In vitro imaging of primary neural cell culture from *Drosophila*

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Cell culture systems are widely used for molecular, genetic and biochemical studies. Primary cell cultures of animal tissues offer the advantage that specific cell types can be studied *in vitro* outside of their normal environment. We provide a detailed protocol for generating primary neural cell cultures derived from larval brains of *Drosophila melanogaster*. The developing larval brain contains stem cells such as neural precursors and intermediate neural progenitors, as well as fully differentiated and functional neurons and glia cells. We describe how to analyze these cell types *in vitro* by immunofluorescent staining and scanning confocal microscopy. Cell type–specific fluorescent reporter lines and genetically encoded calcium sensors allow the monitoring of developmental, cellular processes and neuronal activity in living cells *in vitro*. The protocol provides a basis for functional studies of wild-type or genetically manipulated primary neural cells in culture, both in fixed and living samples. The entire procedure takes ~3 weeks.

INTRODUCTION

The nervous system of the fruit fly *Drosophila melanogaster* provides an excellent model for studying the formation of neuronal and glial diversity. As *Drosophila* is primarily used as an *in vivo* model system, experimental research using *Drosophila* cell cultures is surprisingly limited. This becomes particularly evident in the context of developmental and cell biological studies of the CNS. Two distinct nervous systems are generated during the biphasic life cycle of *Drosophila*[1]. A first wave of neurogenesis forms the larval nervous system during embryonic development, whereas during larval stages a second wave of neurogenesis builds the CNS of the adult fly. Hence, the larval CNS consists of terminally differentiated neurons and glial cells that function during larval life. At the same time, it harbors precursor cells such as neuroepithelial cells; neuroblasts; intermediate precursors with a restricted mitotic potential, termed ganglion mother cells; and immature neurons and glia cells of the presumptive adult nervous system. Thus, the larval CNS allows investigations of various types of proliferative, immature and terminally differentiated neural cells.

Although a number of different cell culture lines are currently used for *in vitro* studies, the origins of most of these cell lines are not well characterized. Most widely used stable cell lines, such as S2 cells or Kc cells[2–4], provide outstanding experimental models for biochemical analyses, RNAi screens and small compound screens[5–7]. However, similarly to human cell lines, these widely used *Drosophila* cell lines often have genomic abnormalities, and thus a distinct cellular identity cannot be assigned[8]. Some progress has been made in establishing continuous embryonic cell cultures that can be propagated over several passages[9,10]. For developmental studies that focus on a particular organ system, such as the CNS, well-defined cell lines are currently not available. Particularly, for genetic manipulations of undifferentiated cells, it is crucial to know the genotype and cellular origin of a cell line. Abnormalities in karyotypes or acquired mutations in continuous cell lines may cause problems in interpreting experimental results.

In mammalian animal models, primary somatic cell cultures have a long-standing tradition and provide an important complementary approach to *in vivo* studies. The availability of well-defined and characterized culture media and sophisticated culturing techniques led to the generation of primary cell cultures derived from various tissues and with various genotypes. Similarly, in *Drosophila*, the use of primary cell cultures originating from embryonic and from larval tissue has a long-standing tradition, and these cell cultures have been used for more than 40 years (refs. 3,4,11,12). Temporal specification, cell division and neuronal differentiation have been studied in *Drosophila* primary neural cultures deriving from various developmental stages by either fixed- or live-cell imaging[13–18]. Furthermore, primary neural cells have been used to assess neuronal activity by electrophysiological recordings or calcium imaging techniques[19–22]. Yet another study examined the level of reactive oxygen species in *Drosophila* neuronal cells in culture[23].

Although to date in *Drosophila* most primary cell cultures are of embryonic origin, there is wide interest in using primary cell cultures derived from particular organs and tissues with identified genotypes for genetic or biochemical studies and for high-throughput screens using RNAi knockdown. An advantage of the presented system is that it allows the generation of primary neural cell cultures derived from any available genotype, and in principle it can be adapted to other organ systems.

Experimental design

We describe a protocol that provides a basis for culturing neural cells of the postembryonic *Drosophila* brain. Previous studies investigated the cellular behavior of neural cells deriving from dissociated larval brains, but primary cultures were not observed over a longer time period[24,25]. Our protocol allows the analysis of cellular development over the course of at least 2 weeks. The use of an array of cell type–specific molecular markers allows the assessment of cellular identity by immunofluorescent staining and confocal microscopy. Molecular markers are used to reveal the identity of neuroblasts, ganglion mother cells, postmitotic neurons and glia cells. Cell type–specific fluorescent reporter lines allow the

identification and characterization of specific neuronal cell lineages in fixed tissue and by live imaging. A major aim in the field of neural development is to understand and characterize the genetic and molecular mechanisms underlying the regulation of neural stem cell proliferation and differentiation. It is difficult to address this question with stable cell lines, which have genetic alterations that cause them to proliferate indefinitely. Primary cell cultures containing genetically identifiable neural stem cells offer an entry point to study the cellular mechanisms of neural stem cell behavior and neuronal differentiation[26,27].

Comparable to studies on developmental mechanisms in nervous system formation, the functionality of fully differentiated neurons can also be assessed *in vitro*. The use of genetically encoded calcium sensors such as GCaMP allows the functional assessment of identifiable neurons *in vivo* and *in vitro*[21,28]. The protocol described here can, for instance, be applied to assess the neuronal activity in response to exposure to a given neurotransmitter. Thus, the protocol described here further allows combining the wealth of genetic techniques available in *Drosophila in vivo* with subsequent assessment *in vitro*.

MATERIALS

REAGENTS

- Schneider's medium (Gibco, cat. no. 21720-024)
- FBS (HyClone, cat. no. SH30070)
- Penicillin-streptomycin (pen-strep) (Gibco, cat. no. 15070)
- Benzalkonium chloride (microbicidal; Fluka, cat. no. 12060)
  ! CAUTION Benzalkonium chloride is harmful on contact with skin and when swallowed. It causes burns and should be handled using eye and skin protection. Use a fume hood to make the stock solution. It is very toxic for aquatic life and should be disposed of as a hazardous chemical.
- Methyl 4-hydroxybenzoate (antifungal) (Acros Organics, cat. no. 126960025)
- Cornmeal (from any supermarket)
- Sugar (from any supermarket)
- Agar (Difco Laboratories, cat. no. 0812-17-9)
- Sucrose (Difco Laboratories, cat. no. 0229-17-6)
- Glucose (Sigma-Aldrich, cat. no. G5400)
- Apple juice (from any supermarket)
- Collagenase I (Sigma-Aldrich, cat. no. C9722)
- PBS (Gibco, cat. no. 18912-014)
- Formaldehyde, 36.5% (vol/vol) (Sigma-Aldrich, cat. no. 33220)
  ! CAUTION Formaldehyde is a potential carcinogen and should be handled using eye and skin protection. Use a fume hood to make the fixation solution. It should be disposed of as a hazardous chemical.
- NaCl (Sigma Aldrich, cat. no. 71380)
- KCl (Merck, cat. no. 104936)
- $NaH_2PO_4 \cdot H_2O$ (Merck, cat. no. 106346)
- $NaHCO_3$ (Merck, cat. no. 106329)
- HCl (Merck, cat. no. 100317) **! CAUTION** HCl is very hazardous in case of skin or eye contact and ingestion. Handle it only when using skin and eye protection and working in a fume hood.
- BrdU (Fluka, cat. no. 16880) **! CAUTION** BrdU is a carcinogen. Only handle it when using skin and eye protection and working in a fume hood. BrdU should be disposed of as a hazardous chemical.
- Triton X-100 (Fluka, cat. no. 94318)
- Concanavalin A (Sigma-Aldrich, cat. no. C5275)
- Commercially available primary antibodies (**Table 1**)
- Alexa Fluor–conjugated secondary antibodies (Molecular Probes)
- Vectashield mounting medium (Vector Labs, cat. no. H-1000)
- Autolyzed bakers' yeast (Difco Laboratories, cat. no. 0229-17-6)
- Bakers' yeast (from any supermarket)
- Clear nail polish (from any supermarket)
- Ethanol (Honeywell, cat. no. 10311036)
- Acetylcholine iodide (Sigma-Aldrich, cat. no. A7000)
- *Drosophila* stocks of desired genotypes (**Table 2**)

EQUIPMENT

- Siliconized 1.5-ml centrifuge tubes (Fisher Scientific, cat. no. 02-681-320)
  ▲ CRITICAL Use siliconized microcentrifuge tubes to prevent cells from sticking to the plastic walls.
- Siliconized 200-µl pipette tips (VWR International, cat. no. 53503-790)
  ▲ CRITICAL Use siliconized-tip tubes to prevent cells from sticking to the plastic walls.
- Pipette tips, 1,000 µl (Treff, cat. no. 96.01702.6.01)

TABLE 1 | Commercially available antibodies for labeling primary neural cells in culture.

Antibody	Cell type	Dilution	Source/reference	Cat. no.
Mouse anti-Prospero	Ganglion mother cells/immature neurons/glial cell subset	1:10	DSHB	MR1A
Rat anti-Elav	Neurons	1:30	DSHB	7E8A10
Mouse anti-Repo	Glial cells	1:10	DSHB	8D12
Mouse anti-Neuroglian	Motor neurons/sensory neurons	1:30	DSHB	BP104
Mouse anti-Fasciclin II	Motor neurons/sensory neurons/pioneer neurons	1:30	DSHB	1D4
Rabbit anti-phosphorylated histone 3	Mitotic cells	1:1,000	Upstate Biotechnology	06-570
Rabbit anti-cleaved Caspase 3	Apoptotic cells	1:300	Cell Signaling Technology	9661
Mouse anti-BrdU	Replicating cells	1:300	Abcam	ab1893
Rabbit anti-GFP	GFP-expressing cells	1:1,000	Molecular Probes	A6455

TABLE 2 | Useful *Drosophila* fly lines lines to visualize primary neural cells in culture.

Drosophila lines	Visualized cells	Source	Stock no.	Ref. no.
GH146-Gal4	Subset of projection neurons of antennal lobe	Bloomington	BL 30026	30
pdf-Gal4	Main pacemaker neurons of the clock circuit	Bloomington	BL 6899	31
MB247-Gal4	Kenyon cells of the mushroom body (insert in *myocyte enhancer factor 2* locus)	Bloomington		32
elav-Gal4	Postmitotic neurons	Bloomington	BL 8765	
c855a-Gal4	Progenitor cells of the optic lobe	Bloomington	BL 6990	33
Mz1407-Gal4	Neuroblast lineages (insert in *inscuteable* locus)			34
pcna-GFP	Replicating cells	Bloomington	BL 25749	35
UAS-mCD8-GFP	UAS responder line to visualize cell morphology (membrane-tethered GFP)	Bloomington	BL 5137	
UAS-Histone2B-mRFP1	UAS responder line to visualize nucleus (histone2B-RFP fusion)			36
UAS-GCaMP5	UAS responder line to visualize enhanced calcium levels	Janelia Farm		37

- Pipette tips, 20 µl (Treff, cat. no. 96.01701.4.02)
- Sterile pipettes, 2 ml (Axygen Scientific, cat. no. SER-2ML-SI)
- Sterile pipettes, 10 ml (BD Falcon, cat. no. 357551)
- Petri dishes, 46 × 16 mm (Semadeni, cat. no. 1708)
- Petri dishes, 60 × 15 mm (Greiner Bio-One, cat. no. 628103)
- Petri dishes, 94 × 16 mm (Greiner Bio-One, cat. no. 633180)
- Cell strainer, 40 µm (BD Falcon, cat. no. 352340)
- Dispenser (Eppendorf, cat. no. 022230204)
- Filter paper
- Cell culture microplate with lid, 96 wells, flat-bottom wells, Nunclon delta cell culture–treated clear polystyrene, sterile (Nunc, cat. no. 161093)
- Sterile humid chamber (OKT Germany)
- Fine forceps (Dumont 5; Fine Science Tools, cat. no. 11254-20)
- Teflon microscopy slide, eight wells (Menzel-Glaser, cat. no. X2XER201B#MNZ)
- Microscope cover glasses, 22 × 50 mm (Menzel-Glaser, cat. no. BB022050A1)
- Glass-bottom dishes, 35 mm, poly-D-lysine coated (Mat Tek, cat. no. P35GC-1.0-14-C)
- Silicone spreader (any supermarket, baking department)
- Laminar flow cabinet (e.g., Gelaire TC-48)
- Heating plate (e.g., Heidolph MR2002)
- Tabletop centrifuge (e.g., Eppendorf 5424)
- Thermostable cabinet (e.g., Liebherr)
- Binocular microscope for larval brain dissection (e.g., Leica)
- Scanning confocal microscope (e.g., Leica SP5)
- Inverted fluorescent microscope (e.g., DeltaVision, Applied Precision)
- Inverted fluorescent microscope (e.g., Leica CTR 700 HS)

REAGENT SETUP

Heat-inactivating FBS Inactivate FBS by heating it for 30 min at 56 °C according to the manufacturer's instructions. Subsequently, aliquots can be frozen at −20 °C and stored for several months.

Supplemented Schneider's medium Supplement Schneider's medium with 10% (vol/vol) FBS and 1% (vol/vol) pen-strep. Always freshly prepare the medium.

Rinaldini solution with 1% (vol/vol) pen-strep Mix 8 mg ml^{-1} NaCl, 0.2 mg ml^{-1} KCl, 0.05 mg ml^{-1} NaH_2PO_4 H_2O, 1 mg ml^{-1} $NaHCO_3$, 1 mg ml^{-1} of glucose and 1% (vol/vol) pen-strep solution in ddH_2O. The solution can be frozen and stored at −20 °C for several months.

Collagenase I, 0.5 mg ml^{-1}, in Rinaldini solution For 1 ml, add 50 µl of collagenase I stock solution (10 mg ml^{-1} of ddH_2O) to 950 µl of Rinaldini solution. The solution can be frozen and stored at −20 °C for several months.

Egg-collection plates Prepare a mixture of 1,500 ml of water, 70 g of agar and 25 g of sucrose. Autoclave the mixture at 121 °C for 50 min. Add 500 ml of apple juice and mix the contents. When the mixture cools down to 60 °C, add 10 ml of the benzalkonium chloride/methyl 4-hydroxybenzoate stock solution (stock solution: 10 mg ml^{-1} benzalkonium chloride and

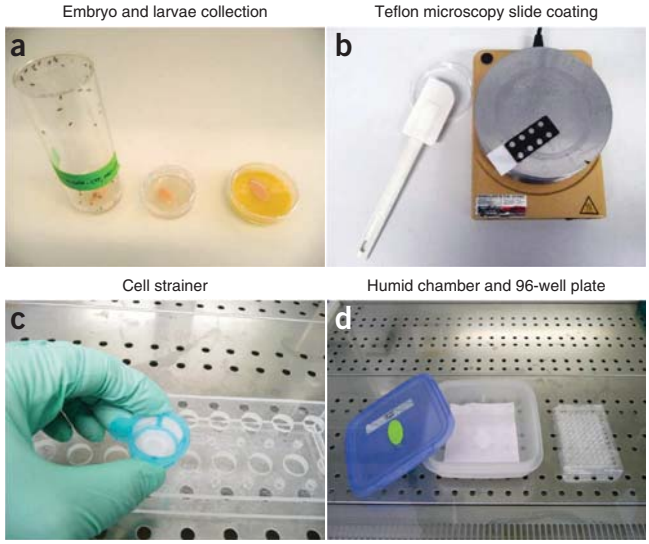

Figure 1 | Illustration of important steps and typical equipment used in the protocol. (**a**) Steps 1–3. Collect embryos on apple juice plates in small fly cages. Transfer freshly hatched larvae to Petri dishes with cornmeal fly food and let them grow to third instar. (**b**) Preparation of Concanavalin A–coated Teflon microscopy slides. Use a silicone spreader to distribute Concanavalin A solution on a Teflon slide. Briefly dry the slide on a heating plate and keep it in a dust-free environment. (**c**) Step 13. To avoid larger cell clusters and tissue debris in culture, use a cell strainer to filter the cell suspension. Press the cell strainer onto a microcentrifuge tube and pipette the cell suspension through the middle of the mesh. (**d**) Pipette the cell suspension into a 96-well plate and place it in a humid chamber. For long-term culture, incubate the cells in a thermostable cabinet at 25 °C.

125 mg ml^{-1} methyl 4-hydroxybenzoate in ethanol). Mix the solution well and pour it into Petri dishes (46 × 16 mm) (**Fig. 1**). The plates can be stored at 4 °C for up to 8 weeks.

Larvae-collection plates Add 7 g of agar and 4 spoons of dry yeast to 1,700 ml of water. Boil the mixture in a pan. Add 200 g of cornmeal and 140 g of sugar. Stir the mixture constantly. Add another 500 ml of water and stir until the mixture starts to boil. Remove the pan from heat and place it into a sink filled with cold water. Cool down the mixture for 10 min while stirring. Add 50 ml of benzalkonium chloride/methyl 4-hydroxybenzoate stock solution (stock solution: 10 mg ml^{-1} benzalkonium chloride and 125 mg ml^{-1} methyl 4-hydroxybenzoate in ethanol) and stir it well. Add food to the Petri dishes (60 × 15 mm) (**Fig. 1a**). Let the Petri dishes cool down for 1–2 h at room temperature (25 °C). Store the dishes at 10 °C for up to 1–2 weeks.

Fixation buffer Fixation buffer is 4% (vol/vol) formaldehyde in PBS. Fixation buffer should be freshly prepared.

PBST PBST is 0.1% (vol/vol) Triton X-100 in PBS. PBST can be stored at 4 °C for up to 8 weeks.

EQUIPMENT SETUP

Concanavalin A–coated Teflon microscopy slides Add 30 µl of Concanavalin A stock solution (5 mg ml^{-1} in PBS) to 10 ml of ddH_2O in a large Petri dish (94 × 16 mm). Dip a silicone spreader in the Petri dish and spread the solution across the microscopy slide once (**Fig. 1b**). Put the slide briefly on the heating plate to dry, and keep the slide dust free. Always freshly prepare the slides.

Humidified culturing chamber with wet filter paper Heat-sterilize filter paper wrapped in aluminum foil in an oven at 200 °C for 2 h. Open the autoclaved humid chamber in the laminar flow cabinet and add the filter paper (**Fig. 1d**). Add ddH_2O to wet the filter paper.

Humidified staining chamber with wet tissue paper An empty mounting media box (Vectashield) or any suitable box with a lid can be used as a staining chamber. Add some wet tissue paper around the edges of the box to keep it humid.

PROCEDURE

Preparation and larval collection ● TIMING ~2 weeks

1| Grow *Drosophila* wild-type strains or the genotype of interest in vials. For laboratory husbandry of *Drosophila melanogaster*, refer to *Drosophila: a Laboratory Handbook*[29].

2| Transfer the adult flies to embryo-collection cages. For the best yield of embryos, use flies that are 2–5 d in age. For staged collections, collect the embryos for 4–6 h on apple juice plates streaked with yeast paste kept at 25 °C and 65% humidity in a 12 h:12 h light-dark cycle (**Fig. 1a**).

3| At 24 h after the midpoint of collection, transfer freshly hatched larvae to food plates and let them grow to desired stages (for example, 72 or 96 h after larval hatching (ALH)).

Dissection of larval brains ● TIMING ~1 h

4| Pick the larvae from the food plate and rinse them first in a Petri dish containing 70% (vol/vol) ethanol and then in a Petri dish containing PBS; repeat the procedure twice to wash off food and yeast.

5| Dissect the larval brains in supplemented Schneider's medium using forceps. Clear off imaginal discs in order to avoid non-neural cells in cell culture. Dissection can be done in glass jars or in drops of medium on the inside of a Petri dish lid.

6| For one primary cell culture sample, dissect 15–20 third-instar brains. After dissection, rinse the brains in a drop of supplemented Schneider's medium before transferring them into a siliconized microcentrifuge tube containing 1 ml of Rinaldini solution.

▲ CRITICAL STEP Rinsing the brains with supplemented Schneider's medium helps wash off non-neural cells, such as fat cells, as well as yeast cells derived from the fly food.

? TROUBLESHOOTING

Preparation of cell suspension ● TIMING ~2 h

7| Centrifuge the brains for 5 min at 300*g* at room temperature. Remove the supernatant and wash the brains two more times in Rinaldini solution.

▲ CRITICAL STEP During this step and subsequent steps, the larval brains should be kept in sterile conditions, and all the steps should be performed in a laminar flow cabinet.

8| Remove the Rinaldini solution and add 1 ml of sterile filtered collagenase I (0.5 mg ml^{-1}) solution. Let the digestion reaction take place at room temperature for 1 h.

9| Spin down the digested brain tissue for 5 min at 300*g* at room temperature and replace the collagenase I solution with 1 ml of supplemented Schneider's medium.

10| Spin down the suspension for 5 min at 300*g* at room temperature and wash it three more times with 1 ml of supplemented Schneider's medium.

Box 1 | BrdU labeling of cells in primary cell culture ● TIMING 2–3 d

1. In Step 14 of the main PROCEDURE, add supplemented Schneider's medium containing BrdU to a final concentration of 15 µg ml^{-1}.
2. Culture the cells for the length of BrdU pulse (Step 15). BrdU-positive postmitotic cells can be observed after ~4 h, which probably reflects the time neuroblasts and ganglion mother cells require for DNA duplication and completion of the cell cycle.
3. Stop the pulse by performing Step 16A(i–iv).
4. Wash the fixed cells three times for 2 min with PBST.
5. To denature DNA, add 2 N HCl for 15 min.
6. Wash the cells three times for 2 min with PBST.
7. Incubate the cells with mouse BrdU-specific antibody at a dilution of 1:300 overnight at 4 °C in a humid chamber.
8. Wash the cells three times for 2 min and twice for 10 min in PBST.
9. Incubate the cells with secondary antibody (for example, Goat anti-mouse Alexa Fluor 488) for 2 h at room temperature or overnight at 4 °C in a humid staining chamber.
10. Wash the cells three times for 2 min and three times for 10 min in PBST.
11. To mount and visualize the cells, perform Step 16A(x,xi).

11| Remove all of the supernatant and add 10 µl of supplemented Schneider's medium per brain. For example, add 200 µl for 20 dissected brains.

12| Set the pipette to half the total volume and use siliconized tips. Pipette the digested brain tissue up and down 100–200 times to prepare a cell suspension.
? TROUBLESHOOTING

13| Pipette the cell suspension through a 40-µm cell strainer (**Fig. 1c**) pressed onto a new microcentrifuge tube to filter out tissue pieces and larger cell clusters.

14| For culturing, pipette 40 µl of cell suspension per well in a 96-well plate and add 160 µl of fresh supplemented Schneider's medium (**Fig. 1d**). For BrdU labeling of cells, see **Box 1**.

15| Place the 96-well plate in the prepared humid chamber and incubate it in a thermostable cabinet at 25 °C for the desired culture period (e.g., 24 h).

Imaging

16| To fix, stain and image the cells, follow option A. For live-cell imaging, follow option B. For calcium imaging, follow option C.

(A) Immunofluorescent staining of fixed primary cell culture ● TIMING 2–3 d

(i) Pipette 200 µl of cell suspension from the 96-well plate into a siliconized microcentrifuge tube and spin down the cells for 8 min at 300*g* at room temperature.
(ii) Remove 160 µl of supernatant, resuspend the cells in the remaining 40 µl and pipette the cell suspension onto one well of the Concanavalin A–coated Teflon slides.
(iii) Allow the cells to settle for 15 min.
▲ **CRITICAL STEP** Increasing the settling time will result in a higher cell density on the slide.
? TROUBLESHOOTING
(iv) Remove the supernatant and fix the cells in 40 µl of fixation buffer (4% (vol/vol) formaldehyde in PBS) for 15 min in a wet box.
(v) Wash the fixed cells on the slide three times for 2 min with 40 µl of PBST.
(vi) Apply primary antibodies diluted in PBST in a total volume of 40 µl per well, and incubate it overnight at 4 °C in a wet chamber.
(vii) Remove unbound antibody by washing the slide three times for 2 min and twice for 10 min in PBST.
(viii) Apply secondary antibodies diluted in PBST and incubate for 2 h at room temperature in a humid staining chamber.
? TROUBLESHOOTING
(ix) Wash the slide three times for 2 min and three times for 10 min in PBST.
(x) Remove all of the supernatant and mount it in Vectashield. Place a coverslip, and after the Vectashield has spread to cover the entire slide seal the edges with clear nail polish.
■ **PAUSE POINT** Slides can be stored at 4 °C for several months.

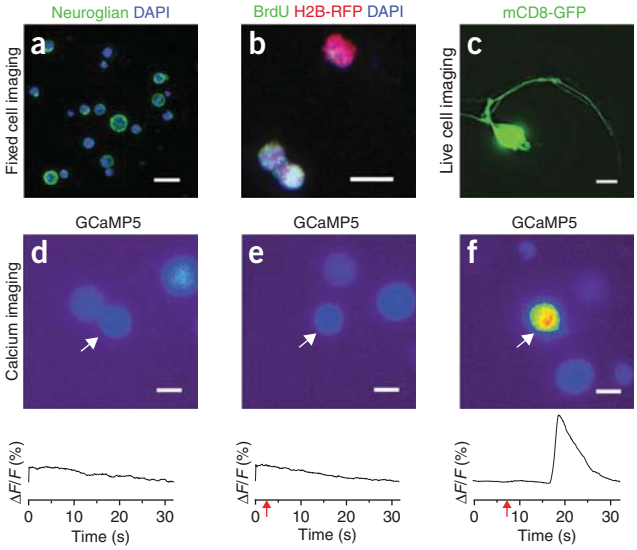

Figure 2 | Primary neural cell cultures derived from *Drosophila* larval brains. (**a**) A typical fixed sample of primary neural cells right after plating it on a Teflon microscopy slide. Cell membranes are outlined by immunofluorescent labeling against the neuronal marker Neuroglian (green); DNA is visualized by DAPI (blue). (**b**) Primary neural cells fixed after 15 h in culture. Some cells express Histone2B-RFP (red) under the control of an optic lobe–specific driver (*c855a-GAL4*). A doublet of RFP-positive cells shows BrdU incorporation (green), which indicates that cells of optic lobe origin are dividing in culture. (**c**) Live primary neural cell expressing membranous mCD8-GFP (green) of *elav-GAL4*. mCD8-GFP reveals cell morphology such as neuronal projections. The image is taken with an inverted microscope (Delta Vision, Applied Precision). (**d**–**f**) Cells expressing the calcium sensor GCaMP5. Arrows show the same cell with different treatments. False-colored images of fluorescence (F) are shown with intensity graphs: nonstimulated control (**d**), 2 µl of Schneider's medium (**e**) and 2 µl of acetylcholine iodide 1 mM (**f**). Red arrows indicate the time point of application. A subset of cells in culture shows a response to acetylcholine stimulus (**f**). Scale bars: (**a**) 10 µm; (**b**–**f**) 5 µm.

(xi) Image the cells using a standard fluorescence microscope or a confocal microscope.

(B) Live imaging of cells in primary neural culture ● TIMING variable

(i) Pipette 200 µl of cell suspension from the 96-well plate into a coated glass-bottom dish.
▲ **CRITICAL STEP** To image neurite outgrowth, use a poly-L-lysine–coated glass-bottom dish. For proper neurite formation in culture, a period of several hours is advantageous (e.g., **Fig. 2** shows neuronal extensions after 24 h in culture).

(ii) Let the cells settle down to adhere to the coated glass-bottom dish.
▲ **CRITICAL STEP** Wait for at least 30 min before starting imaging.

(iii) Mount the glass-bottom dish on the stage of an inverted microscope and image the cells.

(C) Calcium imaging ● TIMING 6 h–2 d

(i) Add 200 µl of the cell suspension on a poly-L-lysine–coated glass-bottom dish.

(ii) Wait for 1 h until the cells are attached at the bottom of the glass dish.
▲ **CRITICAL STEP** To image neural activity, neurons must adhere properly. poly-L-lysine coating is essential.

(iii) Record for ~30 s (92 ms per frame) live with the inverted fluorescence microscope (×63 objective).

(iv) After 10 s, carefully pipette 2 µl of control substances with the pipette in the drop of cell culture. As control substances, use culture medium and solvent (e.g., water).

(v) After 10 s, carefully pipette 2 µl of a test substance (e.g., acetylcholine, 1 mM) to the drop of cell culture.

? TROUBLESHOOTING

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

TABLE 3 | Troubleshooting table.

Step	Problem	Solution
6	Contamination with yeast cells	Rinse brains several times in supplemented Schneider's medium to wash off yeast cells deriving from fly food. Some protocols use autoclaved yeast
12	Too many large cell clusters in culture	Use more pipetting up and down to homogenize brain tissue Collagenase I solution might be out of date; use a new lot
16A(iii)	Numbers of cells per slide is smaller than expected	Cells need time to settle down. Wait longer for cells to attach to the slide before removing the supernatant
16A(viii)	Background in staining is high	Primary antibodies are often used at a lower concentration than would be used for staining whole-mount tissue. Test different dilutions for your primary antibody Usually stainings can be improved when cells are incubated with secondary antibodies overnight at 4 °C Blocking with normal goat serum does not improve staining in our hands[38]

● TIMING

Steps 1–3, preparation and larval collection: ~2 weeks
Steps 4–6, dissection of larval brains: ~1 h
Steps 7–15, preparation of cell suspension: ~2 h
Step 16A, immunofluorescent staining of fixed primary cell culture: 2–3 d
Step 16B, live imaging of cells in primary neural cell culture: variable; depends on imaging time
Step 16C, calcium imaging of primary cells in culture: 6 h–2 d
Box 1, BrdU labeling of cells in primary cell culture: 2–3 d

ANTICIPATED RESULTS

The protocol presented here describes how to make primary neural cell cultures from larval brains that can be observed for several days and up to several weeks. Approximately 15–20 dissected third-instar larval brains will yield ~800–1,000 µl of cell suspension. Antibodies against lineage-specific markers such as Neuroglian or Elav for neurons and Repo for glial cells should be used to determine cell identity (**Fig. 2a**). The monoclonal antibodies from the Developmental Studies Hybridoma Bank (DSHB) (**Table 1**) work very well on fixed cells in culture, and they can be used as positive controls when testing the protocol with new antibodies. In principle, the system allows the generation of primary neural cell culture from larval brains of any genotype to assess cell morphology, proliferation and differentiation. BrdU incorporation showed that at 45 h in culture 8% of cells had gone through S phase[26]. Cultures can be generated from brains in which specific subtypes of neuronal lineages are genetically labeled by using the *Gal4/UAS* system (**Table 2**). We used, for example, a lineage-specific *Gal4* driver line to express histone2B-RFP in optic lobe precursor cells (**Fig. 2b**). This experiment revealed that about half of all replicating cells in culture originate from the developing optic lobes[26]. A useful transgene for studying cell morphology is UAS-*mCD8-GFP,* which codes for a membrane-tethered GFP reporter. It can reveal neurite extensions such as those shown in **Figure 2c**. To assess neural activity in primary cell culture, GCaMP5 can be expressed in neurons of interest. We used the pan-neuronal driver *elav-Gal4* to express GCaMP5 in all postmitotic neurons in the nervous system. We stimulated neuronal activity by exposing cultures to the neurotransmitter acetylcholine (**Fig 2f**). As expected, we find that only a subset of neurons is responsive to acetylcholine. Thus, the protocol can be applied to the study of both developmental and functional processes *in vitro*.

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