Video Article

High Throughput Assay to Examine Egg-Laying Preferences of Individual *Drosophila melanogaster*

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Abstract

Recently, egg-laying preference of *Drosophila* has emerged as a genetically tractable model to study the neural basis of simple decision-making processes. When selecting sites to deposit their eggs, female flies are capable of ranking the relative attractiveness of their options and choosing the "greater of two goods." However, most egg-laying preference assays are not practical if one wants to take a systematic genetic screening approach to search for the circuit basis underlying this simple decision-making process, as they are population-based and laborious to set up. To increase the throughput of studying of egg-laying preferences of single females, we developed custom chambers that each can simultaneously assay egg-laying preferences of up to thirty individual flies as well as a protocol that ensures each female has a high egg-laying rate (so that their preference is readily discernable and more convincing). Our approach is simple to execute and produces very consistent results. Additionally, these chambers can be equipped with different attachments to allow video recording the egg-laying animals and to deliver light for optogenetics studies. This article provides the blueprints for fabricating these chambers and the procedure for preparing the flies to be assayed in these chambers.

Video Link

The video component of this article can be found at https://www.jove.com/video/53716/

Introduction

Drosophila melanogaster is a powerful genetic model organism to study the neural basis of behaviors. The rapid developments of genetic tools to manipulate neurons in a targeted manner and the emergence of sophisticated behavioral analysis tools have significantly improved our ability to dissect the circuit mechanisms that underlie the sensory-motor transformation processes of several innate and learned behaviors[1-3].

Drosophila egg-laying is a suitable model to study the neural basis of simple decision making processes. In particular, *Drosophila* females have been shown to possess the ability to compare and rank their options before "committing" to depositing an egg onto a given option[4-8]. For example, when given only a plain (sucrose-free) substrate or only a sucrose-containing substrate, females readily accept either option for egg-laying. However, when presented with both options, females robust reject the sucrose substrate in some contexts[7,9,10]. Relatively little is known about the neural mechanisms that allow females to "choose the greater of two goods", however. A major obstacle has been the lack of an efficient method to assay egg-laying preferences such that one can use a systematic genetic screening approach to study this problem.

In this report, we describe the protocol we developed that allows egg-laying preferences of females to be assayed at single-animal resolution and with substantially improved throughput and consistency over previous methods. Specifically, we provide the blueprints for constructing the chambers we designed, the protocol for preparing the females so that each is primed to lay many eggs, and the protocol for using the chambers.

Protocol

1. Preparing Flies to be Assayed

1. Culture flies on standard molasses/cornmeal media at incubator set at 25 °C and 65% humidity. Take care to not overcrowd the vials. For example, put 8 females and 6 males into a narrow food vial.
   Note: The "narrow food vial" used here has an inner diameter of 2.3 cm. We typically dispensed about 10 ml of fly food into each vial. The fly food recipe we used is described here: http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/molassesfood.html.

2. 2 - 3 days after females eclosion, prepare vials with fresh yeast paste as shown in **Figure 1E**. Make fresh yeast paste by mixing 6 g of active yeast with 10 ml of 0.5 % proprionic acid. Use a spatula to apply the yeast paste onto the sidewall of the food vials. Collect 30 - 35 eclosed females together with 20 - 25 males into the vials.
    1. In general, collect the males from the same vials where the females are collected (so as to save time and effort). However, if there is concern about the fertility of males, use WT males instead. Also, yeast paste is important for stimulating egg production therefore prepare it fresh daily.
3. Maintain the collected females/males at 25 °C, 65% humidity, unless the flies are temperature sensitive.
4. After ~4 - 5 days, check the vials to see if females are ready for egg-laying experiments. They are ready when the surface of the food medium is wet from larvae actively burrowing in the food (see **Figure 1E - F**).
   Note: Females typically withhold laying eggs once the food surface becomes wet and crawling with larvae. This step ensures that females would be ready to lay many eggs when they encounter substrates with desired texture (*e.g.*, ~1% agarose).

2. Chamber Construction, Assembly, Assay Setup

1. Have a machine shop build acrylic egg-laying chambers (**Figure 1A - D**). The engineering drawing of different pieces were shown in **Supplementary Figure 1 - 3**. High resolution pictures can also be found here (http://www.rebeccayang.org/pdf/chamber%20design.pdf).
2. Insert plastic sheets into loading (top) piece of the chamber as shown in **Figure 1C**. This serves as the bottom surface while loading flies into individual egg-laying arenas.
3. Anesthetize females on a CO_2 pad and load them individually into each egg-laying arena. Allow ~30 min for flies to recover from the CO_2 and to become acclimated to the new environment.
4. Prepare the agarose substrates.
    1. For convenience, keep a premade bottle of melted 1% agarose in a 55 °C water bath.
    2. Add the desired amount of stock sucrose solution (2 M) into a 50 ml conical tube and mixing it with appropriate amount of agarose. For example, to prepare the 150 mM sucrose substrate, place 750 µl of 2 M sucrose solution into the tube and then fill the tube with agarose to the 10 ml mark.
    3. Prepare the plain substrate in the same manner but add distilled water instead of sucrose solution.
       Note: The final concentration of agarose in this protocol is slightly less than 1%. In our experience, the exact concentration of agarose does not matter so long as it is controlled to be within ~0.9 - 1.1% and that the two substrates are of the same agarose concentration.
5. Take the substrate (bottom) piece of the chamber and pipette 1,000 µl of agarose substrate into each trough as seen in **Figure 1D**.
6. Allow agarose to solidify for ~30 min.
7. Once the agarose substrates and flies are ready, assemble all three pieces of the egg-laying chamber and then take out the plastic sheets.
8. Place the chambers in fly incubators.
   Note: Length of egg-laying experiments can vary depending on experimental needs. We typically run the experiment O/N (14 - 16 hr). Also, no significant impact of circadian timing on egg-laying preferences was observed.
9. Anesthetize females by injecting CO_2 into the chamber. Disassemble the chamber, discard the anesthetized flies into fly morgue (*i.e.*, an empty coffee can filled with some corn oil). Take pictures of the results for recordkeeping (see **Figure 2**).
10. Count the number of eggs manually and calculate preference indices for analysis. Calculate preference index as $(N_a - N_b)/(N_a + N_b)$ where N_a and N_b represent number of eggs on site a vs. site b, respectively.

Representative Results

The egg-laying chambers are composed of several pieces: a substrate (bottom) piece, a divider (middle) piece, a loading (top) piece, and 2 sliding doors (**Figure 1A - D**). These pieces are used to independently setup flies and substrates before egg-laying experiments. **Figure 1F** shows how vials should look when female flies are ready for egg-laying. When flies are given a choice between a plain substrate and a sucrose-containing substrate, females robustly preferred the plain substrate for egg-laying as shown in **Figure 2**.

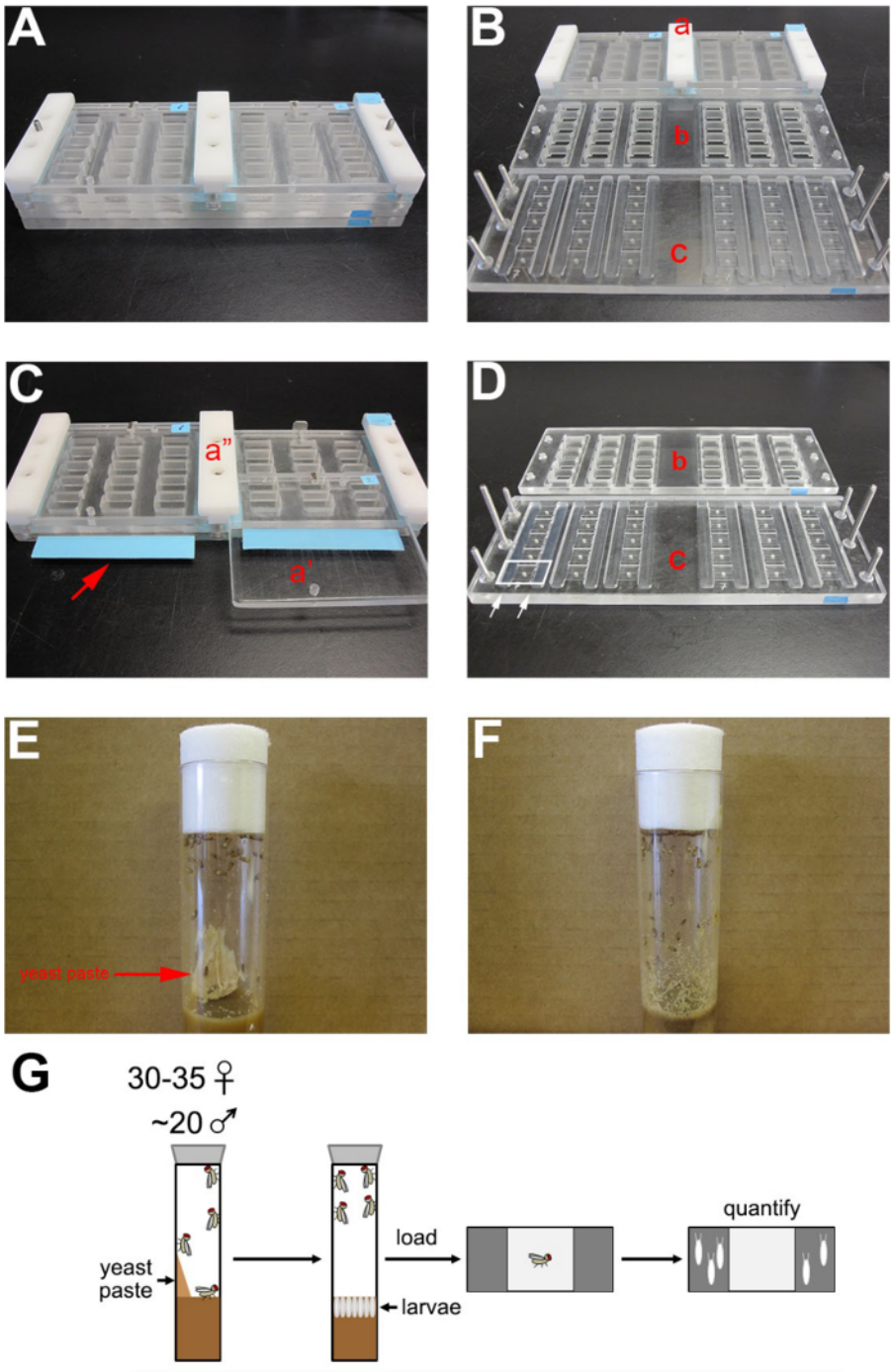


Figure 1. Egg-laying Chambers and Egg-laying Protocol. (**A**) Fully assembled egg -laying chamber. (**B**) Disassembled pieces. a: the loading piece (top), b: the divider piece (middle), c: the substrate (bottom) piece. Engineering drawings of these pieces are shown in **Supplementary Figure 1 - 3**. (**C**) The loading piece of the chamber. a': sliding doors, a": rails. The plastic sheets are inserted into the loading piece to serve as a floor to keep the loaded flies in place. We typically put color tapes onto the edge of the plastic sheets (red arrow). (**D**) The substrate (bottom) and the divider (middle) pieces of the chamber. Agarose is deposited into individual troughs to serve as egg-laying substrates (arrows). Quadrilateral outlines the egg-laying arena for a single fly. (**E**) Day 0 of collected females/males in a yeasted vial. (**F**) Day 4 - 5 of collected females/males in a yeasted vial. Note that larvae and adult females have eaten most of the yeast and the surface of the food has become occupied by larvae. The wet surface food crawling with larvae prevents females from laying more eggs in the vial. (**G**) Schematic depicting the protocol for setting up egg-laying behavior experiments. (Please click here to view a larger version of this figure)

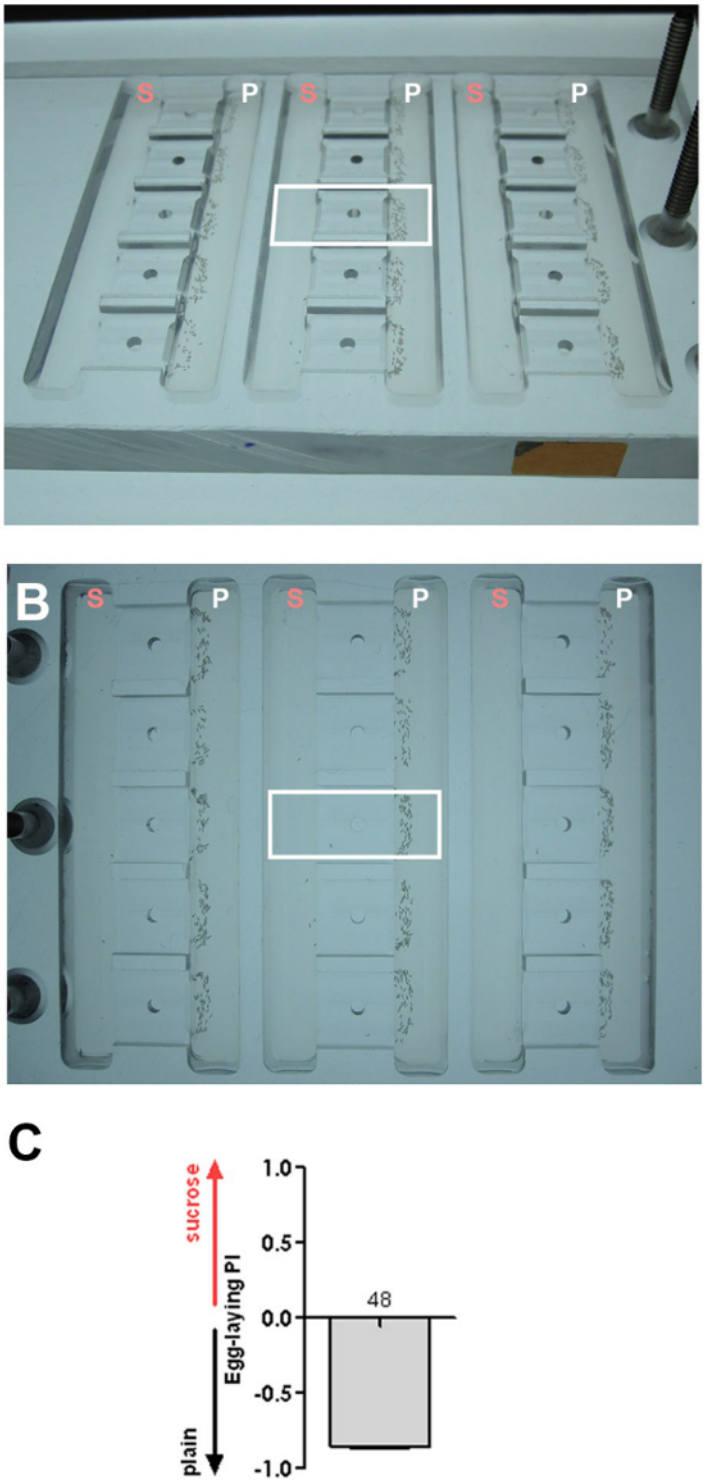
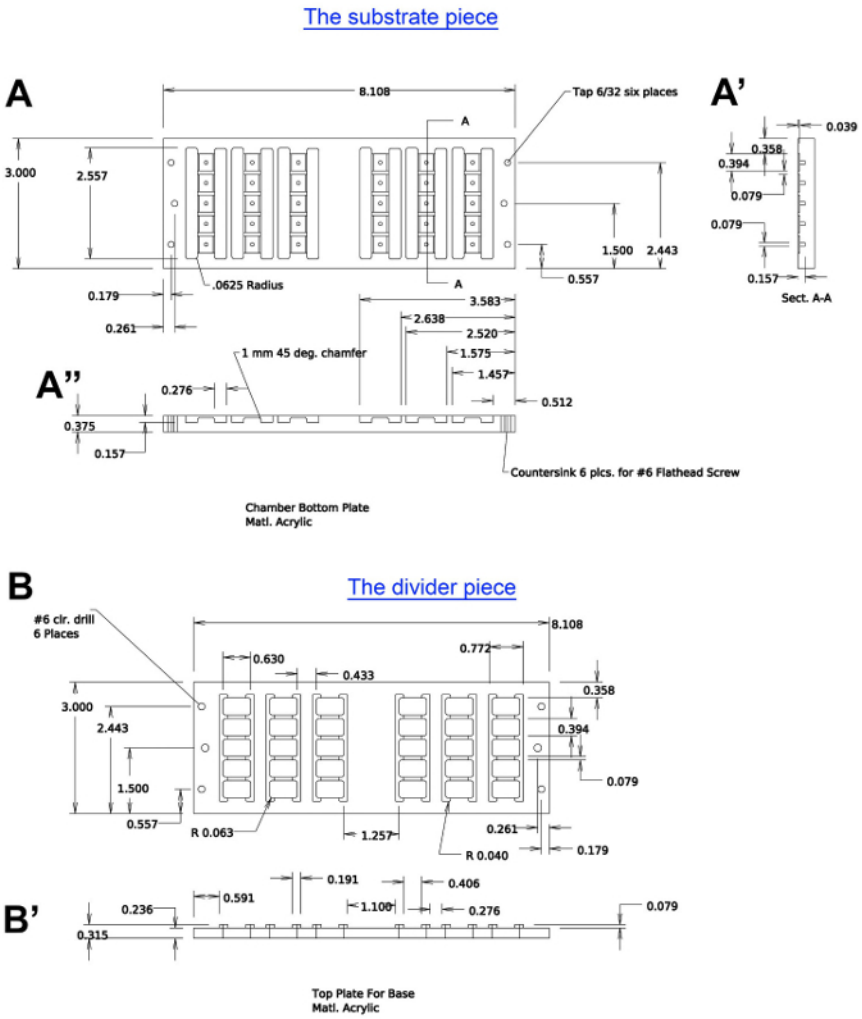
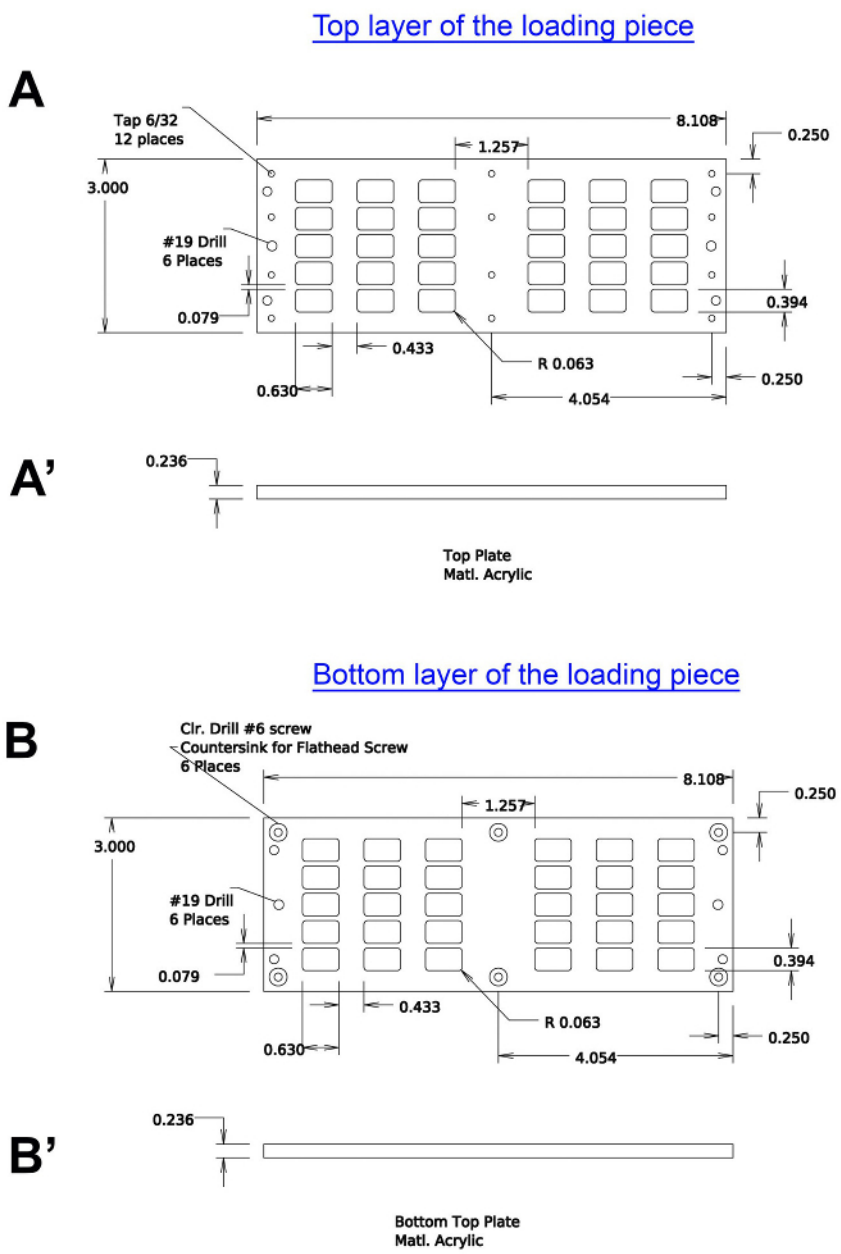


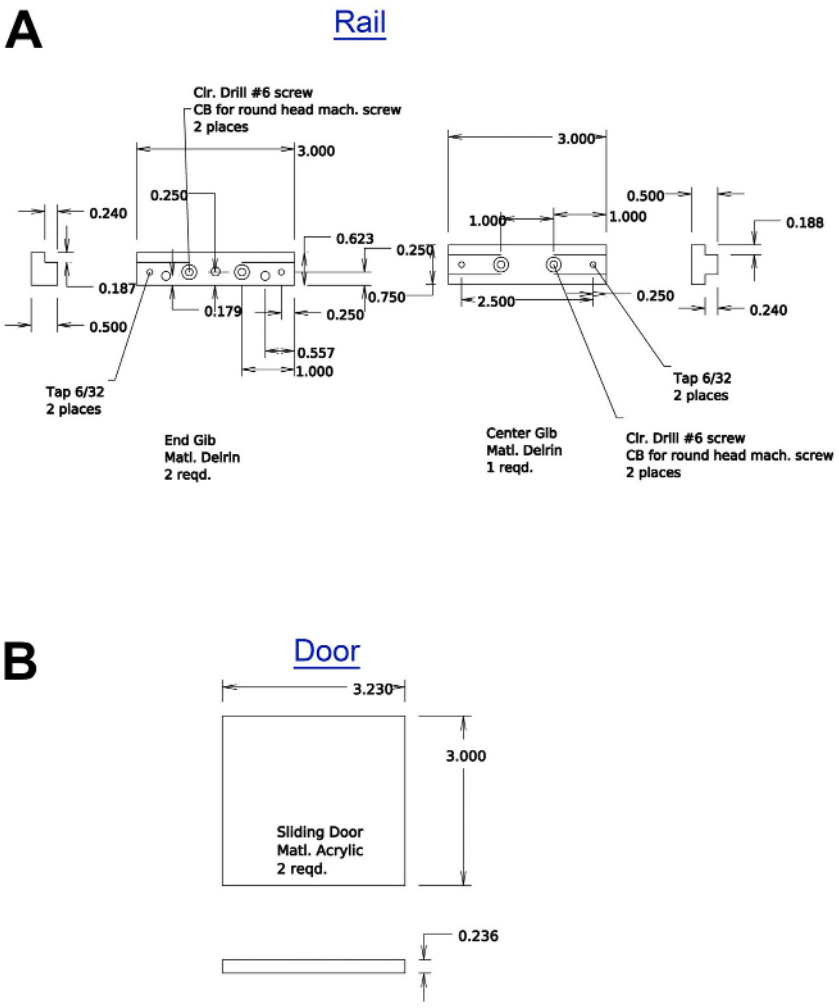
Figure 2. Representative Egg-laying Results for Flies Choosing between Sucrose and Plain Substrates. (**A**) Side view of egg-laying results of wild type flies when they were given a choice between sucrose (150 mM) and plain substrates. White box outlines one egg-laying arena for a single fly. (**B**) Top view of egg-laying results. White box outlines one egg-laying arena for a single fly. (**C**) Preference index (PI) of wild type flies when asked to choose between a sucrose-containing substrate and a plain substrate. PI for each female is calculated as follows: (number of eggs on sucrose substrate - number of eggs on the plain substrates)/total number of eggs. Error bar indicates SEM. (Please click here to view a larger version of this figure)



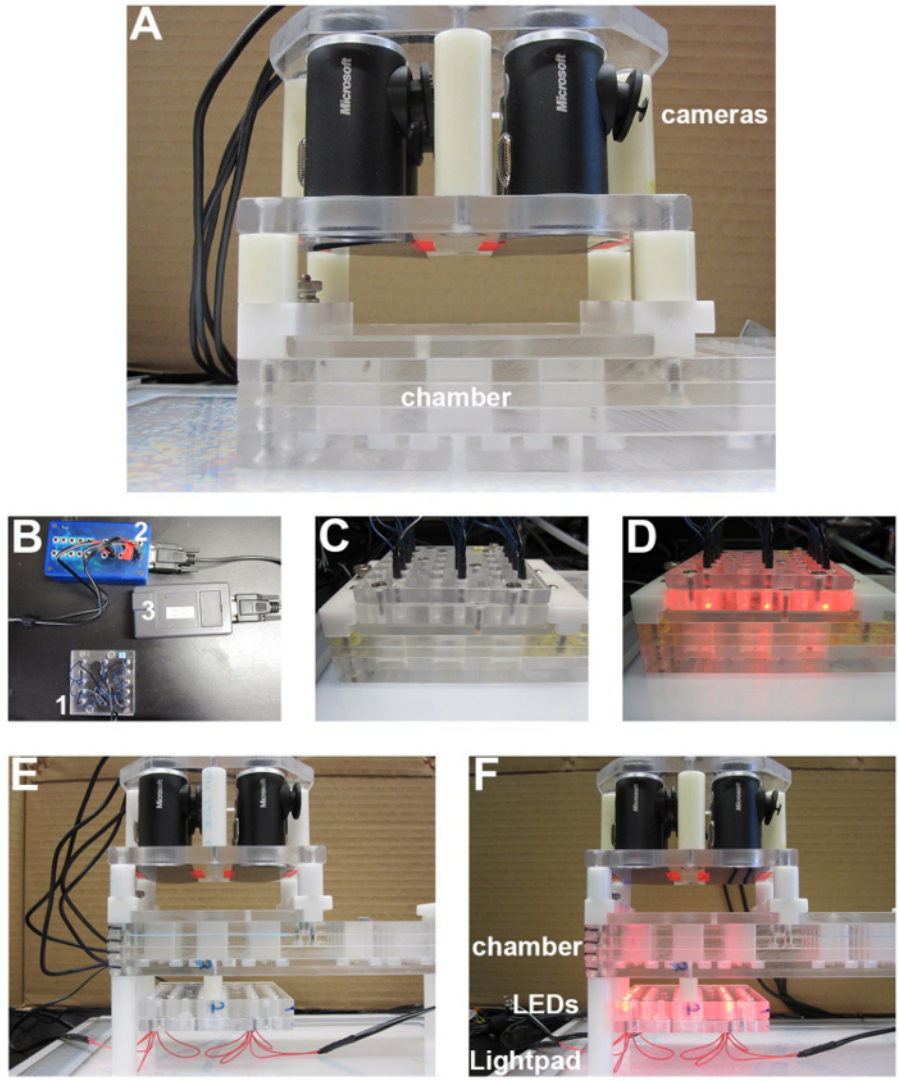
Supplementary Figure S1. Engineering Drawings for the Divider and the Substrate Piece of the Chamber. (**A - A"**) Different views of the substrate piece of the chamber. (**B - B'**) Different views of the middle divider piece of the chamber. (Please click here to download this file)



Supplementary Figure S2. Engineering Drawings for the Loading Piece of the Chamber. (**A - A'**) Different views of the upper layer of the loading piece of the chamber. (**B - B'**) Different views of the bottom layer of the loading piece of the chamber. (Please click here to download this file)



Supplementary Figure S3. **Engineering Drawing for the Sliding Door and the Rail on the Chamber.** (**A**) The rail fixed onto the top piece of the chamber. (**B**) The sliding door for the chamber. (Please click here to download this file)



Supplementary Figure S4. Accessories and Customization of Egg-laying Chambers. (**A**) Egg-laying chambers equipped with cameras to video record flies during egg-laying experiments[13]. Ctrax[14] with extension[12] is used to track the flies and custom MATLAB code is used to plot the trajectories. (**B**) Setup to illuminate egg-laying substrates with red light. LEDs (1) are connected to a LED driver (2). LED intensity is controlled by microcontroller (3). For more information about the camera, light delivery system and tracking animals in abnormal lighting conditions, see Zhu *et al*. and Stern *et al*. (**C**) Red LEDs (light off) attached to top of egg-laying chambers. (**D**) Red LEDs (light on) attached to top of egg-laying chambers. The LED intensity of red LEDs is typically set to be around 7 - 10 µW/mm when CsChrimson[15] is used to activate the neurons. (**E**) Egg-laying chambers equipped with cameras and LEDs to video record flies during experiments with red light (light off). (**F**) Egg-laying chambers equipped with cameras and LEDs to video record flies during experiments with red light (light on). (Please click here to download this file)

Discussion

The chambers and protocols described here have several improvements over previous egg-laying assays. First, they increase the throughput of assaying preferences of single animals significantly. Each chamber can assay 30 single females and it takes less than an hour to set up. Second, they increase the consistency of the egg-laying preferences over previous methods. The standardization of the dimensions of the arena, size of the egg-laying substrates, and distance between substrates makes it easier to compare the results between different experimental days and from different research groups. Third, the chambers can be fitted with attachments to accommodate additional analysis one wants to pursue (**Supplementary Figure 4**). For example, for behavioral analysis, video cameras can be attached to the top of the chamber to record the flies[7,11-13]. Most importantly, this approach is scalable. These chambers are relatively inexpensive to produce with the help of a machine shop. Also, it typically takes a skilled worker less than 2 hr to set up 5 chambers (150 single females) worth of egg-laying assays.

One of the most critical factors when studying egg-laying preference of single females is to ensure that each female is primed to lay many eggs. (A preference of "50 vs. 0 egg" is much more convincing than a preference of "1 vs. 0 egg"). This protocol, when properly executed, should allow regular wild-type flies (Canton S and w1118) to lay at least 50 eggs/per female O/N. Several factors contribute to producing females with high egg-laying rate. First, one should take care to not overpopulate the vials/bottles when culturing the females to be assayed. Overcrowding often produces smaller larvae, which would grow into smaller females that lay fewer eggs. Second, one should make sure that females to be assayed

have access to plenty of yeast paste when mixed together with males in the food vials. It is recommended that 0.5% proprionic acid instead of water be used to prepare yeast paste because proprionic acid not only increases egg-laying but also reduces fungal infection in vials. Third, one should not assay the females until the larvae/food surface of the food vials look like the picture shown in **Figure 1F**; if not sufficiently egg-laying deprived, females would not lay as many eggs when assayed in the chambers. Also, note that the food vials used here are the "narrow vials". If one uses vials with larger diameter, one needs to increase the number of females/males to be placed in the vial so that the females would be ready to be assayed in 4 - 5 days.

While the protocol described here specifically focuses on examining females' preferences between sucrose and plain substrates, it can be adapted to study females' preference in other conditions. For example, these chambers can be used to assay flies' preference when choosing between harder and softer agarose (*e.g.*, 1% vs. 1.5% agarose), as well as substrates that differ in other chemosensory cues (*e.g.*, 3% acetic acid). If fitted with lid that contains LED to illuminate one or both substrates, the chamber may also provide an efficient platform to conduct optogenetic studies (**Supplementary Figure 4).**

Finally, it is worth noting that although this setup allows higher throughput for analyzing egg-laying preferences of *Drosophila*, it has some important limitations. First, the fixed dimension of the chambers limits the flexibility of the behavioral assay. For example, one will need to make new chambers in order to test how other parameters (*e.g.*, distance between substrates) may affect the egg-laying preferences of flies. In addition, this system does not capture the complex environment *Drosophila* encounter in the wild. It is very rare that flies will have to decide between a pure sucrose substrate vs. a pure plain substrate for egg-laying in nature. Thus while our assay is efficient in studying the neural basis of a simple decision task, one needs to be aware that the specific "decisions" females make in our chambers may not be ethologically relevant.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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