin layer coating a good portion of the center of the slide. Place the slide into a slide book or slide box to dry for a minimum of 24 h before use. Store the slides at room temperature, protected from dust, for up to 6 months.

! CAUTION Prepare the slides in a laminar flow hood and wear gloves.

PROCEDURE

Washing and bleaching ● TIMING ~20 min

1| Collect the larvae and age them to the developmental stage desired.

2| Transfer the yeast and larvae to a collection basket using a micro-spatula or paintbrush, and a squirt bottle of deionized water.

3| Rinse any yeast in the basket off under running deionized water.

4| Place the basket into a dish of 100% bleach so that the larvae are completely covered. Bleach for 10 min at room temperature, agitating occasionally.

5| During the bleaching step, fill a 2-ml Eppendorf tube with 1.5 ml of CCD buffer (5-ml tube with 4 ml of CCD, for larger specimens).

▲ **CRITICAL STEP** The CCD buffer should be ready before Step 8 so that the larvae do not dry out.

6| Remove the basket from the bleach and rinse the larvae thoroughly for several minutes under running deionized water until no bleach can be detected by smell.

7| Blot dry the basket, disassemble it, and blot dry the bottom of the mesh containing the larvae.

Chitinase–chymotrypsin digestion ● TIMING ~70 min

8| Place the larvae (from Step 7) into the CCD buffer (from Step 5) by dipping the mesh into the buffer and/or by using a small paintbrush, taking care to retrieve any animals from the wall of the collection basket with the wet paintbrush.

9| Seal the tube with Parafilm and incubate it in CCD buffer at 37 °C in a water bath for 1 h.

10| Remove the tube from the water bath and place it in a rack to allow the larvae to settle.

11| Remove as much CCD buffer as possible without losing animals (CCD buffer may be reserved, refrozen, and used again with success at least once).

! CAUTION CCD buffer should be disposed of as 'toxic' waste similar to phenol or chloroform.

▲ **CRITICAL STEP** Do not wash or rinse—move directly to the first fixation phase; some CCD buffer will remain in the tube.

First fixation ● TIMING ~40 min

12| Add 1 ml of 9% (vol/vol) fixative 1 solution (paraformaldehyde) to the 2-ml tube from Step 11 (add 2 ml of 9% (vol/vol) fixative if using a 5-ml tube).

13| Add 1 ml of heptane to the 2-ml tube (add 2 ml of heptane if using a 5-ml tube).

14| Place the tube on a rocker or rotator at room temperature for 25–35 min.

15| Remove the tube from the rotator and place it in a rack to allow the larvae to settle before continuing with the next step.

Cracking ● TIMING ~10 min

16| Once the larvae have settled, remove as much of the upper heptane layer and the lower fixation layer as possible, avoiding the animals; a little fixation solution and/or heptane will remain in the tube.

17| Add 1 ml of fresh heptane to the 2-ml tube (add 2 ml of fresh heptane if using a 5-ml tube).
▲ **CRITICAL STEP** Heptane must be added first—otherwise, 'cracking' will not occur.
▲ **CRITICAL STEP** Water-free heptane is important for efficient cracking; do not reuse the fixative heptane. Prepare aliquots from your stock bottle, and use a fresh aliquot each week.

18| Add 800 ml of methanol to the 2-ml tube—one sample at a time (add 1,800 ml of 100% methanol if you are using a 5-ml tube).

19| Immediately close the tube and strike hard and vigorously on a solid tabletop for 1–2 min. Allow the larvae to settle for 1–2 min, repeat the striking, allow the larvae to settle, and repeat the striking (a total of three rounds of striking).
▲ **CRITICAL STEP** Methanol must always be added after heptane; otherwise, the 'cracking' will not occur.
▲ **CRITICAL STEP** Water-free methanol is important for efficient cracking; prepare aliquots from your stock bottle, and use a fresh aliquot each week.
▲ **CRITICAL STEP** Add the methanol just before striking on the table, and do not allow the sample to sit in methanol without striking; otherwise, 'cracking' will not occur.
? TROUBLESHOOTING

20| After allowing the larvae to settle, remove as much supernatant liquid as possible, avoiding all settled larvae. Remove all floating animals, which are not properly fixed/cracked. Some liquid will remain in the tube with the settled larvae; leave it and do not wash or rinse.

Second fixation (MOF) ● TIMING 3–7 d

21| Add MOF solution to the sample tube from Step 20 until it is 90% full.

22| Add a 10% volume of heptane, cap the tube, seal it with Parafilm, and place it on its side at –20 °C for a minimum of 3 d (7 d for largest larvae).
▲ **CRITICAL STEP** The optimal duration of the second fixation will need to be determined for each antibody and for each larval stage; generally, the older and/or larger the larvae, the longer they should stay in MOF solution.
■ **PAUSE POINT** Once the second fixation incubation period is complete, it is possible to replace MOF solution with 100% methanol and store the larvae at –20 °C for weeks/months. However, we have not fully tested the effects of long-term methanol storage on the outcome of experiments.

Removal of the second fixation (MOF) solution ● TIMING ~20 min

23| Decant or use a cut transfer pipette to transfer the entire sample to a large-volume tube such as a 15-ml conical tube, 50-ml conical tube, or a small beaker.

24| Flood the sample with 100% methanol.

25| Allow all animals to settle to the bottom of the tube or beaker.

26| Once settled, transfer the settled animals back to a 1.5-ml Eppendorf tube (or appropriate size for your sample) using a cut transfer pipette.

27| Wash the sample twice by filling the tube with 100% methanol, allowing the larvae to settle, and then removing as much methanol as possible without losing larvae.

28| Wash the sample by filling the tube with 100% (vol/vol) ethanol and allowing the larvae to settle.

29| Remove as much ethanol as possible without losing the sample. If Pro-K treatment is required (e.g., to get better staining of deep structures), proceed to Step 30. Otherwise, proceed directly to Step 35 for blocking.

(Optional) Pro-K treatment ● TIMING ~40 min

30| Fill the tube from Step 29 with Pro-K solution.

31| Slowly and gently rock the tube for 30 min at room temperature.

32| Remove the tube from the rocker and allow the sample to settle for 1–2 min.

33| Remove the Pro-K solution without losing larvae.

34| Rinse twice by adding HLB, allowing the larvae to settle, and then removing as much HLB as possible without losing larvae.

Blocking ● TIMING ~12–24 h

35| Fill the tube (from Step 29 or 34) with blocking buffer and place it on its side at 4 °C overnight.

▲ **CRITICAL STEP** Do not rock, as rocking can decrease the binding efficiency of blocking proteins to nonspecific binding sites.

■ **PAUSE POINT** Blocking can be done for a duration ranging from overnight to 36 h at 4 °C.

36| If sonication is required to get better staining of deep structures, proceed to Step 37; if not, proceed directly to Step 41 for primary antibody incubation.

(Optional) Sonication ● TIMING ~20 min

37| Remove the old block solution, and add 250 µl of ice-cold fresh block solution to a 1.5-ml tube containing an ~50-µl volume of larvae; close and place the tube on ice to chill for 5 min, leaving it on ice at all times until Step 39.

38| Give a 3-second pulse with the tip sonicator set on 100% duty cycle—its lowest output setting.

39| Repeat the pulse three to four times (three times for L1 and early L2 larvae, four times for late L2 and L3 larvae), putting the tube back on ice to rest for 1 min between pulses.

40| Fill the tube with blocking buffer and proceed to Step 41.

Primary antibody incubation ● TIMING 3–7 d

41| Dilute the required primary antibodies in PBS (**Table 2**).

▲ **CRITICAL STEP** If using prelabeled primaries for MCFO work, avoid direct light to limit fluorophore bleaching.

42| Remove the blocking buffer from the sample from Step 35 or 40; no rinsing or washing is required.

43| Apply 500 ml of primary antibody solution (scale up as needed for larger samples) and briefly invert to mix.

44| Place the tube on its side at 4 °C for 3–7 d to incubate, depending on the size of the animals; note that some antibodies may require longer incubations for optimal staining, regardless of larval size.

▲ **CRITICAL STEP** Do not rock, as rocking can decrease antibody binding efficiency and remove bound blocking proteins.

Washing ● TIMING 2–4 d

45| Remove primary antibody, and reserve for reuse if desired. Many antibodies improve with reuse; add 0.02% (wt/vol) sodium azide to preserve the mix.

46| Gently fill the tube with wash buffer, and briefly invert to mix.

▲ **CRITICAL STEP** Do not add the wash buffer directly to the larvae; add it to the sidewall of the tube to avoid agitating the larvae too roughly.

47| Allow the larvae to settle, and then remove the solution (without losing larvae).

48| Repeat Steps 45 and 46 twice more (three washes in total).

49| Fill the tube a fourth and final time with wash buffer and place on its side at 4 °C for 2–4 d; do not rock the tube.
▲ **CRITICAL STEP** Do not rock the tube, or the bound antibody may be dislodged if the binding affinity is weak.
▲ **CRITICAL STEP** This wash between the primary and secondary incubations decreases the background staining—the larger the size of the larvae, the longer this wash should be.
▲ **CRITICAL STEP** If using primary antibodies directly conjugated to a fluorophore (e.g., for the MCFO stains), there is no need for a secondary antibody, and you should proceed directly to Step 54.

Secondary antibody incubation ● TIMING 6–8 h
50| Dilute the secondary antibodies of choice in wash buffer.

51| Place the tube from Step 49 upright and, after the larvae settle, remove the wash buffer.

52| Apply 500 µl of secondary antibody solution (scale up as needed for larger samples) and briefly invert to mix.

53| Lay the tube on its side and incubate for 6–8 h at room temperature without rocking, protected from light.
▲ **CRITICAL STEP** Do not rock the tube, or the antibody may be dislodged if the binding affinity is weak.

Washing ● TIMING 12–18 h
54| Place tube in a rack and allow the sample to settle; then remove and discard the antibody solution.

55| Gently fill the tube with wash buffer and invert to mix.

56| Allow the larvae to settle, and then remove the wash buffer.

57| Gently fill the tube with wash buffer and lay it on its side at 4 °C overnight—do not rock the tube.

DPX mounting ● TIMING ~3 h
58| Remove the wash buffer after allowing the sample to settle.

59| Fill the tube with 30% (vol/vol) ethanol, lay it on its side, and incubate it for 10 min, protected from light.

60| Place the tube upright, allow the sample to settle, and remove the solution.

61| Fill the tube with 50% (vol/vol) ethanol, lay it on its side, and incubate for 10 min, protected from light.

62| Fill the tube with 50% (vol/vol) ethanol, lay it on its side, and incubate for 10 min, protected from light.

63| Place the tube upright, allow the sample to settle, and remove the solution.

64| Fill the tube with 70% (vol/vol) ethanol, lay it on its side, and incubate for 10 min, protected from light.

65| Place the tube upright, allow the sample to settle, and remove the solution.

66| Fill the tube with 90% (vol/vol) ethanol, lay it on its side, and incubate for 10 min, protected from light.

67| Place the tube upright, allow the sample to settle, and remove the solution.

68| Fill the tube with 100% (vol/vol) ethanol (first of two rinses), lay it on its side, and incubate for 10 min, protected from light.

69| Place the tube upright, allow the sample to settle, and remove the solution.

70| Fill the tube with 100% (vol/vol) ethanol (second of two rinses), lay it on its side, and incubate for 10 min, protected from light.

71| Place the tube upright, allow the sample to settle in the tube, and move it into the laminar flow hood area to work.
▲ **CRITICAL STEP** All subsequent steps should be done in a laminar flow hood using double gloves.

72| Using a cut transfer pipette, transfer the ethanol and larvae to a 4-ml glass culture tube.
▲ **CRITICAL STEP** Xylene will dissolve the plastic Eppendorf tubes and turn the solution to milky white clumps, and thus glass tubes must be used for all remaining steps.

73| Fill the tube with 2.0 ml of xylene (first of two rinses) and incubate for 15 min, protected from light. Remove the xylene.
▲ **CRITICAL STEP** A portion of xylene should be poured out into a 'working aliquot' jar to avoid absorbing water in your main stock bottle due to the hygroscopic nature of xylene.

74| Fill the tube with 2.0 ml of xylene (second of two rinses) and incubate for 15 min, protected from light; then continue with the next step, leaving the xylene in the tube.

75| Place a silicone-coated slide onto a dark surface for ease of sample viewing.
▲ **CRITICAL STEP** The sample will be extremely fragile, brittle, and subject to rapid, irreversible dehydration at this point; keep it in the xylene until the slide is out and the DPX is ready to be used.

76| Open the DPX container and using a transfer pipette, fill the pipette with DPX, avoiding bubbles, and let it remain in the bottle so that it is ready.

77| Cut the tip off of a plastic transfer pipette so that it has a large enough opening for the sample to pass through without damage.
▲ **CRITICAL STEP** DPX will dry rapidly, so prepare only one slide at a time.

78| Using the plastic transfer pipette, suck up some of the sample, along with some xylene, and carefully move it over the silicone-coated slide, allowing the larvae to settle into the end of the transfer pipette tip. Gently extrude the larvae onto the slide and set aside the transfer pipette.
▲ **CRITICAL STEP** Do not put too many animals on one slide; use multiple slides for one sample if there are many animals. Work in small batches and add more xylene to the sample tube if needed.

79| Take a piece of ripped Kimwipe and wick off some of the excess xylene along the edge of the sample without touching it.
▲ **CRITICAL STEP** Wick off excess xylene but do not let the larvae dry out completely, or they will be unusable.

80| Scrape the excess DPX off the transfer pipette before removing it from the bottle. Using the transfer pipette, place two drops of DPX on top of the sample on the slide.

81| If necessary, use a clean toothpick to gently separate animals and disperse them into the drop of DPX.
▲ **CRITICAL STEP** Do not expose the larvae for more than 5–10 s without xylene or DPX, or the sample will be ruined.
▲ **CRITICAL STEP** DPX will dry quickly, so work carefully, efficiently, and quickly.

82| Using a pair of small forceps, take a round 22-mm diameter coverslip and place it on top of the drop of DPX.

83| Protect the mounted sample from light and allow it to dry in the laminar flow hood for a minimum of 24 h before attempting to image it on a microscope—DPX fumes are toxic until DPX is fully dried.
▲ **CRITICAL STEP** Do not press down on the coverslip; the DPX will naturally fill the area under the coverslip—there should be a bit of excess around the edges.
▲ **CRITICAL STEP** Use a slight excess of DPX so that it comes out the edges of the coverslip to allow for shrinkage during drying.
▲ **CRITICAL STEP** Do not use coverslips larger than 22 × 22 mm, as DPX will not harden in the center.

Imaging ● TIMING variable
84| Acquire images using a suitable microscope and objectives. We use a Zeiss 710 confocal microscope with 405 nm, 488 nm, 561 nm, and 633 laser lines using 10×, 20×, 40×, or 63× objectives.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reasons	Possible solution
19	Larvae fail to settle to the bottom of the tube, indicating that the cracking step has failed	Addition of methanol before heptane in Steps 17 and 18	Always add heptane first at Steps 17 and 18
		Waiting too long after addition of methanol to heptane before tapping on benchtop at Step 19	Tap immediately following the addition of methanol to heptane at Step 19
		Heptane and/or methanol at Steps 17 and 18 have taken on too much water and are failing to create the proper hydrophobic–hydrophilic interaction needed to 'crack' the sample	Always have a capped sealed stock bottle of both methanol and heptane that you remove a fresh aliquot from each week. Replace stock bottles of both at six-month intervals
84	High background staining	Nonspecific binding of antibodies	Preadsorb antibody to larvae overnight at 4 °C, and/or reuse the antibody for multiple stainings
		Low signal-to-noise ratio, giving high background	Not blocking long enough and/or using agitation/rocking during blocking or any step from 35 onward
		Rinse duration too short at Step 49	Extend rinse time between primary and secondary antibody incubation (Step 49) up to 7 d
	Weak staining	Failure of cracking step (Step 19)	See solutions to problems at Step 19, above
		Rocking, rotation, or some form of agitation during blocking or primary antibody or secondary antibody incubation (Steps 35–83)	Lay tube on its side without rocking for the blocking step and for primary and secondary antibody incubation
		Excessive or aggressive application of solutions (Steps 35–83)	Gently apply all solutions, aiming at the sidewalls of the sample tube, from Step 35 through Step 83
	Poor staining with a new antibody	Failure to optimize conditions for that particular antibody	Optimize conditions by changing first and/or second fixation concentration, first and/or second fixation duration, primary and/or secondary antibody concentration, primary and/or secondary antibody incubation duration, or wash duration.
	White/solid samples that look 'crystalline'; light does not penetrate	Sample dehydrated during Steps 74–80 (during xylene washes or mounting)	Keep the sample moist with xylene at all times, only wicking off the excess at Step 79, allowing the sample to remain wet with some xylene. Xylene evaporates very rapidly—use caution, and work one sample at a time

● TIMING

Steps 1–7, washing and bleaching: ~20 min
Steps 8–11, chitinase–chymotrypsin digestion: ~70 min
Steps 12–15, first fixation: ~40 min
Steps 16–20, cracking: ~10 min
Steps 21 and 22, second fixation: 3–7 d
Steps 23–29, removal of the second fixation solution: ~20 min
Steps 30–34, (optional) Pro-K treatment: ~40 min
Steps 35 and 36, blocking: ~12–24 h
Steps 37–40, (optional) sonication: ~20 min
Steps 41–44, primary antibody incubation: 3–7 d
Steps 45–49, removal of the primary antibody solution: 2–4 d
Steps 50–53, secondary antibody incubation: 6–8 h
Steps 54–57, removal of the secondary antibody solution: 12–18 h
Steps 58–83, DPX mounting: ~3 h
Step 84: imaging: variable

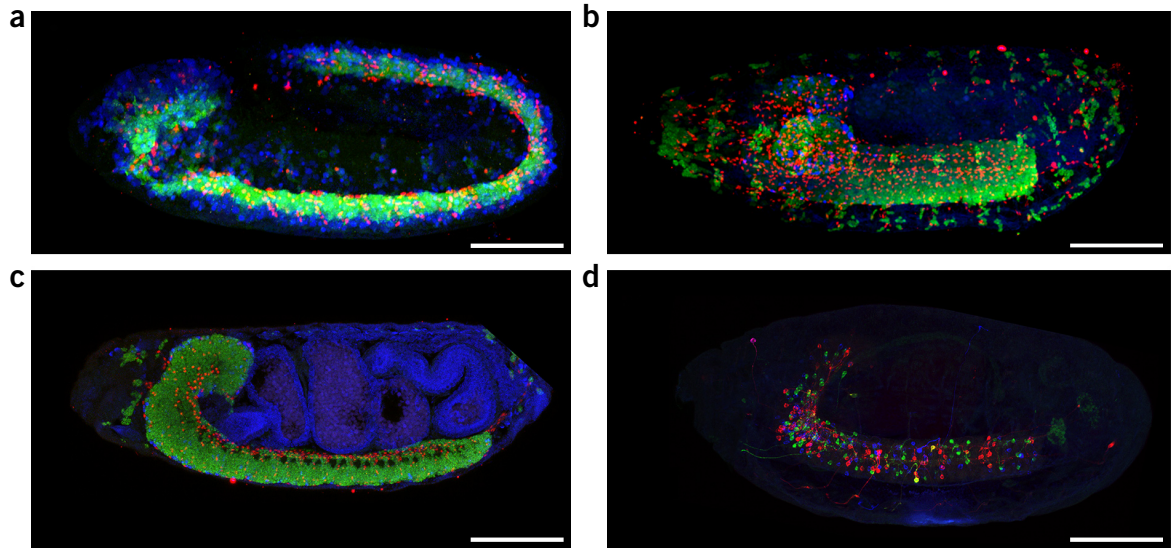

Figure 6 | Staining of embryos. (**a**) Stage 11 embryo stained for the nuclear proteins Dpn (blue), Elav (green), and Repo (red). (**b**,**c**) Stage 16 embryos stained for the nuclear proteins Dpn (blue), Elav (green), and Repo (red). (**b**) Oblique view; (**c**) lateral view. (**d**) Stage 17 embryo stained for the multicolor flip-out antigens that decorate neuronal membranes of a subset of Elav-gal4-57C10-MCFO neurons. Scale bars, 50 µm. Additional details regarding antibodies used can be found in **Table 2**.

ANTICIPATED RESULTS

On successful completion of this protocol (using appropriate antibodies), it should be possible to visualize nuclear or transmembrane antigens in all cells—deep or superficial—and in all larval stages (L0–L3) (**Figs. 2**–**6**). This can be illustrated by MCFO antigen staining of neurons in deep CNS tissue in all larval stages: L0 (**Fig. 2c**,**e**), L1 (**Fig. 3a**,**b**), L2 (**Fig. 4a**,**b**), and L3 (**Fig. 5c**). In addition, we can trace neurons from peripheral locations to central targets (e.g., sensory dendrites to the ventral nerve cord; **Figs. 2g**, **3a**, **4a**, or **6d**). Of course, the goals of each experiment are unique, and they may benefit from modifications to the protocol, such as changing the length of primary antibody incubation or using optional treatments such

TABLE 4 | Tested modifications and ideas for future improvements.

Step	Treatment	Outcome
Worked but at a cost		
12	4% (vol/vol) paraformaldehyde fixation solution plus 0.25% ZnCl for 30 min at room temperature	Excellent staining; lower percentage yield of stained animals
21	4% (vol/vol) paraformaldehyde MOF step	Better penetration, higher percentage yield of stained animals, decreased intensity of staining
22	MOF step without heptane	Lower percentage yield of stained animals
30	Pro-K digestion after MOF	Excellent deep tissue staining, but loss of peripheral staining (epidermis, sensory neurons)
37	Sonication after MOF	Higher percentage yield of stained animals and quality of nuclear antigens, but disruption of tissues
Did not work		
8	CCD without 1–2% (vol/vol) DMSO	Poor antibody penetration
8	Chitinase ≤ 2 units per ml; chymotrypsin ≤ 10 units per ml	Poor antibody penetration
8 and 12	CCD digestion after first fixation	Total failure to stain
8, 12, and 16	*C. elegans* freeze/crack method	Total failure to stain

(continued)

TABLE 4 | Tested modifications and ideas for future improvements (continued).

Step	Treatment	Outcome
9	CCD incubation at room temperature	Poor antibody penetration
12	Addition of 0.1% (vol/vol) glutaraldehyde to first fixation solution	Total failure to stain
21	MOF with 2% formaldehyde, 50% MeOH in PBS	Total failure to stain
23	1,000 units per ml collagenase treatment after MOF	Total failure to stain
Not tried yet		
8	Addition of 7 M urea to CCD	Possibly better antibody penetration
8	Addition of 1% SDS to CCD	Possibly better antibody penetration
12	First fixation with >9% formaldehyde	Possibly better antigen preservation
12	First fixation with 1% paraformaldehyde + 0.25% ZnFa fixation for 2–20 h at room temperature	Possibly better antigen preservation
14	Initial fixation longer than 20 min	Possibly better antigen preservation
19	Follow cracking with a second CCD digestion	Possibly better antibody penetration
22	Agitation during –20 °C MOF step	Possibly better antibody penetration
All	1% (vol/vol) DMSO instead of Triton X-100 in all solutions	Possibly better antibody penetration

as Pro-K; ideas for modifying the protocol are given in **Table 4**. For example, detection of nuclear antigens in the large L3 CNS may benefit from the optional Pro-K treatment, which improves deep-tissue staining; in this, case the loss of superficial tissue integrity may not matter.

ACKNOWLEDGMENTS We thank E.S. Heckscher for her inspiration to begin this work, and B. Bowerman, K. Sugioka, and C.-H. Chuang for their help on the *C. elegans* protocols. This work was supported by the Howard Hughes Medical Institute, where C.Q.D. is an investigator.

AUTHOR CONTRIBUTIONS L.M. conceived and performed all experiments, collected all data, and wrote the first draft of the paper. C.Q.D. assembled the figures and provided comments on the text.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

1. Nern, A., Pfeiffer, B.D. & Rubin, G.M. Optimized tools for multicolor stochastic labeling reveal diverse stereotyped cell arrangements in the fly visual system. *Proc. Natl. Acad. Sci. USA* **112**, E2967–E2976 (2015).
2. Chung, K. & Deisseroth, K. CLARITY for mapping the nervous system. *Nat. Methods* **10**, 508–513 (2013).
3. Mitchison, T.J. & Sedat, J. Localization of antigenic determinants in whole *Drosophila* embryos. *Dev. Biol.* **99**, 261–264 (1983).
4. Doe, C.Q. Neural stem cells: balancing self-renewal with differentiation. *Development* **135**, 1575–1587 (2008).
5. Homem, C.C., Repic, M. & Knoblich, J.A. Proliferation control in neural stem and progenitor cells. *Nat. Rev. Neurosci.* **16**, 647–659 (2015).
6. Kohwi, M. & Doe, C.Q. Temporal fate specification and neural progenitor competence during development. *Nat. Rev. Neurosci.* **14**, 823–838 (2013).
7. Li, X., Chen, Z. & Desplan, C. Temporal patterning of neural progenitors in *Drosophila*. *Curr. Top. Dev. Biol.* **105**, 69–96 (2013).
8. Parton, R.M., Valles, A.M., Dobbie, I.M. & Davis, I. *Drosophila* larval fillet preparation and imaging of neurons. *Cold Spring Harb. Protoc.* **2010** (2010).
9. Clark, M.Q., McCumsey, S.J., Lopez-Darwin, S., Heckscher, E.S. & Doe, C.Q. Functional genetic screen to identify interneurons governing behaviorally distinct aspects of *Drosophila* larval motor programs. *G3 (Bethesda)* **6**, 2023–2031 (2016).
10. Patel, N.H. Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. *Methods Cell Biol.* **44**, 445–487 (1994).
11. Arata, Y., Lee, J.Y., Goldstein, B. & Sawa, H. Extracellular control of PAR protein localization during asymmetric cell division in the *C. elegans* embryo. *Development* **137**, 3337–3345 (2010).
12. Copf, T., Rabet, N., Celniker, S.E. & Averof, M. Posterior patterning genes and the identification of a unique body region in the brine shrimp *Artemia franciscana*. *Development* **130**, 5915–5927 (2003).
13. Freeman, J.A., Cheshire, L.B. & MacRae, T.H. Epithelial morphogenesis in developing *Artemia*: the role of cell replication, cell shape change, and the cytoskeleton. *Dev. Biol.* **152**, 279–292 (1992).
14. Fidler, A., Bouley, L. & Wawersik, M. Sonication-facilitated immunofluorescence staining of late-stage embryonic and larval *Drosophila* tissue *in situ*. *J. Vis. Exp.* **90**, e51528 (2014).
15. Duerr, J.S. Immunohistochemistry. *WormBook* **19**, 1–61 (2006).
16. Ott, S.R. Confocal microscopy in large insect brains: zinc-formaldehyde fixation improves synapsin immunostaining and preservation of morphology in whole-mounts. *J. Neurosci. Methods* **172**, 220–230 (2008).
17. Horiuchi, D. *et al.* Control of a kinesin-cargo linkage mechanism by JNK pathway kinases. *Curr. Biol.* **17**, 1313–1317 (2007).
18. Pfeiffer, B.D. *et al.* Tools for neuroanatomy and neurogenetics in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **105**, 9715–9720 (2008).