TITLE:
A Rapid Food-Preference Assay in *Drosophila*

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SUMMARY:
We present a protocol for a two-choice feeding assay for flies. This feeding assay is fast and easy to run and is suitable not only for small-scale laboratory research, but also for high-throughput behavioral screens in flies.

ABSTRACT:
To select food with nutritional value while avoiding the consumption of harmful agents, animals need a sophisticated and robust taste system to evaluate their food environment. The fruit fly, *Drosophila melanogaster*, is a genetically tractable model organism that is widely used to decipher the molecular, cellular, and neural underpinnings of food preference. To analyze fly food preference, a robust feeding method is needed. Described here is a two-choice feeding assay, which is rigorous, cost-saving, and fast. The assay is Petri-dish-based and involves the addition of two different foods supplemented with blue or red dye to the two halves of the dish. Then, ~70 prestarved, 2–4-day-old flies are placed in the dish and allowed to choose between blue and red foods in the dark for about 90 min. Examination of the abdomen of each fly is followed by the calculation of the preference index. In contrast to multiwell plates, each Petri dish takes only ~20 s to fill and saves time and effort. This feeding assay can be employed to quickly determine whether flies like or dislike a particular food.

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INTRODUCTION:
Despite dramatic differences in the anatomical structure of taste organs between flies and mammals, the flies' behavioral responses to many tastant substances are strikingly similar to those of mammals. For example, flies prefer sugar[1–8], amino acids[9,10], and low salt[11], which indicate nutrients, but reject bitter foods[12–15] that are unpalatable or toxic. Over the past two decades, flies have proven to be a highly valuable model organism for advancing the understanding of many fundamental questions related to taste sensation and food consumption, including tastant detection, taste transduction, taste plasticity, and feeding

regulation[16–20]. Remarkably, a number of studies have demonstrated that the taste transduction and neural circuit mechanisms underlying taste perception are analogous between fruit flies and mammals. Therefore, the fruit fly serves as an ideal experimental organism, enabling researchers to uncover evolutionarily conserved concepts and principles that govern food detection and consumption in the animal kingdom.

To investigate taste sensation in flies, it is critical to establish a fast and rigorous assay to objectively measure food preference. Over the years, various feeding methods, such as dye-based assays[11–13,21–23], the fly proboscis extension response assay[24], the Capillary Feeder (CAFE) assay[25,26], the Fly Liquid-Food Interaction Counter (FLIC) assay[27], and other combinatorial methods have been developed to quantitatively measure food preference and/or food intake for fruit flies[28–31]. One of the popular feeding paradigms is the dye-based two-choice feeding assay using either a multiwell microtiter plate[12,21,32] or, as described here, a small Petri dish[11,22] as the feeding chamber. This assay is designed based on the transparency of the fly's abdomen. During this assay, flies are placed into the feeding chamber and presented with two food options mixed with either red dye or blue dye. Once the assay is complete, fly abdomens appear red or blue depending on which food they have consumed.

Both the Petri-dish and the multiwell-plate dye-based feeding assays are highly robust and yield approximately the same results. Using these two assays, numerous important discoveries and breakthroughs have been made toward deciphering the highly diversified receptors and cells responsible for sensing food tastes and food texture[11,12,21,22,32,33]. In the dye-based assay, one experimental step requiring considerable time and effort is preparing and loading food into the feeding chamber. To reduce the food preparation and loading time, this assay was modified by replacing the multiwell microtiter plate with a small Petri dish, which is divided into two equal compartments. In the Petri-dish-based assay, two different foods supplemented with blue or red dye are added to the two halves of the dish. Then, ~70 prestarved, 2–4-day-old flies are placed in the dish and allowed to choose between blue and red foods in the dark for about 90 min. The abdomen of each fly is then examined, and the preference index (PI) is calculated.

This Petri-dish-based two-choice feeding assay is affordable, simple, and fast. One multiwell plate requires approximately 110 s to fill, whereas each Petri dish takes only ~20 s. In addition, the multiwell plate requires pipetting small volumes of food into a large number of small wells (e.g., 60 or more wells per plate), which demands considerable precision and attention. Conversely, the Petri-dish-based assay requires only two actions per plate. As the feeding assay can involve a large number of replicates, the Petri-dish-based assay saves a nontrivial amount of time and effort. This assay gives results equivalent to those from the multiwell-based assay and has proven successful in addressing many fundamental questions in taste sensation, including salt taste coding[11], taste plasticity modified by food experience[22], and the molecular basis of food texture sensation[33]. In summary, this Petri-dish-based two-choice assay is a powerful tool to investigate how flies perceive external and internal nutrient milieus to elicit appropriate feeding behavior.

PROTOCOL:

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1. Assembling the assay chambers

NOTE: While this protocol describes the use of a 35 mm Petri dish (**Figure 1A**), the desired effect can be achieved using any watertight, smooth-bottomed vessel that can be bisected and covered.

1.1. First, bisect a lidded 35 mm Petri dish by fixing a length of plastic (5 mm in width and 3 mm in height) down the midline with waterproof adhesive, forming two watertight compartments. Confirm that the seal is complete to avoid leakage that can lead to mixing of the two food substrates being assayed.

NOTE: After assembly, reuse this apparatus as long as the seal holds.

2. Preparing starvation vials

2.1. Prepare a sufficient number of empty plastic fly vials; then, loosely compact a piece of tissue paper at the bottom. Compress the tissue paper enough that it fills the space, but not so much that it forms a dense mass.

NOTE: Make sure that there are no deep crevices or folds in the tissue, as this can lead to flies getting trapped.

2.2. Add ~3 mL pure water to the vial so that the tissue is completely saturated, but there is no standing water. Ensure that there are no large droplets of excess water on the wall of the vial. Alternatively, substitute agarose for the soaked paper by preparing a 1% w/v agar solution (without sucrose) by adding 5 mL of 1% agarose to each empty vial and allowing the agarose to solidify at room temperature.

3. Wet starvation of flies prior to the experiment

3.1. Initiate starvation 24 h before the time of the experiment. Under CO_2 anesthesia, sort groups of ~70, 2–4-day-old flies into the prepared starvation vials, labeling each vial with the genotype and time of starvation.

4. Reagent setup

4.1. Preparation of dyes

NOTE: Prior to performing any experiments, it is important to perform a preliminary control assay to determine the correct concentrations of red and blue dyes to use.

4.1.1. For the control assay, prepare a range of dilutions for each dye, and perform the feeding assay with the same food with a different dye color. Use the results to identify two-dye

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concentrations (one red, one blue) that yield a PI of ~0 when no experimental compound is added (see section 7).

NOTE: For example, the final blue dye concentration was fixed at 50 µM and tested against a series of red dye concentrations. Based on the red dye dosage curve, the optimal red dye concentration was 210 µM, which gave minimal dye bias (**Figure 1B**). A higher red dye concentration drives flies to prefer red food, whereas a lower concentration drives flies to prefer blue food. Carefully refine blue or red dye concentrations in increments of 1 µM, as differences of this magnitude and greater can affect experimental outcomes.

4.2. Preparation of 1% agarose

4.2.1. Combine 0.5 g agarose and 50 mL of pure water (or some multiple thereof) in a microwave-safe vessel. Microwave the agarose solution until dissolved, stirring it as needed.

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4.3. Preparation of other food components

4.3.1. Dissolve each food component, including sucrose and any experimental compounds, in water at a 100-fold or higher concentration of the final tested concentration.

NOTE: The total volume of each food ingredient added to 1% agar should not exceed 1 mL per 10 mL molten agar. Otherwise, the agarose may be too dilute and will not solidify appropriately.

4.4. Preparation of food media

4.4.1. Mix agar, dye, and the desired experimental compound in conical polypropylene centrifuge tubes (15 or 50 mL); use water instead of the experimental tastant in the control food. Do this while the agar is still completely liquid, and mix thoroughly using a vortex mixer. Keep the tubes in a 60 °C water bath while not in use to prevent the agarose from hardening before being distributed into dishes.

4.5. Preparing dishes for the experiment

NOTE: Ensure that all dishes are completely dry before starting.

4.5.1. Pipette 1 mL of red experimental food medium into one side of the assay dish (**Figure 1A**); repeat for the desired number of dishes. Allow the agarose to cool until firm (3–5 min), and then pipette 1 mL of blue control food into the other side of the dishes (**Figure 1A**). Repeat this process with the control red/experimental blue pair.

NOTE: Make sure all dishes are fully set before beginning the experiment. Use the dishes within 30 min.

5. Initiating the two-way feeding assay

5.1. Temporarily paralyze experimental fly lines on ice until no obvious motor activities such as flying and climbing are observed. Once the flies are immobilized, gently invert the vial, and tap to transfer all the flies into the assay chamber.

NOTE: Cold shock takes ~3–5 min. Prolonged exposure to cold may affect the fly's physiology and health and should therefore be avoided.

5.2. Quickly place the cover on the chamber and set it aside. Once all the flies have been transferred, move all chambers to a dark, enclosed space. Allow the assay to run for 90 min.

NOTE: A dark environment minimizes the influence of the fly's visual pathway on feeding behavior and removes any environmental cues from outside the dish.

6. Terminating the two-way feeding assay

6.1. After 90 min have elapsed, transfer the chambers to a -20 °C freezer to sacrifice the flies. After ~1 h, count the flies.

NOTE: Invert each Petri dish before placing the dish in the freezer to ensure that no flies will be frozen onto the food.

7. Assigning a preference index (PI) to determine food preference

7.1. Under a standard dissection microscope, examine the flies' abdominal color in each individual dish. Count the flies as either red, blue, or purple according to the color of their abdomen (**Figure 2A**). Count the fly if its abdomen is more than 50% colored, indicating robust feeding (**Figure 2B**). Exclude the fly if its abdomen contains only a tiny food spot, indicating poor eating (**Figure 2C**).

7.2. After the numbers of flies eating blue, red, or both blue and red foods have been counted, use the following equation to assign each Petri dish a preference index (PI):

$$PI = \frac{(Number\ of\ flies\ eating\ experimental\ food) - (Number\ of\ flies\ eating\ control\ food)}{(Number\ of\ flies\ eating\ experimental\ food) + (Number\ of\ flies\ eating\ control\ food) + (Number\ of\ flies\ eating\ both)}$$

PI > 0 indicates a preference for the experimental compound, PI < 0 indicates an aversion to the experimental compound, and PI = 0 indicates no effect of the compound on feeding behavior.

8. Cleaning the assay chambers

8.1. Promptly clean the Petri dishes by scraping out the food substrate and rinsing them with unscented soap and water. Soak the Petri dishes overnight in distilled water. Check that the dividing seal in each dish is still watertight, then let the dish air dry.

NOTE: After ensuring that there is no residual agarose or dye staining, the Petri dishes are ready to use again.

REPRESENTATIVE RESULTS:

In this assay, a 35 mm dish was divided into two equal feeding compartments, with each half of the dish containing agarose food coupled with either blue or red dye (**Figure 1A**). To exclude dye bias, the blue and red dye concentrations were carefully refined to yield an approximate "0" PI when only these two dyes were added (**Figure 1B**). Once the Petri dish was loaded with tested food, ~70 wet-starved, 2–4-day-old adult flies were transferred to the dish, allowing them to choose between the two food options in the dark. After 90 min, the flies' abdominal color was examined with a dissection microscope. Typically, the fly abdomen appears blue or red if the animal predominantly consumes blue or red food (**Figure 2A**), respectively. If the fly consumes both blue and red, its abdomen turns purple (**Figure 2A**).

The flies ingesting considerable amounts of food were scored (**Figure 2B**), while skipping the flies with insufficient food intake (**Figure 2C**). This Petri-dish-based assay was compared to the multiwell-plate-based assay. The results show that these two feeding methods give essentially the same results in assaying feeding responses to sweet, bitter, or salty food in wild-type flies (**Figure 3A–C**). Notably, it is much faster to prepare and distribute food in the Petri dish than in the multiwell plate containing 60 wells (**Figure 3D**). Altogether, the Petri-dish-based assay is a robust and fast feeding method that can be used to quickly determine the food preference for flies.

FIGURE LEGENDS:

Figure 1: Two-choice assay device and dye dosage curve. (**A**) Two halves of a Petri dish are used to present two different food options. One half of the dish contains blue-dyed food, and the other half contains red-dyed food. Prestarved flies are placed into the dish to allow them to consume whichever food they prefer. (**B**) Food preference for wild-type flies choosing between 1% agarose plus 2 mM sucrose containing either 50 µM blue dye or varying concentrations of red dye. The optimal red dye concentration is 210 µM. Data represent mean ± standard error of the mean. For each data point, n = 6 trials. Approximately 70 flies were tested in each trial.

Figure 2: Fly abdominal color after eating blue, red, or both blue and red foods. (**A**) Representative images of flies after having ingested blue food (top right), red food (top left), or both, making the abdomen appear purple (bottom). (**B**) A fly showing sufficient consumption of blue food. (**C**) A fly after ingesting a small amount of blue food.

Figure 3: Feeding responses to different tastants in wild-type flies, and the food-loading time for the 60-well-plate vs Petri-dish-based feeding device. (**A**) Food preference for wild-type flies choosing between 2 mM sucrose and 10 mM sucrose. n = 12 trials, unpaired Student's *t*-tests. (**B**) Food preference in wild-type flies for food containing 2 mM sucrose with or without 10 mM caffeine. n = 10 trials, unpaired Student's *t*-tests. (**C**) Food preference in wild-type flies for food

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containing 2 mM sucrose with or without 20 mM NaCl. n = 10 trials, unpaired Student's *t*-tests. (**D**) Time spent filling food into a 60-well plate and a Petri dish. n = 12 plates or dishes, $*p < 0.0001$, unpaired Student's *t*-tests. Data represent mean ± SEM. Abbreviations: n.s. = not statistically significant; SEM = standard error of the mean; NaCl = sodium chloride.

DISCUSSION:

This method involves several crucial steps where problems can occur. First, make sure flies ingest a sufficient amount of food to provide stable data. If flies eat poorly, ensure that the flies have been wet-starved for at least 24 h and that the experimental media contain at least a minimal sucrose concentration (2 mM). To further stimulate food consumption, prolong the wet-starvation period beyond 24 h, depending on the flies' physiological condition. If too many flies fail to survive the prolonged starvation, ensure that enough water is added to the tissue paper when performing wet-starvation in vials. Avoid excessive water that may drown the flies. Second, flies tend to show feeding bias toward either blue or red dye if their concentrations are not carefully balanced. Small variations in dye concentration can have profound feeding effects (**Figure 1B**). Thus, to prevent dye bias, dye concentration should be precise. If flies are influenced by the dye, carefully refine the dye concentration at increments of 1 µM, and then test different dye combinations to identify the red/blue dye concentration pair that yields a PI = 0 when no experimental compound except a low concentration of sucrose (e.g., 2 mM) is added. The optimal red or blue dye concertation should be readjusted when testing new fly lines or after making new dye stocks. Third, make sure the assay is constrained to 90 min. According to a previous study[22], prolonged feeding can lead to taste adaptation or desensitization.

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Compared with other feeding techniques, such as FLIC[27] or CAFE[25] assays, this Petri-dish-based two-choice assay has the following features and advantages: (1) Simplicity: this device comprises only a small Petri dish bisected with a plastic divider. Because the dishes and plastic dividers are inexpensive and easy to assemble, an entire experiment requires only minimal investment. (2) Expediency: the Petri-dish-based device considerably speeds up the feeding assay (**Figure 3D**). The color-scoring process is also fast and straightforward using a regular dissection microscope. With this method, the flies' taste preference toward a particular food ingredient can be quickly tested. Thus, it is suitable for both small-scale research and large-scale genetic screens. (3) Stability: in contrast to other feeding methods that analyze only a few flies in each device, this method allows the quantification of feeding responses for a large number of adult flies at one time, which significantly minimizes the effects of feeding variations among individual flies. This dye-based two-choice feeding assay has proven to be rigorous and reproducible and has been used to isolate important fly mutants with defects in perceiving food tastes and textures[11,22,33].

As demonstrated by these results, the Petri-dish-based assay produces essentially the same results as the multiwell-based feeding assay for sweet, bitter, and salty taste responses, although the Petri-dish-based assay tends to have smaller variations (**Figure 3A–C**). One time-consuming step of the dye-based feeding assay is the discharge of food into the feeding chamber. The multiwell plate, which contains 60 or more wells, can be laborious to set up due

to the requirement of precisely loading melted agarose food into 60 or more wells per plate. It is much faster to load food in the Petri dish than in the multiwell plate, as the Petri dish contains only two separate compartments (**Figure 3D**). Thus, this Petri-dish-based method not only maintains the robustness of the dye-based assay, but also significantly reduces the time and effort spent in assay preparation, thereby significantly scaling up the capacity and speed of the feeding assay. Consequently, it can be readily employed to analyze a large number of fly lines, such as in a genetic screen project.

While dye-based assays provide a high-throughput avenue of study due to their simplicity and speed, they cannot capture information about more detailed quantitative aspects of feeding such as duration or volume. To overcome this issue, a high-speed camera can be installed above the dish, which reveals more detail of the feeding process, such as the feeding duration and frequency in each chamber. Moreover, several other feeding paradigms can be used to supplement data gathered from the dye-based experiments. Automatic feeding devices, such as the FLIC[27] and the fly proboscis and activity detector (FlyPAD)[34], can record the temporal dynamics of feeding. The CAFE assay[25] or manual feeding assays[35] can measure the volume of food consumed. Nevertheless, these approaches have their own caveats. For example, compared with the Petri dish or the multiwell plate, automatic feeding devices are very expensive to set up in the lab. Additionally, each device assays only a few flies at a time, making it more vulnerable to variability in individual animals.

As the CAFE assay relies on the flies' ability to maneuver their bodies up to the end of the capillary tube hanging inside the feeding chamber, the results can be confounded by motor impairments unrelated to taste sensation. Although other approaches are powerful in their own right, dye-based assays can be a more efficient tool to rapidly discover and analyze food preference in flies. Furthermore, the two-choice setup can be integrated with cutting-edge techniques such as optogenetics[36] to selectively and acutely manipulate the fly's feeding behavior. This can be done using one half of the dish for light activation and the other half as a light-inactive control. Direct activation or inactivation of specific neurons helps determine whether they have a role in regulating feeding behaviors. In summary, these results show that the Petri-dish-based two-choice feeding assay is a rapid and robust feeding method that can help researchers analyze feeding behavior under different physiological and metabolic states.

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DISCLOSURES:

The authors declare no conflicts of interest or competing financial interests.

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