

Technique Notes



Primary culture of *Drosophila* larval neurons with morphological analysis using NeuronMetrics.

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Primary dissociated cultures of developing CNS neurons provide a powerful experimental system with which to investigate the effects of genetic or chemical manipulations on neuronal differentiation, morphology, and function. A number of methods, based on dissociating CNS tissue from *Drosophila melanogaster* during development, yield cell cultures of primarily neuronal lineage, *i.e.*, neurons and neuroblasts, with few if any glia (Wu *et al.*, 1983; O'Dowd and Aldrich, 1988; Kraft *et al.*, 1998; Küppers-Munther *et al.*, 2004; Sanchez-Soriano *et al.*, 2005; Sicaeros *et al.*, 2007; Moraru *et al.*, 2012). By choosing a specific developmental stage, one can study the neuronal plasticity by which the CNS matures and undergoes remodeling during metamorphosis. By choosing the plating density, one can study individual neurons in isolation or, alternatively, neurons engaged in cell-cell interactions. These approaches are further enhanced by the use of genetic markers that allow neural subtypes to be identified in cell culture (Egger *et al.*, 2013), such as mushroom body neurons (Kraft *et al.*, 1998; Su and O'Dowd, 2003).

One of the great advantages afforded by low-density primary neuronal culture is the ability to reveal **cell-autonomous** characteristics of neurons under physiological and pharmacological conditions. This has been particularly important for identifying intrinsic properties, such as regional specialization of axonal compartments (Katsuki *et al.*, 2009), the localization and transport of ribonucleoprotein particles (Barbee *et al.*, 2006), temperature-dependent membrane excitability (Peng *et al.*, 2007), and spontaneous calcium transients (Jiang *et al.*, 2005) in dissociated neurons differentiating *in vitro*. Similarly, steroid hormone-dependent developmental transitions, such as neurite outgrowth of interneurons undergoing remodeling (Kraft *et al.*, 1998) and segment-specific programmed motor neuronal cell death (Winbush and Weeks, 2011) do not require any cell-cell contact *in vitro*. Neurons with distinctive axonal and dendritic arborization morphologies *in vivo* recapitulate those morphogenetic features when cultured *in vitro* (Kraft *et al.*, 1998, 2006; Sanchez-Soriano *et al.*, 2005). Selective vulnerability to specific neurotoxins and pharmacological rescue thereof can also be demonstrated *in vitro* (Wiemerslage *et al.*, 2013). Furthermore, isolated neurons can reveal mutant phenotypes, such as initial events of neurite outgrowth (Kim and Wu, 1987, 1996; Sánchez-Soriano *et al.*, 2009), neurite-arbor morphology (Kraft *et al.*, 2006; Peng *et al.*, 2007), ion channel physiology (O'Dowd *et al.*, 1989), and toxicology (Suzuki and Wu, 1984; Martin *et al.*, 2000).

When cultured at high density, dissociated *Drosophila* neurons form networks connected by chemical synapses, gap junctions, and neurite fasciculation (Rohrbough *et al.*, 2003; Oh *et al.*, 2008; Saad *et al.*, 2012). Classical physiology and pharmacology approaches along with genetic markers and mutations have been used to characterize excitatory and inhibitory synaptic transmission (Lee *et al.*, 2003; Su and O'Dowd, 2003), as well as synaptic plasticity (Lee and O'Dowd, 2000; Campuzano *et al.*, 2007). Importantly, some aspects of synaptic function, such as action-potential-independent neurotransmitter release mediated by Ca(v)2-type calcium channels, have been revealed by cultured brain neurons but not at the neuromuscular junction (Gu *et al.*, 2009). This is particularly relevant for studying genes whose human orthologs are mutated in developmental brain disorders that cause cognitive and behavioral phenotypes (Inlow and Restifo, 2004; Restifo, 2005; Bolduc and Tully, 2009; Gatto and Broadie, 2011).

One of the most promising applications of primary neuronal culture in the *Drosophila* genetic system is for drug discovery, in particular with the goal of treating disorders of brain development. We have already reported results of a first-of-its-kind small-molecule drug screen using *singed*-mutant cultured neurons as a cell-based assay for fascin function (Kraft *et al.*, 2013). Making use of the dramatic “filagree” neurite-trajectory phenotype (Kraft *et al.*, 2006), we identified chemically diverse blockers and enhancers of the fascin pathway and formulated several structure-activity relationship hypotheses. That same drug screen revealed a striking toxic effect of statin compounds (HMG-CoA reductase inhibitors) on neurite outgrowth, the “beads-on-a-string” morphogenesis defect, that is enhanced by lack of the actin-bundling protein fascin (Kraft *et al.*, 2013). This and other examples of drug-induced morphological neurotoxicity set the stage for use of *Drosophila* neuronal cultures to drive the identification of Gene- \times -Environment interactions that disrupt brain development (Halladay *et al.*, 2009).

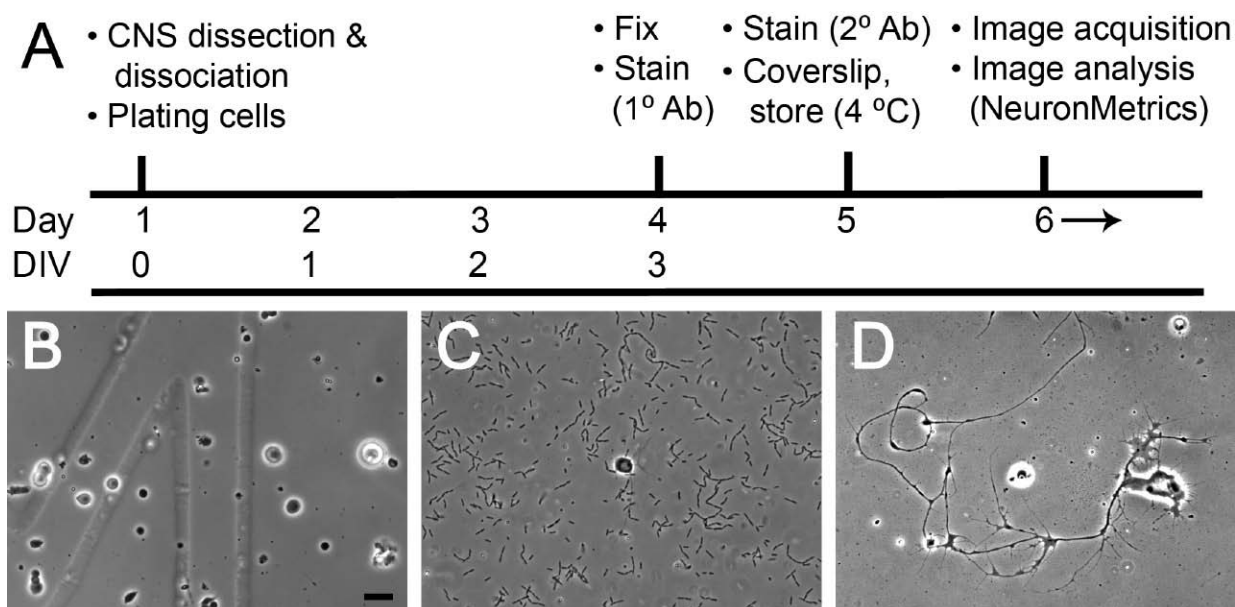


Figure 1. Primary neuronal culture timeline and outcomes. (A) Diagram of a typical experiment. On day 1, the CNS from a w3L is dissected and dissociated, and the cells are plated. The dishes are flooded with culture medium. On day 4, after the neurons have spent 3 div, the cultured cells are chemically fixed and incubated with a primary antibody (*i.e.*, anti-HRP) overnight. On day 5, the neurons are stained with a fluorescent secondary antibody, mounted, coverslipped, and stored at 4°C in the dark. From day 6 onward, neuron images can be acquired using fluorescence microscopy and analyzed quantitatively using NeuronMetrics. (B-D) Phase-contrast images acquired through a 60 \times objective, with same magnification. (B) Dissociated CNS cells immediately after flooding (2 hr after plating), showing the desired density. Part of the alphanumeric grid (top of “4”) is visible under the cells. (C) A 1-div culture, showing yeast contamination, with numerous yeast cells, many in chains, present throughout the field. A single neuron, with a modest neurite arbor, is seen in the center of the field. (D) Wild-type neuron from a 3-div culture, with extensive neurite outgrowth and branching, and growth cones still visible. A long neurite from a nearby cell enters the field from the top.

In this protocol we detail methods for dissociation of enzyme-treated larval CNS tissue by manual mechanical dissociation into individual neurons (Figure 1). The cultured neurons elaborate complex neurite arbors on a laminin-coated substrate over several days. By 3 days *in vitro* (div), differences between wild-type and mutant neurons are often evident by phase-contrast microscopy. For larval cultures prepared with this protocol, neuroblast proliferation is complete and no Repo-positive cells are detected by 3 div (Luedeman,

Chuang, Levine, and Restifo, unpublished observations). Immunofluorescent staining for a neuronal membrane marker, recognized by anti-HRP antisera, generates high-contrast, low-noise images that faithfully reproduce the morphology of living *Drosophila* neurons (Kraft *et al.*, 1998). For quantification of neurite-arbor size and shape, we further demonstrate the use of NeuronMetrics software, a set of plugin modules for ImageJ that carry out semi-automated morphometric analyses of digital 2D images of fluorescently labeled neurons (Narro *et al.*, 2007). NeuronMetrics is available without charge to academic users through Tech Transfer Arizona ([Technology #ua07-056](#)).

Protocol

1) Advance preparations

1.1) Prior to the day of the dissection

1.1.1) Review the overall timeline (Figure 1), recipes (Reagents and Supplies, Table 1), and the dissection protocol detailed below (Section 2). Make sure you have all of the necessary tools, reagents, and that wandering larvae will be available on the day of dissection.

1.1.2) Prepare culture dishes. For homemade dishes, drill 8-mm holes into the centers of 35-mm polystyrene culture dishes. Create a well by securing a glass coverslips (plain or gridded) to the bottom of the dish with a thin layer of Sylgard. Alternatively, pre-made dishes can be purchased from MatTek with 7- or 10-mm wells. Sterilize the dishes by UV exposure for two hours.

1.1.3) Coat the glass-bottom wells with Concanavalin A and laminin (“ConA/laminin”; see Materials, Table 2) to provide a substrate that promotes cell adhesion and neurite outgrowth. Thaw fresh aliquots of ConA (1 mg/ml) and Laminin (1 mg/ml) slowly at 4°C. Add 400 µL ConA to 2 mL sterile water in a small sterile tube. The ConA stock solution is very viscous; flush the pipette tip a few times and mix. Add 4 µL laminin; vortex to mix. Split coating solution into two sterile 1.5-ml tubes and spin in microcentrifuge (16000 × g) at 4°C for 10 minutes. Combine supernatants in a new sterile tube. Use sterile tips to pipette coating solution into wells (100 µL per 8-mm well or 150 µL per 10-mm well) and to spread it carefully to the edges. Do not touch the glass bottom with the pipette tip. Leave dishes in 37°C humidified incubator for 2 hours, then rinse without touching the glass bottom by gently pipetting 6 mL of sterile water onto the well and simultaneously suctioning off the water with a sterile Pasteur pipette attached to a vacuum line. Store coated dishes at room temperature protected from light. For critical experiments, dishes should be used within 2 weeks of coating.

1.1.4) Prepare fresh culture medium by adding FBS, insulin, and NaOH to Schneider’s *Drosophila* Medium (“S10-I” in Materials Table 2). This can be used for up to three weeks when stored at 4°C protected from light.

1.2) On the day of dissection (Day 1)

1.2.1) In the dissection area, clean the entire work surface thoroughly with 70% ethanol. Set aside a test tube rack (rinsed with ethanol) to hold solutions used for dissection. Soak two pairs of forceps (Dumont #5, Figure 2), a sable paintbrush, two applicator-stick-mounted minuten pins, and a 10-well glass dish with 70% ethanol between paper towels.

1.2.2) In the tissue culture hood (ideally, a biosafety cabinet), clean the entire work surface thoroughly with 70% ethanol, preferably wearing gloves sprayed with 70% ethanol. If you are not using gloves, wash your hands with soap and water, then rub your hands with gel alcohol. Use sterile technique and supplies (Pasteur

pipettes, microcentrifuge tubes, volumetric pipettes, and pipette tips). All sterile tubes, solutions, or reagents should be opened only in the hood.

1.2.3) Prepare a working dilution of enzymes (Liberase DH, collagenase with a high concentration of dispase) in Rinaldini's saline (0.21 Wunsch units/ml; see Recipes). For each CNS sample to be dissociated, place 300 μ l of enzyme solution in a labeled 1.5-ml microcentrifuge tube and place at the dissection station. These enzymes digest the extracellular matrix (ECM) to facilitate mechanical dissociation of the tissue into individual cells.

1.2.4) Assemble in the hood *Drosophila* S10-I culture medium, sterile purified (18-M Ω) H₂O, and sterile screw-top tubes and sterile volumetric pipettes. For each sample, you will need 3 mL of water and 8.25 – 12.25 mL culture medium, as follows: 2.5 mL for dissection, 2 mL for wetting pipettes, 2.75 mL for dissociation, and 1 mL per dish for flooding (each sample will be distributed into 1-6 dishes; see Section 4).

1.2.5) Assemble the following at the dissection area next to the microscope (Figure 2): (a) one autoclaved Pasteur pipette per sample (can be placed in a sterile plastic tube for transport to the dissection area); (b) one multi-well dish for washing and dissecting larvae (e.g., a 10-well glass Boerner slide); (c) one paintbrush (red sable bristles work well); (d) dissection forceps; (e) two dissecting pins for final trimming (e.g., wooden applicator sticks, each with a minutien pin embedded in one end); (f) one sterile 1.5-mL microcentrifuge tube with 300 μ l of Liberase DH enzyme solution for each sample; (g) one tube containing 3 mL sterile purified H₂O per dissection (for washing larvae); (h) one tube containing 2.5 mL of culture medium; (i) one tube containing 1 mL of 95% ethanol. These volumes provide sufficient fluids for a single CNS sample; scale up as needed.

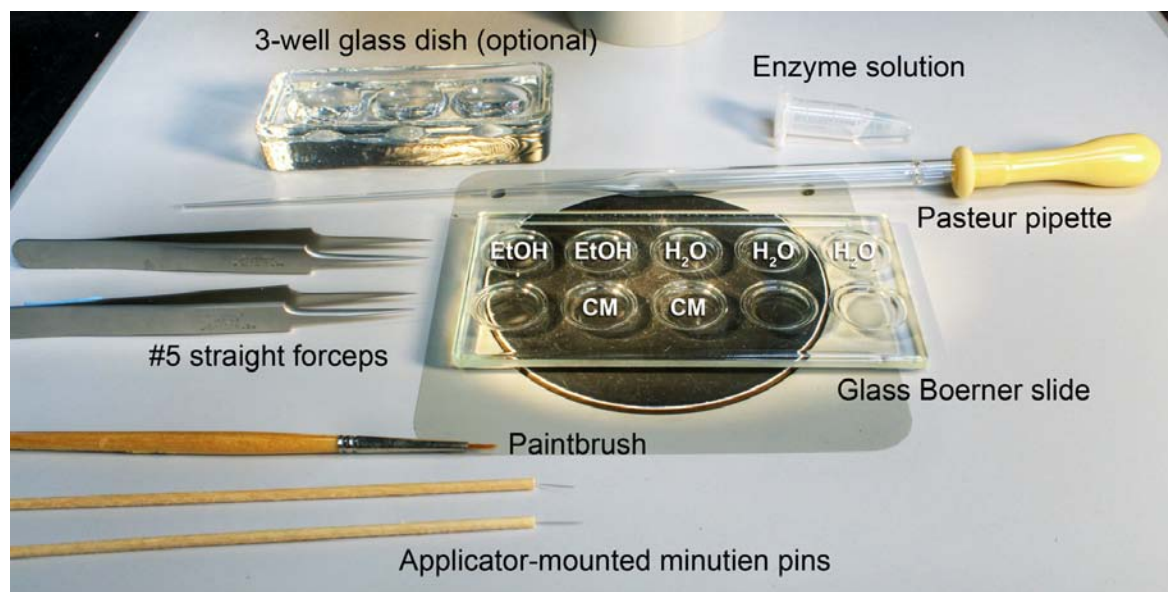


Figure 2. Tools and materials used during the *Drosophila* larval CNS dissection, shown on a stereomicroscope stage. The 3-well glass dish is used for examining larvae and making final selections. The shallow wells of a glass Boerner slide are convenient for cleaning and dissecting the larva. The paintbrush is used to clean and transfer the larva between wells (EtOH, ethanol; H₂O, water; CM, culture medium). The Dumont #5 forceps are used for microdissection, to expose and explant the CNS. The wooden applicator-mounted minutien pins are used to remove non-neuronal tissue (e.g., imaginal discs and the ring gland) and to trim the peripheral nerves. The CNS can then be transferred into the tube of enzyme solution using a Pasteur pipette.

2) CNS dissection

2.1) Selection of larvae.

This may be done at a non-sterile lab bench. Use a paintbrush to carefully remove individual wandering third instar larvae (w3L) from the wall of their culture vial, avoiding any undersized, prematurely wandering larvae. Within the wandering stage, fast-moving larvae are younger while older w3L are slower-moving. If comparing multiple conditions or genotypes, match the larvae for developmental stage, sex, culture density, diet (“fly food”), and rearing conditions such as light and temperature. Transfer each selected larva from the paintbrush to a small tube or Petri dish (but do not seal tightly), using separate tubes or dishes for each genotype or condition.

2.2) Clean each larva before dissection.

Fill two wells of the 10-well dish with 95% ethanol, starting at the top left (Figure 2). Fill the next three wells with sterile purified H₂O. Fill two wells in the bottom row with culture medium. Using a brush wetted with water, transfer the larva to an ethanol-containing well and soak for 1 minute. Gently brush the length of the larva to loosen any surface debris. Roll the larva and repeat on the other side. Transfer the larva to purified H₂O to rinse off the ethanol, using the brush to move the larva to each of the three wells, and for gently brushing if needed. Use forceps to transfer the clean larva to a well containing culture medium for dissection.

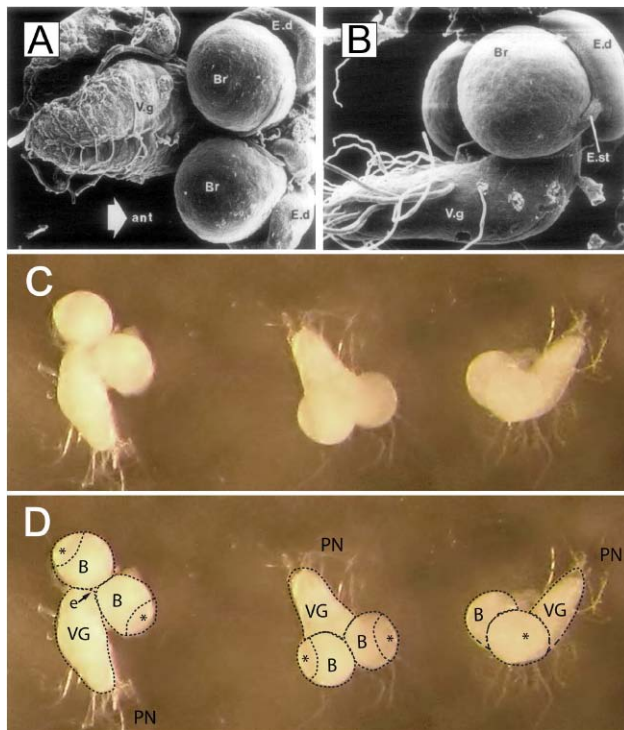


Figure 3. Larval CNS gross anatomy. (A, B) Scanning electron micrographs of larval CNS, with attached eye imaginal discs (E.d), viewed from above (A) and from the side (B); anterior is to the right. Numerous peripheral nerves are seen extending from the ventral (thoracoabdominal) ganglion (V.g). Each eye disc is very closely apposed to the ipsilateral brain lobe (Br), connected by a short stalk (E.st). (Reprinted from *Developmental Biology*, Vol. 65, K. White and D.R. Kankel, Patterns of cell division and cell movement in the formation of the imaginal nervous system in *Drosophila melanogaster*, Pages 296-321, 1978, with permission from Elsevier.) (C) Three freshly microdissected larval CNS in a culture well, each one at a slightly different orientation, viewed through the stereomicroscope. Note that all of the peripheral nerves have been cut short. (D) Same image as in (C), but with an overlay of dotted lines that represent visible anatomical boundaries of interest. Asterisks (*) indicate the optic lobes. B, brain; VG, ventral ganglion; PN, peripheral nerves; e, approximate location of esophageal canal.

2.3) Explant the CNS (Figure 3).

Use two pairs of fine forceps (Dumont #5) to remove the posterior quarter of the larva: clamp with the forceps on the anterior side and pull with the other forceps. Expose the CNS by inverting the anterior portion over the forceps (this is analogous to turning a sock inside out). Explant the CNS by carefully severing the peripheral nerves connecting the CNS to the larval body wall. To avoid damaging the CNS, clamp the peripheral nerves with the forceps adjacent to the CNS and pull with the other forceps, or use one blade of the forceps on the distal side to slice through the nerve. Carefully remove the imaginal discs as well, avoiding damage to the optic lobes when severing the short stalk that connects the eye/antennal disc to the CNS. Use forceps to transfer the CNS to the next well containing fresh culture medium. Use the minutien pins, mounted on wooden applicator sticks, for precision cleanup. Do not leave long pieces of peripheral nerve, which readily stick to

surfaces (see Figure 3 for guidance). Failing to cut them short can result in loss of the sample prior to or during the dissociation process.

2.4) Optional regional dissection (Figure 3B)

Minutien pins embedded in the ends of wooden applicators can be used for fine dissection to separate CNS regions, *i.e.*, in order to isolate the optic lobes or segmental ganglia (ventral ganglion plus subesophageal ganglion) or the brain, as needed.

2.5) Enzyme treatment of the CNS

Use a sterile Pasteur pipette that has been pre-wetted with culture medium to transfer the CNS to the microcentrifuge tube containing the enzyme solution. (Pre-wetting is essential to prevent tissue from sticking to the pipette.) Place the tube containing freshly explanted CNS and enzyme to the tissue culture hood. Incubate for 1 hour.

2.6) Repeat the above steps for each larva.

Dispose of the larval carcass according to the biosafety protocols approved by your institution. Between dissections, clean the multi-well glass dish, the paintbrush, forceps, and minutien pins with ethanol and dry with Kimwipes. Use a new Pasteur pipette for each larva.

3. CNS dissociation by mechanical trituration (These steps take place in the hood. Remember to spray your gloves with ethanol each time you begin work in the hood or return to the hood.)

3.1) Verify that the following are available in the hood:

S10-I culture medium, sterile microcentrifuge tubes, Pasteur pipettes, a micropipettor, and 200- μ L micropipette tips.

3.2) Wash the tissue to remove the enzyme.

Use a sterile Pasteur pipette, wetted with culture medium, to transfer the CNS to a microcentrifuge tube containing 200 μ L of culture medium. The goal is to minimize the transfer of enzyme solution. Soak the tissue for 1-2 minutes before transferring the CNS to a new microcentrifuge tube containing 200 μ L of fresh culture medium.

3.3) Alternative tissue-washing technique.

Wash the tissue by adding 1 mL of culture medium to the enzyme-incubation tube, then centrifuging the tissue at $\sim 1,200 \times g$ for 10 seconds, in a small bench-top mini-microcentrifuge that is kept in the hood. Carefully remove most of the liquid, leaving $\sim 200 \mu$ l behind to avoid disrupting the pelleted CNS tissue. Repeat the wash once more.

3.4) Dissociate the enzyme-treated tissue by mechanical trituration.

This can be done using a micropipettor (*e.g.*, Pipetman) and a sterile 200- μ L tip or a Pasteur pipette that has been flame-polished to smooth the tip and reduce the diameter by about half. In either case, it is imperative to pre-wet the pipette with culture medium. To triturate, hold the pipette tip against the bottom of the tube. Aspirate the fluid into the pipette (tip) and dispense forcefully about 80 times, at which point the tissue should be thoroughly dissociated. Within the first 5-6 cycles, the CNS tissue will be sufficiently disrupted that it is no longer visible to the naked eye. About midway through, close the tube and use a stereomicroscope to confirm that no pieces of tissue are stuck to the pipette tip or wall of the tube. If they are, try to wash them loose with focused pipetting. For best results, minimize foaming or bubbles which decrease recovery. Some evaporation does take place during the trituration, leaving about 180 of the original 200 μ L. At this point, the tube contains a suspension of cells (mostly neurons) and numerous tiny bits of neuropil debris. The cells will start to settle right away; it will be necessary to resuspend them immediately before distributing the cells into culture dishes.

4) Plating and culturing the neurons

4.1) Assemble in the tissue culture hood:

coated culture dishes, culture medium for flooding, a container to hold the cultures and create a humid chamber (*e.g.*, a 150-mm glass Petri dish containing an open culture dish or bottle cap with sterile water), and strips of Parafilm to seal the dishes. Label both the top and bottom of each culture dish to indicate the genotype, date, and experimental condition (*e.g.*, a numeric code that can be used to reference the experimental details).

4.2) Plate the dissociated cells.

4.2.1) For culture dishes with 8-mm wells, fill each well with 75 μL of culture medium. Then use a pipette tip pre-wetted with culture medium to mix the cell suspension thoroughly by pipetting up and down. Carefully add 25 μL of the cell suspension to the medium in the well of each dish, distributing the fluid as evenly as possible within the well and bringing the total volume to 100 μL . The medium forms a dome-shaped droplet bounded by the edge of the well. Use the pipet tip to ensure that the fluid extends all the way to the edge.

4.2.2) For culture dishes with 10-mm wells, fill each well with 95 μL of culture medium and add 40 μL of cell suspension, for a total volume of 135 μL .

4.2.3) If the dissociation is complete and recovery of cells is good, the suspension from a whole w3L CNS can be distributed into six dishes with 8-mm wells or 4 dishes with 10-mm wells. The resulting density will promote elaboration of complex arbors (at least from wild-type neurons) but still allow individual neurite arbors to be analyzed. If density is too low, neurite outgrowth will be impaired (see below).

4.3) Allow the cells to settle and adhere.

Place covered culture dishes in humid chamber (see Section 4.1). Cover the humid chamber and transfer it to a 25°C incubator (without CO₂) for 2-3 hours to allow the cells to settle and adhere to the substrate (*i.e.*, the ConA/laminin coating on the glass coverslip).

4.4) Examine the cells.

At the end of this settling period, use a compound microscope with phase-contrast optics and a 40 \times objective to confirm that the cells are present, adherent to the floor of the well, and at an acceptable cell density (Figure 1B). It is particularly important that the cell densities of cultures to be compared to each other are about the same. Adjustments in cell density can be made if some of the cell suspension is kept in reserve. To supplement a low-density well, resuspend the dissociated cells using a new, pre-wetted pipette tip, and carefully add 5-10 μL to the fluid already in the well. Add 2 hours to the settling time after more cells are added. This is a Go/No-Go point: if there are too few cells, it is better to start over than to proceed. The dissociated neurons will generally be spherical, with some having a short neurite that either survived trituration or extended during the settling period. Expect to see numerous tiny fragments of tissue debris, especially near the center of the well.

4.5) Flood the dishes.

Transfer the humid chamber to the tissue culture hood and flood each culture by carefully adding 900 μL of culture medium to each dish using a barrier tip. This is the point at which hormones, drugs, and so forth, are typically added to the culture. Dispense the media at the side of the dish, away from the well, to prevent the cells from detaching from the glass coverslip. Rock the dish gently to ensure the culture medium covers the entire dish. Seal each culture dish by wrapping it with Parafilm to limit evaporation. Return the culture dishes in their humidity chamber to the 25°C incubator (without CO₂).

4.6) Culture the neurons.

Check the cultures daily using a compound microscope and phase-contrast optics. By the end of 1 div, neurons should have started extending multiple neurites. Signs of trouble are a) lack of neurite outgrowth; b) microbial contamination (yeast is the most common; Figure 1C); c) cell bodies detaching from the substrate.

4.7) Optional. If the cells cultured longer than 3 div, replace 500 μ L of the media with fresh culture medium every four days.

5) Immunofluorescent staining of the cultured neurons

5.1) Assemble at the bench (see Materials Table): buffered saline (either PBS or Ikeda Ringer), freshly made 4% formaldehyde in PBS, primary and secondary antibodies, wash buffer (PTN), polyvinyl alcohol (PVA) with DABCO (or similar mounting media with fade retardant).

5.2) Fix the neurons.

Remove all tissue culture medium from each culture dish using a glass pipette, making sure not to touch the well. Carefully rinse each culture dish with 1 mL of saline. Add \sim 1 mL of 4% formaldehyde in PBS and incubate for 10 minutes at room temperature (RT). Remove the fixative and wash neurons with 1 mL PTN three times for 10 min each at 4 $^{\circ}$ C.

5.3) Incubate with primary antibody, goat anti-HRP antiserum at 1:250 to 1:500 dilution (total volume for each dish can be as low as 250 μ L, if the dishes are in a humid chamber to minimize evaporation). Incubation time can vary from 2-3 hours at RT to 12-16 hours (overnight) at 4 $^{\circ}$ C.

5.4) Remove the primary antibody solution from each dish and add 1 mL of PTN. Wash the neurons five times for 5-10 minutes each with 1 mL PTN at RT.

5.5) Incubate in secondary antibody, either Alexa Fluor 488- or Alexa Fluor 568-conjugated donkey anti-goat antisera at a dilution of 1:500 in PTN. Note: If the neurons are expressing a GFP-tagged reporter gene, choose the fluorophore that does not overlap with GFP. Incubate at 4 $^{\circ}$ C for 2-3 hours in the dark. A light-tight box or aluminum foil should be used to protect your samples from the light.

5.6) Remove the secondary antibody solution from the dishes and add 1 mL of PTN. Wash the dishes five times in 1 mL PTN for 5-10 minutes each at RT in the dark.

5.7) Mount the preparations with cover glass.

Before applying mountant, rinse each dish with 0.1 M Tris-HCl, pH 8.0 and remove all liquid. Apply \sim 200 μ L of PVA/DABCO mountant directly to the center of the well without touching the bottom. Avoid creating bubbles; remove any that do occur. Cover with an 18 \times 18-mm coverslip. Return the dishes to a light-tight box and allow the mountant to set overnight in the dark at 4 $^{\circ}$ C (make sure the box is on a level surface). If the neurons are not to be examined within a day or two, wrap the dishes with Parafilm to prevent the PVA from drying out. At this point the preparations are quite stable and may be stored (4 $^{\circ}$ C in the dark) for many weeks.

6) Acquisition of fluorescent images

6.1) Microscopy and image acquisition.

Use an inverted compound microscope with fluorescence optics and high-magnification oil-immersion objective (60 \times or 100 \times). In order to generate high-contrast images for analysis with NeuronMetrics, use exposure times long enough to obtain a signal of sufficient intensity to visualize the entire neurite arbor. This exposure will typically saturate the neuronal cell body.

6.2) Sampling of neurons.

For systematic sampling of fluorescent neurons across the gridded dish, use the “Stair-Step” or “Parallel Track” schemes shown in Figure 4A-B). Phase-contrast microscopy can be used to record the location of each neuron using the alphanumeric codes on the gridded coverslip.

6.3) Sample size.

Images of 50 or more neurons across the dish should be acquired for adequate statistical power.

6.4) For very large neurons

If the neurite arbor extends beyond the image frame, acquire multiple overlapping images and assemble them into a single image using software capable of image stitching. Software options include Panavue ImageAssembler and Adobe Photoshop.

7) Neurite-arbor morphology quantification

7.1) Download and install ImageJ and NeuronMetrics.

On [NeuronMetric's Tech Transfer Arizona webpage](#), download the NeuronMetrics User Manual and order the software (registration is required). Download ImageJ version 1.36b for PC from the ImageJ website: <http://rsbweb.nih.gov/ij/>. Download the FeatureJ plugin from the ImageJ website. When you receive an approval notice, download the NeuronMetrics plugins. Carefully follow the installation instructions in the User Manual.

7.2) Rules for image format and file names.

All images must be in 8-bit format. If the images were acquired in a different bit depth, use “batch converter” in ImageJ to convert your images to 8-bit tiff format. Image file names must not end with a letter. To prevent troubleshooting issues with directory names, we **STRONGLY** advise making a new folder in the root of your C: drive, and giving it a simple name like “images.” This folder should contain the input-images folder for NeuronMetrics (see the video for a demonstration).

7.3) Configure NeuronMetrics in ImageJ (Plugins → NeuronMetrics → 2 Setup).

See the NeuronMetrics User Manual for detailed instructions.

7.3.1) Calibrate the scale. Set “Scale” and “Units in pixels”, a calibration that is obtained from “1 Save Scale”; it is essential for obtaining accurate length units from NeuronMetrics output. Scale calibration is performed only once for each microscope/objective used to acquire images.

7.3.2) Define the “Neuron Folder” that contains all of your image files. The “Destination Folder” will be designated automatically when you define the “Neuron Folder.”

7.3.3) Select “Neuron Signal” – Uniform or Non-uniform.

7.3.4) Select “Optional Features.” Select “Noise ROIs” to eliminate background bright spots. Select “Length Correction” if neurites have regions of self-fasciculation, which NeuronMetrics may interpret as a single neurite. Select “Dominant Neurite” if you want to calculate the Polarity Index, for which you need to determine the primary neurite with the largest total length; if you do not need to calculate Polarity Index, make sure it is not selected. Select “Territory” to calculate the area and perimeter of the neurite-arbor footprint.

7.3.5) Select the “Optional Output Images” you would like to save.

7.4) Run NeuronMetrics.

7.4.1) Open “Neuron and Cell Body” (Plugins → NeuronMetrics → 3 Neuron and Cell Body). As each input image (in the folder defined in Setup) is sequentially opened and you are prompted to circle the region of interest (ROI), click and drag the mouse to circumscribe the entire neuron with all of its neurites. Press the space bar. When prompted to define the cell body, click the center of the neuronal soma. Press the space bar again. The image will automatically close and the next image will appear; this sequence repeats until all images in the folder are done.

7.4.2) Open “Skeletonize and Improve” (Plugins → NeuronMetrics → 4 Skeletonize and Improve). This is an automated process and does not require user input. This module functions to eliminate background noise, fill short gaps, and generate the skeleton. See Narro *et al.* (2007) for information on the algorithms used. If the improved skeleton, when overlaid on the original image, shows that there are errors (*e.g.*, part of a neurite was

not included because there was a large gap in the signal, or a false neurite was created from background noise), it is possible to make changes at this point. Open the skeleton in ImageJ and use the Pencil and Eraser tools to fill the large gap or remove the noise. Delete the improved skeleton and improved skeleton overlay folders. Run Improve Skeleton again.

7.4.3) If desired, open “Length Correction” (Plugins → NeuronMetrics → 5 Length Correction). Draw a free-form curve to indicate the length that should be added to a region of self-fasciculation. To visualize the skeleton, click the “Toggle Skeleton” button in the Length Correction Manager; if needed, use the zoom tool to better visualize the skeleton. To add a neurite, click and drag the mouse to draw it, making sure the neurite drawn makes contact with the original skeleton. Then click the “Add” button and designate the weighting to be used (in the example shown in the video a weight of 1 is used). When you are finished, click the “Finished Image” button. The length correction is used by the “Dominant Neurite” and “Measure Skeleton” Plugins, but not by the “Territory” Plugin.

7.4.4) If you have chosen to compute Polarity Index, open “Dominant Neurite” (Plugins → NeuronMetrics → 6 Dominant Neurite) to indicate the dominant neurite, *i.e.*, the primary neurite with the largest arbor.

7.4.5) Open “Measure Skeleton” (Plugins → NeuronMetrics → 7 Measure Skeleton) to automatically compute all the morphometric parameters. NeuronMetrics creates, in the Destination Folder, a folder with a name ending in “_dat” which contains a text file (.txt) with the numerical data from all images. Specifically, the data file contains the number of primary neurites, total branch number estimate, total neurite length, Polarity Index (if selected), and Territory perimeter and area (if selected) for each neuron image. Import these tab-separated (a/k/a tab-delimited) data into an MS Excel spreadsheet or other programs for statistical analysis or graphing. The time required to analyze a folder of ~60 neurons will depend on individual user speed, choice of optional features, and computer processing speed.

7.5) If (and only if) you have problems running NeuronMetrics, you may have to download Java 1.5.0-03 (J2SE Development Kit 5.0 Update 3) from: http://www.oracle.com/technetwork/java/javasebusiness/downloads/java-archive-downloads-javase5-419410.html#jdk-1.5.0_03-oth-JPR

Representative Results

This protocol is most successful when carried out with advanced planning, attention to detail, and practice of the manual techniques (Figures 1A, 2). Microdissection of the *Drosophila* larval CNS (Figure 3C-D) is relatively easy, making it suitable for teaching to novices. The relevant anatomy is well appreciated in scanning electron micrographs of the larval CNS (Figure 3A-B) from a classic study of postembryonic neurogenesis (White and Kankel, 1978). These images reveal two potential dissection-related challenges. First, precision is required to sever the short stalk (labeled E.st), between the eye-antennal imaginal disc and the optic lobe, without tearing the CNS. Second, the peripheral nerves are numerous and, because the cut ends are sticky, must be cut close to the CNS (Figure 3C-D) to prevent the CNS from getting stuck inside pipets. Contaminating microbes will not be evident at this magnification, but note that the larval gut is a potential source of yeast contamination. And, as in all arthropods, the esophagus runs through a narrow canal in the center of the CNS (Figure 3D). Hence, cleanliness of the dissection station (Figure 2) and careful removal of the gut early in the dissection process are important.

When viewed at high-magnification after the dissociated cells have settled and the dish has been flooded, one should see multiple spherical cells per field, with no clumps (Figure 1B). Clumps indicate inadequate dissociation, which could result from reduced activity of old enzymes or insufficient pressure during manual trituration. Some of the cells will not survive or differentiate, and not all are neurons. For example, neuroblasts, neural stem cells whose proliferation during the first 1-2 div can be demonstrated by BrdU incorporation (Luedeman, Levine, and Restifo, unpublished observations). Tiny phase-dark dots represent subcellular debris, most likely bits of neuropil; debris is most common in the center of the well. If

the cell yield is extremely low, the CNS tissue was most likely lost (stuck to a pipet wall) early in the dissociation. The most common errors leading to this result are failing to cut the peripheral nerves short or to pre-wet pipettes with culture medium. If cells are present but the density is low, the problem could be inadequate resuspension of the dissociated cells prior to plating. Reserving some of the cell suspension for density adjustments can help ensure that cultures being compared in a given experiment have similar densities. For this purpose, it is important to check the cell density before flooding, and supplement the dishes as needed.

Neurons cultured from the CNS of *Drosophila* larvae extend neurites within the first few hours. By 1 div, neurite outgrowth should be readily apparent. This is a good time to check for microbial contamination, of which the most common culprit is yeast from the larval gut (Figure 1C). During the next several days the arbors become progressively larger and more complex through neurite extension and branching (Figure 1D). Small neurite arbors may be due to low cell density or problems with the substrate (coating) or culture medium, such as expired or incorrectly handled ingredients. On the other hand, reduced neurite outgrowth can be a *bona fide* mutant phenotype or an indication of neurotoxicity of a chemical added to the culture medium. Hence, good controls are essential for correct interpretation of reduced neurite outgrowth. At 1 div and

thereafter, dead neurons are typically phase-dark spheres with no neurites, or with blebbed neurites that tend to lift off the substrate after a few days. Excessive cell death is generally due to problems with the culture medium, substrate, or exposure to toxins. When testing the effects of drugs or other chemicals, the use of barrier tips during handling of reagents is highly recommended to minimize the risk of cross-contamination.

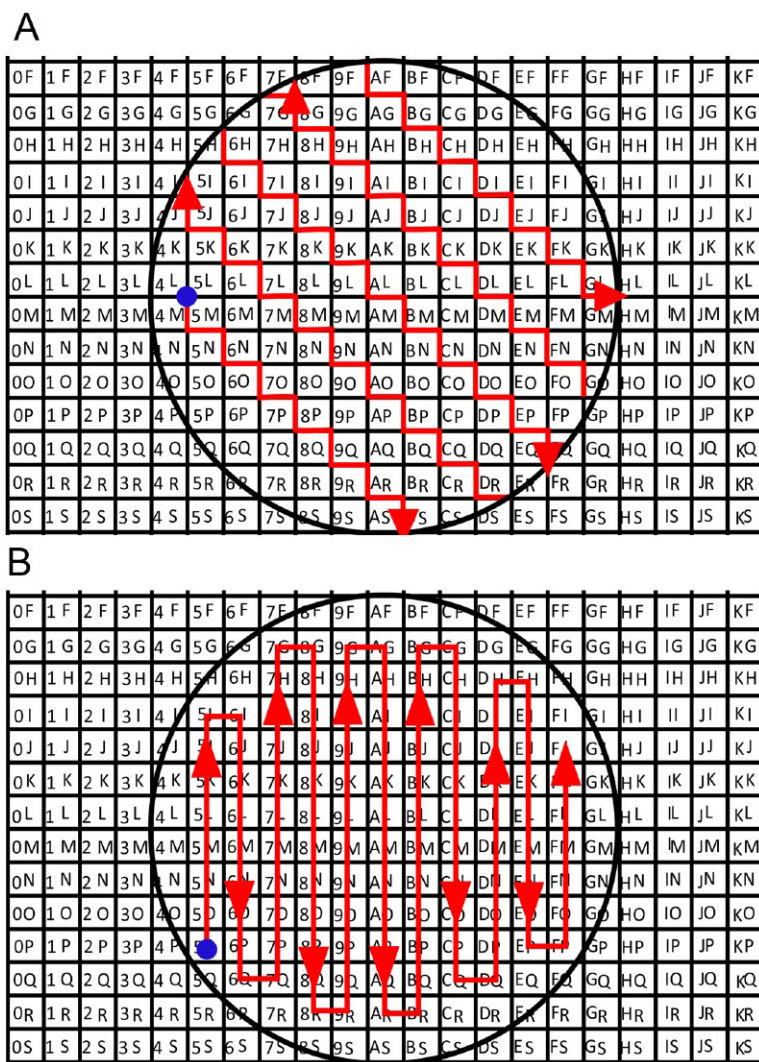


Figure 4. Neuron-sampling strategies using a photoetched grid in the floor of the culture well. Circle, representing the 8-mm culture well, superimposed on a Bellco grid at the same size scale. (A) The “Staircase” sampling method, wherein the experimenter uses the grid to find a standard starting point (blue dot), e.g., at “9 o’clock” in the well, and systematically follows a staircase track (red lines, note directional arrows) during image acquisition. Each diagonal sweep across the dish is two columns away from the adjacent ones, which minimizes the risk of double counting neurons. (B) The “Parallel Track” sampling method whereby, from a standard starting point (blue dot), the experimenter moves up and down each column of the grid.

Optimum cell density will allow for survival and outgrowth of individual neurons whose non-overlapping neurite arbors can be analyzed readily with NeuronMetrics. Two alternative strategies for sampling of neuron images from a single culture dish are diagrammed in Figure 4. The “Staircase” sampling method (Figure 4A) is optimal when the neuron density is high. The “Parallel Track” sampling method

(Figure 4B), which covers a larger fraction of the culture well, is useful when the neurons of interest are in the minority, such as when selection is based on expression of a specific marker. The cell density and neurite-arbor size will determine the specific rules (*e.g.*, all neurons in contact with each stair tread) that are applied to ensure systematic sampling across the dish. In no case should neurons with weak or spotty staining (suggestive of cellular pathology or impending death) be imaged.

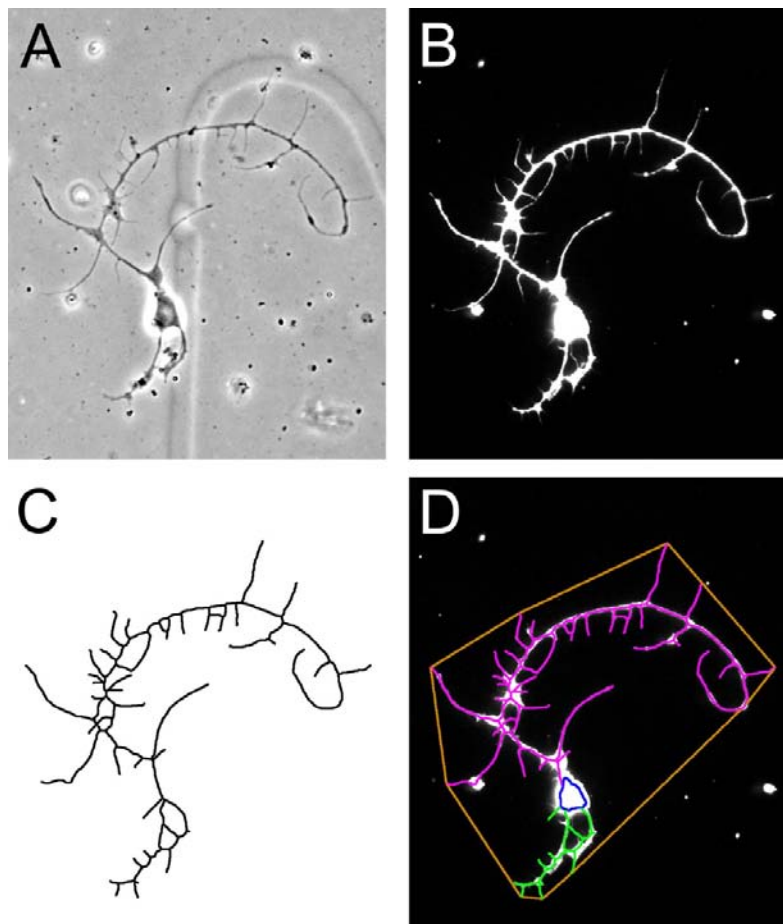


Figure 5. 2D neuron-image analysis with NeuronMetrics. Successive steps in image analysis of a neuron cultured for 3 div. Red asterisks mark the neuronal cell body. (A) Representative phase-contrast image of a live neuron acquired with 60 \times objective (numerical aperture 1.4). Part of the alphanumeric grid is visible underneath the neuron. Scale bar = 20 μ m. (B) The same neuron after fixation and immunofluorescent labeling with anti-HRP. (C) Computer graphics-assisted drawing (using Adobe Photoshop) of the neurite-arbor skeleton computed by NeuronMetrics; thickening of the NeuronMetrics-generated 1-pixel-wide skeleton aids in visualization. The cell body region has been drawn in for orientation. (D) Final graphic output from NeuronMetrics software, thickened in Photoshop to improve visibility. The neuron territory is represented by the orange polygon (a convex hull) and the neuronal cell body in dark blue. The neurite skeleton is now divided into the dominant, *i.e.*, largest, primary neurite and its branches in magenta, and the other primary neurites in green).

A representative example of an immunostained neuron suitable for NeuronMetrics image analysis is shown in Figure 5. High contrast is essential for automated skeletonization of neurons by NeuronMetrics (Narro *et al.*, 2007). Because the antigen detected by anti-HRP immunostaining is present at high levels throughout the entire neuronal membrane, the fluorescent signal is strong and uniform (Figure 5B), which is optimal for this software. In contrast, neuronal expression of cytoplasmic GFP may not give a strong enough signal unless amplified by anti-GFP labeling. In our experience, transmembrane GFP (*i.e.*, CD8-GFP fusion protein) is problematic in cultured *Drosophila* neurons because it does not yield a smooth membrane signal.

One particularly valuable module within NeuronMetrics' tool set is the assessment of neurite arbor polarity, quantified as Polarity Index (PI). High PI values indicate that the arbor has a major, or dominant, primary process, usually highly branched, with relatively few or no other primary processes. Like cultured pupal gamma mushroom body neurons, the cell shown in Figure 5 has a high PI (Figure 5D). This may reflect distinct axonal and dendritic compartments of the arbor.

Discussion

One somewhat surprising feature of our neuron culture system is the virtual absence of Repo(+) glial cells after 3 div. Because there are very few glial cells present at the time of plating (data not shown), the

problem is mainly lack of recovery rather than lack of survival. In contrast, CNS glial cells from developing brains of both mammalian and other insect species can be studied in dissociated culture, allowing the investigation of neuron-glia interactions (*e.g.*, Tucker *et al.*, 2004; Jacobs and Doering, 2010). It is possible that the mechanical trituration necessary to release neurons deep in the *Drosophila* CNS is too traumatic for the vast majority of differentiated glia to tolerate, and the small number that do survive the dissociation do not have sufficient access to glial-specific trophic factors to survive. Hence, identifying mechanical and biochemical modifications to the protocol that would permit glial cell recovery and survival remains an important goal.

To our knowledge, there has been no successful mechanical dissociation protocol developed for adult *Drosophila* CNS using trituration of enzyme-treated tissue. A single publication of a vibration-based dissociation technique reported successful isolation of adult CNS neurons for acute electrophysiological studies, but no culture for neurite outgrowth or other features of differentiation was attempted (Wu *et al.*, 2001). The lack of established methods for dissociation and culture of adult neurons limits the ability to study aging, or effects of genes or drugs on adult brain function independent of development. Hence, future efforts to adapt our protocol for studies of brain aging would allow the neuron culture system to take full advantage of *in vivo* aging neuroscience research in *Drosophila* (*e.g.*, Robertson and Keene, 2013).

Future development goals for NeuronMetrics include greater automation, *e.g.*, for cell body identification, which would speed data analysis, and a version for Mac OS. In addition, enhanced signal-detection capabilities, that would allow use of phase-contrast images or low-level fluorescent protein expression without introducing artifacts, would enable time-course data collection and eliminate immunostaining for some single-time-point experiments.

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References: Barbee, S.A., P.S. Estes, A.M. Cziko, J. Hillebrand, R.A. Luedeman, J.M. Collier, N. Johnson, I.C. Howlett, C. Geng, R. Ueda, A.H. Brand, S.F. Newbury, J.E. Wilhelm, R.B. Levine, A. Nakamura, R. Parker and M. Ramaswami 2006, *Neuron* 52: 997-1009; Bolduc, F.V., and T. Tully 2009, *Fly (Austin)* 3: 91-104; Campusano, J.M., H. Su, S.A. Jiang, B. Sicaeros and D.K. O'Dowd 2007, *Dev. Neurobiol.* 67: 1520-1532; Egger, B., L. van Giesen, M. Moraru, and S.G. Sprecher 2013, *Nat. Protoc.* 8: 958-965; Gatto, C.L., and K. Broadie 2011, *Curr. Opin. Neurobiol.* 21: 834-841; Gu, H., S.A. Jiang, J.M. Campusano, J. Iniguez, H. Su, A.A. Hoang, M. Lavian, X. Sun, and D.K. O'Dowd 2009, *J. Neurophysiol.* 101: 42-53; Halladay, A.K., D. Amaral, M. Aschner, V.J. Bolivar, A. Bowman, E. DiCicco-Bloom, S.L. Hyman, F. Keller, P. Lein, I. Pessah, L. Restifo, and D.W. Threadgill 2009, *Neurotoxicology* 30: 811-821; Inlow, J.K., and L.L. Restifo 2004, *Genetics* 166: 835-881; Jacobs, S., and L.C. Doering 2010, *J. Neurosci.* 30: 4508-4514; Jiang, S.A., J.M. Campusano, H. Su, and D.K. O'Dowd 2005, *J. Neurophysiol.* 94: 491-500; Katsuki, T., D. Ailani, M. Hiramoto, and Y. Hiromi 2009, *Neuron* 64: 188-199; Kim, Y.-T., and C.-F. Wu 1987, *J. Neurosci.* 7: 3245-3255; Kim, Y.T., and C.-F. Wu 1996, *J. Neurosci.* 16: 5593-5602; Kraft, R., M.M. Escobar, M.L. Narro, J.L. Kurtis, A. Efrat, K. Barnard, and L.L. Restifo 2006, *J. Neurosci.* 26: 8734-8747; Kraft, R., A. Kahn, J.L. Medina-Franco, M.L. Orłowski, C. Baynes, F. López-Vallejo, K. Barnard, G.M. Maggiora, and L.L. Restifo 2013, *Disease Models & Mechanisms* 6: 217-235; Kraft, R., R.B. Levine, and L.L. Restifo 1998, *J. Neurosci.* 18: 8886-8899; Küppers-Munther, B., J.J. Letzkus, K. Luer, G. Technau, H. Schmidt, and A. Prokop 2004, *Dev. Biol.* 269: 459-478; Lee, D., and D.K. O'Dowd 2000, *J. Neurosci.* 20: 2104-2111; Lee, D., H. Su, and D.K. O'Dowd 2003, *J. Neurosci.* 23: 4625-4634; Martin, R.L., B. Pittendrigh, J. Liu, R. Reenan, R. French-Constant, and D.A. Hanck 2000, *Insect Biochem. Mol. Biol.* 30: 1051-1059; Moraru, M.M., B. Egger, D.B. Bao, and S.G. Sprecher 2012, *Neural Dev.* 7: 14; Narro, M.L., F. Yang, R. Kraft, C. Wenk, A. Efrat, and L.L. Restifo 2007, *Brain Res.* 1138: 57-75; O'Dowd, D., and R. Aldrich 1988, *J. Neurosci.* 8: 3633-3643; O'Dowd, D.K., S.E. Germeraad, and R.W. Aldrich 1989, *Neuron* 2: 1301-1311; Oh, H.W., J.M. Campusano, L.G. Hilgenberg, X. Sun, M.A. Smith, and D.K. O'Dowd 2008, *Dev. Neurobiol.* 68: 281-294; Peng, I.F., B.A. Berke, Y. Zhu, W.H. Lee, W. Chen, and C.F. Wu 2007, *J. Neurosci.* 27: 12611-12622; Restifo, L.L., 2005, *Ment. Retard. Dev. Disabil. Res. Rev.* 11: 286-294; Robertson, M., and A.C. Keene 2013, *Gerontology* 59: 334-339; Rohrbough, J., D.K. O'Dowd, R.A. Baines, and K. Broadie 2003, *J. Neurobiol.* 54: 254-271; Saad, Y., M. Anabosi, S. Anava, G. Nadav, Y. Yerushalmi, and A. Ayali 2012, *J. Mol. Histol.* 43: 421-430;

Sanchez-Soriano, N., W. Bottenberg, A. Fiala, U. Haessler, A. Kerassoviti, E. Knust, R. Lohr, and A. Prokop 2005, *Dev. Biol.* 288: 126-138; Sanchez-Soriano, N., M. Travis, F. Dajas-Bailador, C. Goncalves-Pimentel, A.J. Whitmarsh and A. Prokop 2009, *J Cell Sci* 122: 2534-2542; Sicaeros, B., J.M. Campusano, and D.K. O'Dowd 2007, *J. Vis. Exp.* 200; Su, H., and D.K. O'Dowd 2003, *J. Neurosci.* 23: 9246-9253; Suzuki, N., and C.F. Wu 1984, *J. Neurogenet.* 1: 225-238; Tucker, E.S., L.A. Oland, and L.P. Tolbert 2004, *J. Comp. Neurol.* 472: 478-495; White, K., and D.R. Kankel 1978, *Dev. Biol.* 65: 296-321; Wiemerslage, L., B.J. Schultz, A. Ganguly, and D. Lee 2013, *J. Neurochem.* 126: 529-540; Winbush, A., and J.C. Weeks 2011, *Neural Dev.* 6: 15; Wu, C.-F., N. Suzuki, and M.-M. Poo 1983, *J. Neurosci.* 3: 1888-1899; Wu, L.J., Y. Lu, and T.L. Xu 2001, *J. Neurosci. Methods* 108: 199-206.

Table 1. Ordering Information for Supplies and Reagents.

Name of Item	Company	Catalog Number	Comments/Description
Antibody, Goat anti-HRP	Cappel	55973	Primary antibody, for detection of abundant <i>Drosophila</i> neuronal membrane antigen
Antibody, Donkey anti-Goat IgG Alexa Fluor 488	Molecular Probes – Life Technologies	A-11055	Secondary antibody, fluorescently tagged; adsorbed against rabbit, rat, mouse, human IgG
Antibody, donkey anti-goat IgG Alexa Fluor 568	Molecular Probes – Life Technologies	A-11057	Secondary antibody, fluorescently tagged; adsorbed against rabbit, rat, mouse, human IgG
Concanavalin A, Type IV	Sigma-Aldrich	C-2010	Dilute to 1 mg/ml in sterile purified H ₂ O, aliquot and store at -20 °C.
Coverslips, gridded	Bellco Glass	1916-91818	Photoetched, square, 18 x 18 mm, glass, #2 thickness
Coverslips, plain	Bellco Glass	1943-00012	Round, 12-mm diameter, glass, #1 thickness
DABCO	Sigma-Aldrich	D2522	1,4-Diazabicyclo[2.2.2]octane
Dishes, culture	Corning	430588	35-mm diameter x 10 mm, polystyrene, sterile
Dishes, glass-bottom	MatTek	P35G-1.5-7-C	Pre-made 35-mm culture dish with 7-mm diameter well (also comes with 10- or 14-mm); glass #1.5 thickness
Dishes, glass-bottom with grid	MatTek	P35G-2-14-CGRD	Pre-made 35-mm culture dish with grid-etched 14-mm diameter well; glass #2 thickness. Custom orders for other diameters.
FBS (fetal bovine serum)	Hyclone (sold by Fisher)	SH30071	Characterized, heat-inactivated. This reagent is the most variable from lot to lot. Request aliquots of several lots for screening to find one that supports neuronal survival and outgrowth well.
Filter Sterilization Units	Millipore	SCGPU02RE	0.22-µm, GP Express Plus Membrane
Forceps, Dumont #5	Fine Science Tools	11295-00	Standard tips, 0.1 mm x 0.06 mm, Dumostar
Forceps, fine, Dumont #5	Fine Science Tools	11252-40	Biologie tips, 0.05 mm x 0.02 mm, Titanium
Formaldehyde	Ted Pella	18505	16%, EM-grade, 10-ml glass vials
Insulin, bovine	Sigma-Aldrich	I6634	From bovine, suitable for cell culture. Alternatively, human recombinant insulin (I3536) can be used.

Laminin	VWR	47743-734	Dilute to 1 mg/mL in sterile Tris-buffered saline, pH 7.4. Aliquot and store at -80°C.
Liberase DH Research Grade (replaces Liberase Blendzyme 1)	Roche	5401054001 (2 vials X 5 mg each)	Collagenase I and II, with D ispase at a H igh concentration (hence the term "DH" in the product name). "5 mg" refers to weight of collagenase, and provides 26 Wünsch units of collagenase activity. Dissolve in 1 mL sterile water on ice, aliquot, and store at -20°C.
Minutien pins	Fine Science Tools	26002-15	0.15-mm minutien pins
Paintbrush	Ted Pella	11807	Red Sable, size 3/0 (other sizes available as well)
Parafilm M	VWR	52858-000	4" x 125 ft. roll
PVA	Sigma-Aldrich	P8136	Polyvinyl alcohol
Schneider's Drosophila Medium	GIBCO (Invitrogen Life Technologies)	21720-024	500-ml bottle. Wrap in foil and store at 4°C. Do not use after expiration date or if precipitate has formed.
Slide, Boerner	Thomas Scientific	6690-K10	10-well glass dish for dissection
Sylgard 184	Dow-Corning	4026148	Silicone elastomer kit
Triton X-100	Sigma-Aldrich	T8787	<i>t</i> -Octylphenoxypolyethoxyethanol (CAS 9002-93-1)

Table 2. Solutions.

Solution	Amount	Ingredient (*qs = quantity sufficient)	Final concentration	Instructions (*qs = quantity sufficient)
ConA/Laminin [substrate for culture dishes]	For 2.4 ml: 2 mL 400 µL 4 µL	sterile purified H ₂ O Concanavalin A, 1 mg/ml Laminin, 1 mg/ml	167 µg/ml 1.67 µg/ml	<ul style="list-style-type: none"> • Use fresh aliquots of ConA and Laminin; thaw slowly at 4°C. This batch size will coat ~24 8-mm or 16 10-mm wells (100 or 150 µL/well, respectively).
Culture medium ["S10-I"]	For 100 mL: 87.5 mL 10 mL 2.5 mL 200 µL	Schneider's Insect Medium FBS Insulin, 2 mg/mL [see recipe below] 1 N NaOH	10% 50 µg/ml	<ul style="list-style-type: none"> • Check pH of ~100 µL on test strip; should be 7.0-7.2. If needed, adjust with 1 N NaOH drop-wise [rarely necessary]. Filter sterilize through 0.22-µm filter under vacuum. • Store at 4°C for up to 3 weeks, protected from light.
Enzyme solution [to digest ECM prior to tissue dissociation]	8 µL 1 mL	Stock solution of Liberase DH (26 Wünsch units/ml) Rinaldini's saline	0.21 Wünsch units/ml	<ul style="list-style-type: none"> • See Ordering Info for stock solution instructions and storage. Make fresh; 300 µL enzyme solution per CNS sample.
Formaldehyde, 4% [fixative]	For 20 mL 5 mL 2 mL	16% Formaldehyde 10X PBS, pH 7.4	4% 10 mM NaPO ₄ 137 mM NaC	<ul style="list-style-type: none"> • qs* with purified H₂O to 20 mL • store at 4°C for ≤ 1 mo.

Ikeda Ringer's saline, calcium-free	For 1,000 mL: 7.6 g 0.35 g 0.37 g 0.1 g 0.05 g	NaCl KCl MgCl ₂ •6H ₂ O KH ₂ PO ₄ NaHPO ₄ qs* with purified H ₂ O to 1000 mL	130 mM 4.7 mM 1.8 mM 0.74 mM 0.35 mM	<ul style="list-style-type: none"> • Filter sterilize through 0.22-μm filter under vacuum. • Store at 4°C.
Insulin solution additive for culture medium]	For 2 mg/ml: 100 mg 50 ml	Insulin Water, acidified [freshly made]	2 mg/mL	<ul style="list-style-type: none"> • Aliquot (e.g., 2.5 ml x 20). • Store at -20°C.
10X PBS (Phosphate-buffered saline) [stock solution]	For 100 mL: 50 mL 8 g	0.2 M Sodium Phosphate, pH 7.4 NaCl qs* with purified H ₂ O to 100 mL	For 10X: 100 mM 1.37 M	<ul style="list-style-type: none"> • Autoclave
PTN [wash buffer]	For 200 mL: 100 mL 2 mL 20 mL	0.2 M Sodium Phosphate, pH 7.4 10% Triton X-100 1% Sodium Azide (NaN ₃) qs* with purified H ₂ O to 200 mL	0.1 M 0.10% 0.10%	<ul style="list-style-type: none"> • Store at 4°C. Note that NaN₃ is a highly toxic compound
PVA with DABCO [mounting medium with anti-fade agent]	For ~65 mL: 8 g 40 mL 20 mL 1 g	Polyvinyl alcohol 0.2 M Tris-HCl, pH 8.5 Glycerol DABCO	~12% w/v ~125 mM ~30% v/v ~1.5% w/v	<ul style="list-style-type: none"> • Stir PVA in buffer gently overnight to dissolve. • Add glycerol and DABCO, mix. Centrifuge, 1500 x g, 15 min, to pellet any undissolved PVA. • Aliquot supernatant into 1.5-mL tubes. Store at -20°C.
Rinaldini's saline [to dilute enzymes]	For 100 mL: 800 mg 20 mg 5 mg 100 mg 100 mg	NaCl KCl NaH ₂ PO ₄ •H ₂ O NaHCO ₃ D-glucose, anhydrous qs* with purified H ₂ O to 100 mL	140 mM 2.7 mM 0.35 mM 12 mM 55 μ M	<ul style="list-style-type: none"> • Filter sterilize through 0.22-μm filter under vacuum. Store at 4°C.
Sodium Phosphate buffer, 0.2 M, pH 7.4	For 1,000 mL: 5.57 g 41.2 g 900 mL	NaH ₂ PO ₄ •H ₂ O Na ₂ HPO ₄ purified H ₂ O		<ul style="list-style-type: none"> • Adjust pH to 7.4, then qs* with purified H₂O to 1,000 mL. • Autoclave.
Tris-buffered saline (TBS), pH 7.4 [to dissolve laminin]	For 1,000 mL: 6.05 g 8.76 g ~800 mL	Tris base NaCl H ₂ O, purified	50 mM 150 mM	<ul style="list-style-type: none"> • Adjust pH to 7.4 with 1 M HCl, then qs* with purified H₂O to 1000 mL. • Autoclave
Water, acidified [to dissolve insulin]	0.2 mL 99 mL	5 M HCl sterile purified H ₂ O	~10 mM HCl	<ul style="list-style-type: none"> • Adjust pH to 2.0 with 5 M HCl dropwise.



Two simple jigs for cutting olfactory trap components.

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The olfactory trap is a simple apparatus that can be used to measure innate and learned olfactory preferences (Woodard *et al.*, 1989). Each trap consists of a standard 1.5 mL microcentrifuge tube (the collection vial) with the pointed tip cut off, into which is inserted a cut 200 microliter pipette tip that is in turn wedged into a second tip, forming a double cone-shaped funnel (see Figure 1). During the assembly of many of these traps for use in various projects, we noticed that inconsistencies in the angle and length of the cuts could result in traps that allowed flies to escape the trap.



Figure 1. The olfactory trap apparatus. Flies enter the trap in response to olfactory cues originating from an odorant placed in the cap.

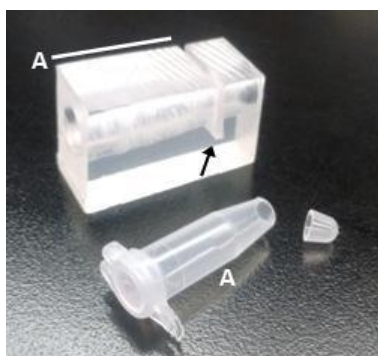


Figure 2. The microcentrifuge tube cutting jig. A 1.5 mL tube is inserted into the hole and cut at the slot indicated by the arrow. Line 'A' indicates the length of the cut.

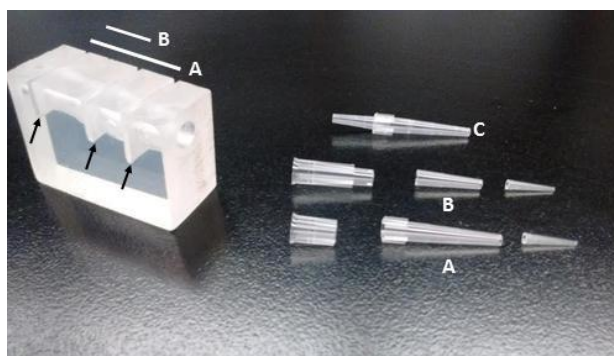


Figure 3. The pipette tip cutting jig. A 200 µL pipette tip is inserted into the hole and cut using two of the three slots (indicated by the black arrows) as guides. Two pipette tips are cut to produce the 'A' and 'B' sections, which are assembled to produce a bidirectional funnel (C). This funnel is inserted into the cut end of the microcentrifuge tube shown in Figure 2 to produce the complete trap shown in Figure 1.

Figures 2 and 3 show two jigs we now use to cut the components of the traps from pipette tips and microcentrifuge tubes accurately and reliably. The microcentrifuge tube cutting jig was made from clear $\frac{3}{4}$ " acrylic stock. After cutting the stock to length, a tapered hole was bored into the block with a series of bits to accommodate the taper of the microcentrifuge tube, and a small rasp was used to smooth the taper. A single slot was then cut through the diameter of the hole to serve as a guide for cutting the tube with a razor blade (see Figure 2). The pipette tip jig was made similarly to the microcentrifuge tube jig, except that a smaller

diameter tapered hole was drilled to accommodate the smaller diameter of the tips, and two slots were cut to allow for cutting the 200 μ L pipette tip into the two sizes required to assemble a trap (see Figure 3).

References: Woodard, C., T. Huang, H. Sun, S.L. Helfand, and J. Carlson 1989, *Genetics* 123: 315–326.



Real-time visualization software for the TriKinetics Environmental Monitor (DEnM).

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TriKinetics behavioral analysis equipment is widely used for small-insect (in particular *Drosophila*) experiments where minute by minute insect locomotor behavior can be easily monitored for days or even weeks (Rund *et al.*, 2012; Cavanaugh *et al.*, 2014). In the case of circadian-biology experiments, this involves changing environmental conditions and monitoring the resulting locomotor response in the animal, or monitoring how behavior changes in the absence of any entraining environmental cues (*zeitgebers*) (Dunlap *et al.*, 2004). However, for any behavioral experiment, verifying that no confounding experimental changes occurred is prudent. For this reason, TriKinetics has developed a *Drosophila* Environmental Monitor (DEnM) which continuously monitors light, humidity, and temperature levels and records these data in real-time to a computer spreadsheet in the same format as their behavioral monitors records locomotor activity. This format

is very useful for data processing, is compatible with the popular ClockLab analysis program, but is still cumbersome to get a real-time reading (*e.g.*, What is the temperature *now* inside the incubator?) or daily verification that conditions were held steady or an anticipated environmental change indeed occurred (*e.g.*, Did the one-hour light pulse occur between 3:00 A.M. and 4:00 A.M. as scheduled?) without navigating through a large multi-column spreadsheet.

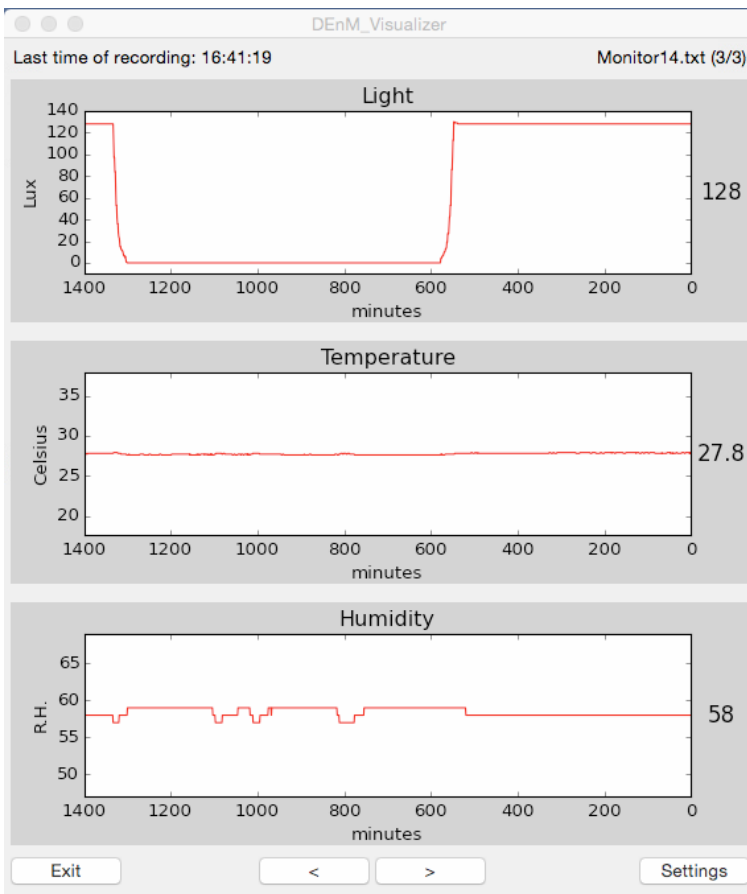


Figure 1. Screenshot of the main window of the DEnM_Visualizer program displaying the current light level (lux), temperature (Celsius), and humidity (%RH) in the incubator along with the previous 36 hours as recorded with the DEnM system.

For these reasons, we developed a tool we have called DEnM_Visualizer. DEnM_Visualizer is installed on the computer collecting data from TriKinetics units and provides real-time temperature, humidity, and light-intensity readings as