

Protocol

Methods for Exploring the Circuit Basis of Ethanol-Induced Changes in *Drosophila* Group Locomotor Activity

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Locomotion is a behavioral readout that can be used to understand responses to specific stimuli or perturbations. The fly Group Activity Monitor (flyGrAM) provides a high-throughput and high-content readout of the acute stimulatory and sedative effects of ethanol. The flyGrAM system is adaptable and seamlessly introduces thermogenetic or optogenetic stimulation to dissect neural circuits underlying behavior and tests responses to other volatilized stimuli (humidified air, odorants, anesthetics, vaporized drugs of abuse, etc.). The automated quantification and readout of activity provide users with a real-time representation of the group activity within each chamber throughout the experiment, helping users to quickly determine proper ethanol doses and duration, run behavioral screens, and plan follow-up experiments.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

All *trans*-retinal (ATR; Sigma-Aldrich, MilliporeSigma)

Store in the dark (photosensitive).

Drosophila adult flies

Ethanol (200 proof, 95%, 70%, and 50%)

Fly food <R>

Odorants (optional, see Step 41; e.g., ethyl acetate [Sigma-Aldrich 270989] or isoamyl acetate [Sigma-Aldrich W205508] dissolved in mineral oil [Sigma-Aldrich M5904] at a ratio of 1 μ L odorant to 108 μ L mineral oil)

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Supplemental material is available at cshprotocols.cshlp.org.

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Equipment

Airflow switches (manual valves; Cole-Palmer)
Air source, compressed air tank, or fish tank pump (Tetra Whisper AP100; but less power could suffice) (see Steps 1 and 7)
Aluminum foil
Arduino integrated development environment (IDE)
Beakers (250-mL)
Behavioral hood (custom-built as needed from cardboard or plastic sheets)
Behavior chambers (custom-built; see Scaplen et al. 2019 for materials and measurements)
Camera (infrared filter)
Computer (Windows operating system [OS], minimum of 8 GB of RAM, 2-GHz processor)
We recommend a minimum of four-core processor (higher clock rate is better) to ensure fast FFmpeg encoding, 16–32 GB of RAM, a high-quality USB camera that supports imaging at least 30 frames per sec and is supported by OpenCV, and Arduino (Uno: store.arduino.cc/product/A000066).
Container for fly waste
Containers (glass or polypropylene) for ethanol and H₂O delivery (custom-made chemical glassware from NDS Technologies; Corning or Fisher 250- to 3000-mL medium bottles)
FFmpeg (64-bit Zerane build, version 2015-06-05 git-7be0f48 or 2017-12-15-2d131fc)
Flow meters (Cole-Palmer)
Fly aspirator
Fly food trays (two, empty; see Step 10)
flyGrAM software and graphical user interface (GUI)
Heating tile (ceramic) with temperature sensor
Humidex sensor
In-line screw valve
Kimwipes
Light-emitting diode (LED) lighting (adjustable)
OpenCV (version 3.1) with Python bindings RC 3.0+
Plastic sheet (folded, corrugated)
Power source (12-V and 9-V)
Python (2.7, 64-bit)
Screws
Stir plate (heated) and stir bar
Thermometer
Timer (optional; see Step 39)
Tubing (primarily 1/4" and 1/16" for inlet into behavior chamber; Nalgene)
Vacuum (active)
Vacuum access (passive)
Vacuum pump
Vials (25-mm × 95-mm with 10 mL of *Drosophila* medium [as referenced in the Recipe section], for fly rearing)



METHOD

Perform all steps at room temperature unless otherwise noted.

Setting up the flyGrAM Apparatus

The flyGrAM provides flexibility for different circumstances and allows the user to define the requisite precision for their research questions. For instance, it is possible to build a fairly sophisticated laboratory apparatus for rigorous

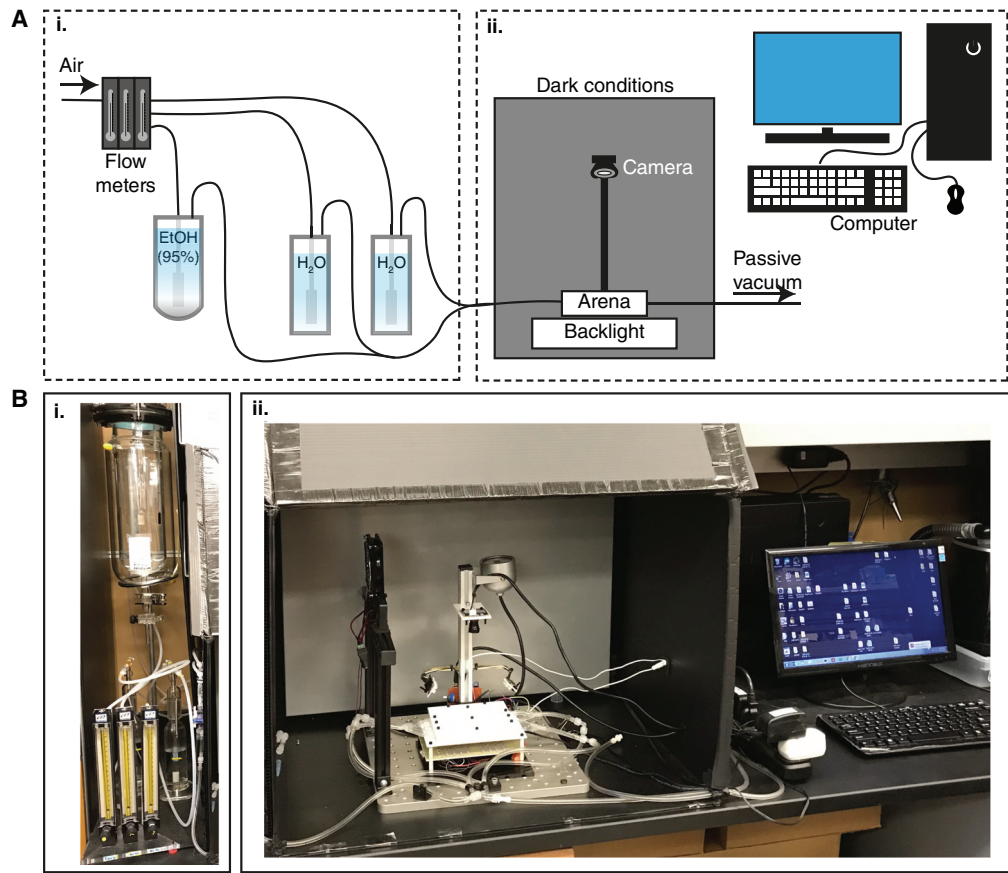


FIGURE 1. Laboratory flyGrAM setup. (A) Schematized version of the “laboratory” flyGrAM setup. (i) Air flows from a compressed air tank into three separate flow meters that control the airflow into three different chambers: one containing 95% ethanol and two different chambers containing H₂O. Ethanol flows from the ethanol-containing chamber and couples with the H₂O-containing chamber to air-dilute the 95% ethanol to desired ethanol concentrations, and to prevent desiccation of the flies within the holding chamber. The second H₂O-containing chamber is used directly to expose flies to air only. (ii) The flyGrAM apparatus itself is self-contained within a dark behavioral hood that is controlled for both temperature and humidity. The infrared filter camera tracks fly movements under dark conditions with the use of the infrared backlight underneath the behavior arenas. Airflow is funneled into the chamber, and a passive vacuum helps disperse the stimuli from the flyGrAM arenas. (B) Example of a “laboratory” flyGrAM setup. (i) A sample of the airflow setup and (ii) the flyGrAM behavior chamber.

analysis (Fig. 1) and to adapt this apparatus for classroom use (Fig. 2). Any setup used requires airflow that is bubbled through H₂O for proper hydration of the flies in the behavior chambers. We provide instructions for a laboratory setup (Steps 1–6) and a classroom setup (Steps 7–11).

Laboratory Setup

1. Using Teflon or polypropylene tubing, individually connect and adjust airflow from a compressed air tank for each stimulus with in-line flow meters.
See the example “laboratory” setup depicted in Figure 1.
2. Connect airflow to be bubbled subsequently through either H₂O (250-mL container) or the 95% ethanol reservoir (3000-mL container).
3. Join the airflow bubbled through the ethanol reservoir with the airflow bubbled through an H₂O reservoir to air-dilute the ethanol stimulus and furthermore to prevent desiccation of flies (Fig. 1Bi). Keep the air-only line separate from the airflow used for baseline, which is also bubbled through a separate H₂O reservoir.

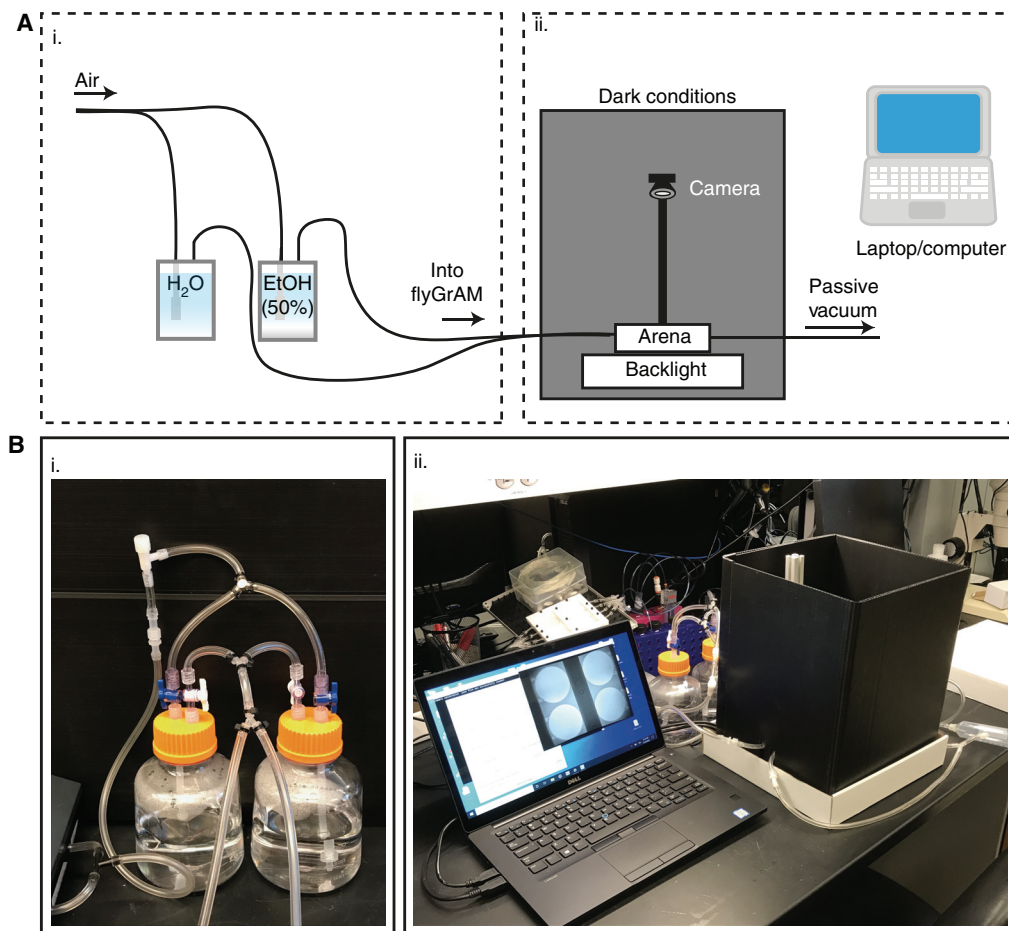


FIGURE 2. flyGrAM “classroom” apparatus. (A) Schematized version of the “classroom” flyGrAM setup. (i) Air flows from an air tank or fish-tank air pump into two separate reservoirs: one containing 50% ethanol and a chamber containing H₂O. Airflows from the ethanol-containing chamber and the H₂O-containing chamber are mixed downstream to prevent desiccation of the flies within the holding chamber. Flow rate is titrated with a flow meter and valve system. Additionally, there is not a separate line used for the air-only baseline, which could lead to contamination from prior ethanol exposures. (ii) The flyGrAM apparatus itself is self-contained within a dark behavioral hood. The infrared filter camera tracks fly movements under dark conditions with the use of the infrared backlight underneath the behavior arenas. Airflow is funneled into the chamber, and a passive vacuum helps disperse the stimuli from the flyGrAM arenas. (B) Example of a “laboratory” flyGrAM setup. (i) A sample of the airflow setup, and (ii) the flyGrAM behavior chamber.

4. Connect air-only or ethanol–air combination lines to the behavioral chambers.

Air and ethanol flowthrough the behavior chambers and are eventually cleared out with the help of a passive vacuum (Fig. 1Aii).

5. Add manual valves to control air or ethanol flow.

Although not depicted in the diagram, all tubes are controlled through manual valves and can be adjusted throughout the experiment.

6. Use a humidex sensor, a ceramic heating tile with a temperature sensor, and adjustable LED lighting (Fig. 1Bii) to create a humidity-, temperature-, and light-controlled behavior environment within a custom-build corrugated plastic behavior environment. Keep the front flap closed for a dark, quiet, humidity- and temperature-controlled environment. *Experiments are typically run under such controlled conditions.*

Proceed to Step 12.

Classroom Setup

The overall concept of the classroom design is similar to the laboratory design and appropriate for addressing how ethanol or optogenetic manipulation affects fly group locomotor activity. For more precise and specific information on apparatus measurements and parameters, please refer to Scaplen et al. (2019).

In Figure 2, we depict an example “classroom” setup that uses an optional in-line flow meter or an in-line screw valve. These rely on measuring the overall air flow rate from the air source before the H₂O or ethanol solutions.

7. Connect airflow from an air tank or fish tank air pump into H₂O and 50% ethanol reservoirs using plastic tubing.
8. Adjust the flow rate by tightening or loosening an in-line screw valve where the tubing divides (Fig. 2Bi).

When only an in-line screw valve is installed, there is not a flow meter that can remain installed with the flyGrAM setup; the overall flow rate should not be adjusted during an experiment. The other main difference here is that this setup uses more dilute ethanol and does not have an additional H₂O reservoir for ethanol hydration/airflow dilution.

9. Connect airflow from the reservoirs into the behavior chambers and from there to a passive vacuum.
10. Sandwich a folded corrugated plastic sheet between two empty fly food trays to create a dark chamber (Fig. 2Bii).
11. Use a humidex sensor, a ceramic heating tile with a temperature sensor, and adjustable LED lighting to create a humidity-, temperature-, and light-controlled behavior environment.

flyGrAM Installation Instructions

Detailed instructions for installing flyGrAM software can be found at github.com/kaunlab and in Supplemental File S1. See Discussion.

12. Install the latest flyGrAM software from GitHub on a computer with sufficient properties (github.com/kaunlab/FlyGrAM-fly_group_activity_monitor):
 - i. fly_activity_experiment_manager.py
 - ii. fly_group_activity_monitor_gui.py
 - iii. roi.py
13. Install the free and open-source Arduino IDE (www.arduino.cc/en/Main/Software).
14. Install Python Anaconda (2.7, 64-bit; repo.anaconda.com/archive/).
15. Connect Arduino “COM” to Python.
16. Use directions from www.wikihow.com/Install-FFmpeg-on-Windows to download and install FFmpeg (64-bit, static, version 2015-06-05 git-7be0f48) for the relevant OS.
17. Set up environment variables.
18. Calibrate the camera and test that the activity of a group of flies can be measured using the flyGrAM software.

Preparing ATR Food Medium (for Optogenetic Experiments)

For optogenetic experiments, prepare fresh ATR food medium and cool it for use directly before fly collection.

19. Dissolve 0.014 g (14 mg) of ATR in 0.5 mL of pure 200-proof ethanol to make a 100 mM ATR solution.

Prepare fresh ATR solution for each experiment.
20. Prepare two beakers, each containing 200 mL of standard cornmeal-based medium (fly food). Heat each beaker at 60°C–70°C on a stir plate. Stir when possible with a stir bar and monitor the temperature with a thermometer to make sure to not exceed a temperature of 70°C.

21. Add 400 μL of 100 mM ATR solution into one beaker containing 200 mL of heated standard cornmeal-based medium and mix to make a final concentration of 0.2 mM ATR food medium. Add 800 μL of 100 mM ATR solution into the second beaker containing 200 mL of heated standard cornmeal-based medium to make a final concentration of 0.4 mM ATR food medium.
The 0.4 mM ATR food medium will be used for the flies on the day before the experiment.
22. Transfer 20 10-mL aliquots of 0.2 mM ATR food medium solution and 20 10-mL aliquots of 0.4 mM ATR food medium solution to empty rearing vials.
The food will dry fairly quickly.
23. Wrap rearing vials with aluminum foil to prevent exposure to light and store them at 4°C in the dark to prevent degradation of ATR until the flies are ready to use for the experiment.
24. Repeat Steps 20–22 without ATR to make standard, cornmeal-based fly food medium. Store vials at 4°C in the dark until the flies are ready to use for the experiment.

Performing a Behavioral Experiment Using the flyGrAM

25. Warm vials to room temperature before placing flies in vials.
26. Collect adult flies under humidified CO₂ anesthesia and place them into fresh food vials ($n = \sim 30$ flies/vial; a set number of flies per vial will control for population-size-dependent effects). Store flies overnight at the same temperature and humidity conditions in which they were raised.
 - For optogenetic experiments, collect flies between 0 and 2 d (days) after eclosion and store them in dark conditions in vials with 0.2 mM ATR food medium. The day before the experiment, transfer flies to vials with 0.4 mM ATR food medium.
 - For all other experiments, collect flies between 2 and 4 d after eclosion and store them under regular lighting in vials with standard, cornmeal-based fly food medium.
Expose flies in the flyGrAM between 4 and 5 d after eclosion, and a minimum of 2 d after collection via CO₂ anesthesia to minimize potential effects that CO₂ exposure has on behavior.
27. Prior to the start of experiments, open the air tank being used to provide airflow for subsequent experiments and turn on the vacuum pump.
The inlet and outlet tubing will not be connected until just before the start of the experiment, during acclimation (mentioned in Steps 32 and 33).
28. Replace the reserves of 95% ethanol and distilled H₂O used for hydration of airflow.
Airflow is funneled in after being introduced to H₂O to prevent dehydration of the flies within the chamber and provide flies with the proper humidity.
29. Use a randomization scheme to place groups of flies within different chambers of the flyGrAM to prevent chamber-specific effects.
The randomization scheme is dependent on the number of groups, the experimental setup, and the number of experiments that will be run sequentially, etc.
30. Make sure that the camera and all flyGrAM components are properly connected to a power source and access the flyGrAM GUI to test the camera.
31. Just before the start of the experiment, gently aspirate ten flies only from a vial of 30 flies into each flyGrAM chamber with a fly aspirator following the randomization scheme.
The analysis code is optimized for ten flies, but we have tested 1–30 flies without much error.
32. Ensure that the flyGrAM behavior arena chamber is connected to the air, stimulus, and vacuum tubing (as described in Steps 1–11) and begin exposing flies to the airflow rate that will be used during the desired experimentation parameters. Adjust flow rates for airflow and stimulus accordingly using in-flow flow meters.
33. Place the flyGrAM chamber within a behavioral hood and prepare to run experiments under dark conditions.

Running experiments under dark conditions provides optimal lighting for computer vision tracking to pick up the contrast with the flies and removes the effect of visual cues on behavior, ensuring that the behavioral effect is only due to the vaporized stimuli.

34. Allow flies to acclimate in flyGrAM chambers for 5–10 min before the start of experiments, while air is being flowed through the chambers and the vacuum is connected and running.
35. During acclimation, open the flyGrAM GUI and preview video to properly center the behavioral apparatus for the camera (Fig. 3A). When centered properly, focus the lens on flies and then close the preview.
36. Take note of the number of flies within each chamber and any abnormalities observed.
The acclimation period is an optimal time to “set ROIs” using the GUI.
37. Click “set ROIs” and manually click and drag to set up rectangular regions of interest (ROI) that fully encompass each separate circular chamber (Fig. 3B). Type “n” to set each ROI. Proceed in the desired order for the ROI, sequentially from 1 to 4, and take note of this order.
Output will use the sequential number of the ROI that was created as its label in the .csv file. For instance, the third chamber that was encompassed within a rectangle and created by typing “n” will be #3 in the .csv output file. Currently four ROIs must be set to run the activity assay.
38. Set the experimentation parameters in the flyGrAM GUI (Fig. 3C).
 - i. Set the timing parameters “Experimentation duration in seconds,” “Time until stimulus onset in seconds,” and “Stimulus duration in seconds.”
We have found that recording for 2 min of baseline activity, 2 min of odor stimulus, and 1 min after the odor stimulus is more than sufficient to observe how odor presentation increases group activity. These setting options affect the final .csv file created and automated real-time feedback on motor activity given. A high-opacity red rectangle will appear during the stimulus window and should give users an idea of when the stimulus was turned on. Make sure to enter values in seconds.
 - ii. For optogenetic experiments, click “use Arduino” and set the stimulation parameters “Opto stim frequency in Hz” and “Opto stim pulse width in ms.”
We recommend trying a frequency of 30–50 Hz and an optogenetic stimulation pulse width of 20–40 msec. A variety of stimulation parameters must be tested to find ones optimal for stimulating the neurons of interest. These setting options are only necessary to link the GUI and LED array used for optogenetic stimulation to the Arduino/Raspberry Pi system for proper stimulus pulse width and frequency during the already designated stimulus period. The array should automatically turn on with the desired parameters during the stimulus window designated above.
 - iii. For video and save parameters, set “Write .AVI video?,” “Write .CSV file?,” and “FPS to enforce” and choose a directory in which to save files from the experiment.
These settings are dependent on the desired output for post hoc analysis. Automatically, most of these settings will start pre-enforced in the GUI, and the output files will be placed in a folder with the experimental time and name in the desired directory.
39. (Optional) Set up a timer.
Of note, before the start of experiment, use a timer to follow along with the experiment. This is only important if the experimental setup relies on manually turning on the stimulus at the desired time window. Although the real-time motor activity-feedback graph readout stimulus time window can be manually shifted based on stimulus, the automated readout may be delayed, depending on other concurrently running programs on the computer and its hardware specifications.
40. Following acclimation, click on “Start activity assay!” and begin the experiment (Fig. 3D).
41. Depending on the desired experiment, manually manipulate onset of the stimulus.
 - If using an ethanol or an odor stimulus, set the desired flow rate and manually turn on the ethanol or odor stimulus. Adjust the air stimulus accordingly to maintain the total flow rate into the flyGrAM chamber. Manually shift the stimulus from airflow to ethanol or odorant using in-flow valves to shift the flow to the ethanol- or odor-containing chamber. Make sure to not create an artificial vacuum by opening the ethanol in-flow valve before closing any other valve.
If any valve is closed before opening another, the pressure change can disturb the flies, disrupt tubing, and break glassware containing ethanol or double-distilled H₂O.

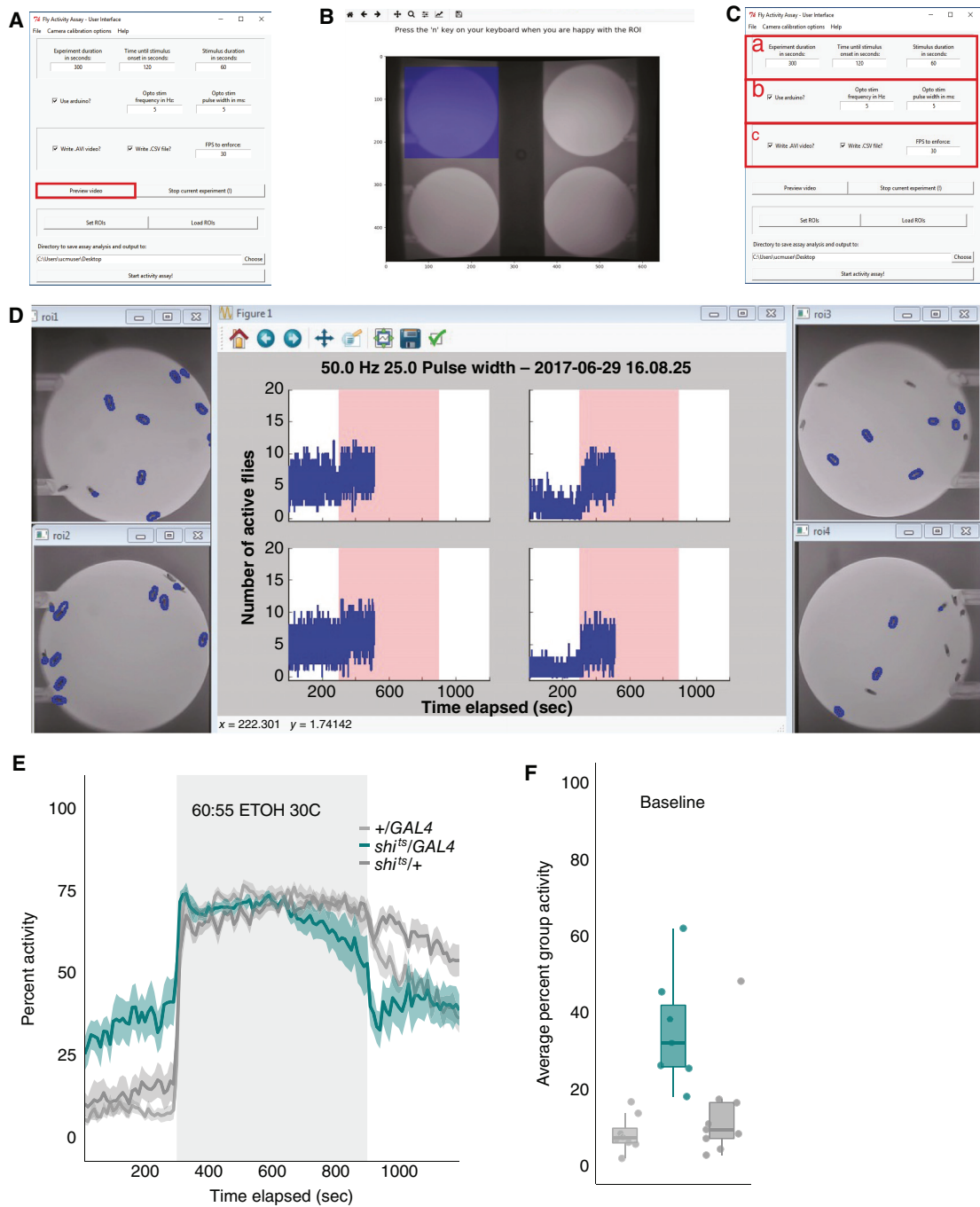


FIGURE 3. flyGrAM software and data output. (A) After placing flies in the behavior arenas, the flyGrAM GUI is opened to preview video. (B) Each region of interest (ROI) is selected by highlighting the read of the video for each arena and pressing “N.” (C) Experimental parameters are set in the flyGrAM GUI including experiment duration, time until stimulus, stimulus duration, optogenetic stimulation parameters, and frames per second to record. (D) The experiment is started and each area is observed with a corresponding timeline of activity. Flies highlighted in blue are active, and the percent of active flies is shown over time for each area. (E) Data over time are plotted using analysis methods available to download from <https://github.com/kaunlab/flyGrAM-Plotting-Analysis>. (F) The data are average over discrete time points to find differences between experimental groups.

- For optogenetic experiments without any additional odorant or ethanol stimuli, allow set parameters in the flyGrAM GUI to automatically trigger LEDs to turn on during the set stimulus time window with the desired optical stimulus frequency and pulse widths.
42. Following completion of the experiment, disconnect flow tubing and dispose of flies in the flyGrAM chamber by proper means.
Typically, we use a separate active vacuum to funnel flies into a fly-waste container with 70% ethanol.
 43. Wipe chambers with wet Kimwipes. Make sure to only use H₂O to clean these chambers.
Do not contaminate chambers with additional odorants or destroy the chamber material. This step is very important if one is running multiple sequential experiments within a day. If running multiple experiments within a day, it is important to also keep track of the time of day that experiments are typically run. Circadian rhythm has a large effect on locomotor behavior and can greatly affect the output if the experimental timing is not consistent.
 44. For subsequent experiments, repeat Steps 31–43. Take note of the time of day, temperature, vibrations, and noise in the room as these factors can all have an impact on behavior.
 45. Once all experiments are completed, disconnect flyGrAM components from their power source. Turn off the vacuum pump and close the air tank.

Data Analysis

Each flyGrAM run will produce a folder that is labeled with the date and time of the experiment. This folder contains two types of files: a recorded .avi video file of the experiment, which can be used for offline postprocessing, and .csv files for each region of interest (ROI), which denote the number of flies moving in each frame for the duration of the experiment.

46. Create a summary .csv file of one or multiple experiments that contain flies of the same genotype and identical manipulations.
47. Create a summary line graph plotting “percent group activity” over time using a custom written program in Python, “flyGrAM_analysis.py”. Save the graph as a .pdf.

Detailed instructions for how to perform this analysis can be found at github.com/kaunlab and in Supplemental File S2. Characteristic data plots and statistics are depicted in Scaplen et al. (2019) and Figure 3E,F.

DISCUSSION

Since the 1970s, *Drosophila* have proven to be an effective model system for studying the genetic mechanisms underlying ethanol preference, metabolism, tolerance, and sensitivity, and other complex behaviors. The ease of use, high-throughput screening capabilities, and the robust nature of ethanol-induced locomotor activity have been particularly helpful for investigating the mechanisms underlying acute intoxication. The remarkable similarity in ethanol-induced behaviors between *Drosophila* and mammals suggests a highly conserved effect of how ethanol affects brain function (David et al. 1976; Moore et al. 1998; Bainton et al. 2000; Scholz et al. 2000; Rodan et al. 2002; Wolf et al. 2002; Berger et al. 2004; Scholz et al. 2005; Bhandari et al. 2009; Devineni and Heberlein 2009; Ogueta et al. 2010; Gakamsky et al. 2013; De Nobrega and Lyons 2016; De Nobrega et al. 2017; Nuñez et al. 2018; Park and Ja 2020; Scaplen et al. 2020; Park et al. 2021).

The flyGrAM assay described here is one of a number of adaptable methods that provides a high-throughput method of studying activity in response to a stimulus. As new tracking software is constantly being published as open-source software or is available commercially, we encourage readers to be on the lookout for more advanced, high-resolution software that could be adapted to this system. For example, behavioral classifier programs like JAABA and individualized fly-tracking computer vision programs like Ctrax and FlyTracker have provided high-content data from flyGrAM experiments using post hoc analysis of the video recordings (Branson et al. 2009; Kabra et al. 2013;

Nath et al. 2013; Berman et al. 2014; Robie et al. 2017; Scaplen et al. 2019; Oepen et al. 2021). These types of innovations are helpful for understanding the acute effects of ethanol intoxication and the behavioral dynamics that mediate the progression toward use disorders.

When initially implementing flyGrAM, it is important to test that the setup is functioning properly and interfacing directly with the flyGrAM computer software and GUI. For ethanol-induced behaviors, we initially recommend validating the system by running a dose curve with increasing air:ethanol flow rates, concentrations, and stimulus durations. Results can be compared to dose–response curves, and to variations in behavior seen in different genetic background strains in Scaplen et al. 2019. Methods can be validated by testing mutants with known phenotypes in this assay, such as those in *amnesiac* (Wolf et al. 2002), *tank* (Devineni et al. 2013), *G-protein-coupled receptor kinase 2* (GPRK2) (Kang et al. 2020), and *alcohol dehydrogenase* (ADH) (Geer et al. 1985; Scholz et al. 2000; Ogueta et al. 2010).

When testing odor-induced changes in activity, we recommend recording for a shorter period of time during the stimulus exposure, and trying a variety of different odors and concentrations to optimize the response. We found that recording for 2 min of baseline activity, 2 min of odor stimulus, and 1 min after the odor stimulus is more than sufficient to observe how odor presentation increases group activity. We also found that both appetitive and aversive odors induce similar initial responses in group activity and that odor concentrations between 1:108 and 1:216 are typically sufficient for observing a range of odor responses. We recommend testing the response in anosmic flies (such as antennae-less flies or Or83b mutants) to ensure that the responses observed are not due to changes in air pressure as one switches from humidified air to the odor as opposed to odor sensitivity.

When testing optogenetically induced changes in activity, we recommend validating the system by testing how altering the light duration, intensity, and intertrial interval affects activity. *GAL4*-driver lines that we previously tested at the Cold Spring Harbor *Drosophila* Neurobiology course using differing stimulation parameters with positive effects include GMR15C01, GMR19G09, GMR23F03, GMR24A06, GMR27G01, and GMR21D07 (McKellar and Wyttenbach 2017). Note that flashing red light typically has effects on group locomotor activity in a variety of wild-type strains (Scaplen et al. 2019); therefore, it is important to plan for this in one's experimental design. If testing how manipulating a circuit can affect ethanol-induced changes in locomotion, we recommend trying an ellipsoid body expressing *GAL4* line (for example, 4–67) (Scaplen et al. 2019).

Validating the system with mutants or driver lines shown to have large effects on the locomotor behavior that is being investigated is important because it will inform the efficacy of the setup and help determine what parameters should be used for follow-up experiments. We have also noticed that baseline group locomotor activity is particularly sensitive to genetic background, lighting, temperature, and humidity; therefore, we recommend trying a number of different parameters until a consistent result is achieved. Startle to ethanol smell or light appears to be much less sensitive to varying environmental conditions; therefore, if there is difficulty getting consistent baseline responses, one may want to try normalizing one's ethanol- or light-induced activity curve to the startle response.

In summary, we briefly describe how to perform real-time fly activity quantitation with the added option to control optogenetic stimulation. flyGrAM allows for quantitation of fly motion (i.e., the number of flies moving in a given frame) during various experimental paradigms. Importantly, this movement quantification is done in real time (or near real time). Features of flyGrAM software include (1) it can communicate with an Arduino to control the onset time, duration, frequency, and pulse width of LED diodes for optogenetic experiments; (2) it can directly write out “raw” (corrected for camera lens barrel distortions) collected video during an experiment to “.avi” format for subsequent analysis with other more sophisticated tracking software packages (i.e., Ctrax, JAABA, etc.); and (3) it can produce real-time activity plots for four different ROIs. We encourage those interested to use new tracking and software analysis methods as they become available to improve the resolution in behavioral feature tracking.

RECIPE

Fly Food

Ingredients	Amounts (for 300-bottle batch)
Water	17 L
Agar	93 g
Cornmeal	1,716 g
Brewer's yeast	310 g
Sucrose	517 g
Dextrose	1033 g
Antifungal reagents	as appropriate ^a
Antibiotics	as appropriate ^b

^aUse 8.69 g/L sodium potassium tartrate and 26.1 mL of a Tegosept (methyl-*p*-hydroxy benzoate) Stock solution. (For the Tegosept stock solution, dissolve the Tegosept in 95% ethanol at 100 g/L.)

^bFor each batch of food, add 15 mg/L tetracycline plus one of the following two antibiotics (alternate with each batch of food): 50 mg/L ampicillin, or 17 mg/L streptomycin. Make up the antibiotics as fresh stock solutions, with tetracycline dissolved in ethanol at 10 mg/mL and ampicillin or streptomycin dissolved in water at 100 mg/mL.

Mix the agar into 13 L of water; use the other 4 L of water to wet the remaining ingredients (excluding antifungals and antibiotics). Cook the medium in a steam kettle at 15 pounds per square inch (psi) for 20 min with constant stirring. Next, switch off the steam and cover the kettle with a lid. Allow the medium to cool to exactly 55°C and then add both the antibiotics and antifungals, stirring well. Dispense medium into vials and bottles, cover with cheesecloth, and allow it to solidify overnight at room temperature before capping.

This recipe is adapted from that of Lewis (1960) and from recipe 4 from the Bloomington *Drosophila* Stock Centre website (flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm).



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