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December 2, 2020

To the editors and reviewers of the *Journal of Visualized Experiments*,

We thank you for providing us with insightful comments on our manuscript titled "A streamlined food-preference assay in *Drosophila*". We are delighted that you expressed positive opinions on our first submission. To address your comments, we added new data, including new **Figures 1B**, **2B, 2C,** and **3A-D,** to the revised manuscript. Below we summarize our responses to each comment or concern:

A. Editorial comments

Comment 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Answer 1: This manuscript has been thoroughly reviewed.

Comment 2. Please provide an email address for each author.

Answer 2: Email address has been added for all authors.

Comment 3. Please include a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Answer 3: This summary has been added.

Comment 4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Answer 4: This formatting change for references has been made.

Comment 5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Answer 5: All trademarks and proprietary names have been removed.

Comment 6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the

imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Answer 6: The instructions have been changed to the imperative and Notes have been added where appropriate.

Comment 7. How old are the flies?

Answer 7: The flies are 2-4 days old. This information has been added.

Comment 8. How are the flies photoentrained? Any specific light source? Humidity and temperature conditions?

Answer 8: We addressed the concern about the photoentrainment in our response to Concern 6 of Reviewer 3. The humidity level is 50-60%, and the temperature is 25 °C.

Comment 9. What concentrations should be tested in the preliminary assay?

Answer 9: Our feeding assay can test a wide range of experimental food concentrations and the preliminary assay is not necessary.

Comment 10. Do you first establish that flies feed in the dark and then transfer the dishes to the dark?

Answer 10: The flies are transferred immediately to a dark place after being added to the Petri dish, and the entire time course of feeding is done in the dark. We have made this clear in the protocol.

Comment 11. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Answer 11: Highlighting and spacing have been added.

Comment 12. After protocol, please include at least one paragraph of text to explain the representative results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Answer 12: We have included the requested representative results.

Comment 13. After the representative results, list the figure legend. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.) and remove the title and figure legend from the uploaded figure.

Answer 13: The figure legends have been added, and the high-resolution psd versions of three main figures have been submitted.

Comment 14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol (please move the problems and solutions into the discussion)
b) Any modifications and troubleshooting of the technique (please move the problems and solutions into the discussion)

c) Any limitations of the technique
d) The significance with respect to existing methods

Answer 14: We have added this information to the Discussion.

Comment 15. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Answer 15: The Disclosure has been included.

Comment 16. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Answer 16: These changes have been made to the references.

Comment 17. Please include volume and issue numbers for all references. Please do not abbreviate journal names.

Answer 17: These changes have been made to the references.

Comment 18. Please sort the Materials Table alphabetically by the name of the material.

Answer 18: This change has been made.

B. Reviewers' comments

Reviewer 1:

Minor concern 1: Overall the description of the method and text is written well and with enough detail to describe the assay. However, I strongly suggest removing the less scientific descriptors or comparisons of their method. The authors don't need to convince anyone to use the assay; just describe the pros and cons as succinctly as possible. Statements like "the field lacks a robust, simple, and high-throughput feeding assay" (line 31), and even the title ("streamlined" food-preference assay) are unnecessary and don't really describe the assay well.

Answer 1: We have removed these words from the text and replaced them with purely descriptive language. Also, we changed the title to "A **rapid** food preference assay in Drosophila".

Minor concern 2: Although it's fine to compare their dye-based food-preference assay with other publications that have done something similar, I suggest not going overboard trying to find advantages for their assay. The authors suggest that the food dries out in multiwell plates. Is this really a problem when the assay is over such a brief time period? I have not had this issue and, more importantly, I think I actually see more food drying when the surface area to volume ratio is greater, as in the larger plates that the authors are using. The authors also suggest that their method is "highly reproducible, simple, and fast" compared to other feeding methods. Unless they rigorously show this comparison, this seems like an overstatement. I like their method, but the multiwell format has advantages as well. Multiwell plates are typically used in larger cages that permit larger numbers of flies. I'd rather not speculate which method would actually provide higher throughput, but if the authors want to show this experimentally rather than claim it, that's fine. The total surface area for fly feeding may actually be less in the authors' petri dish format compared to the multiwell plate, when you sum up all of the wells. I don't think this makes the multiwell plate system better! But again the authors should be careful in presenting differences between the methods.

Answer 2: Both the multiwell-plate and Petri-dish-based feeding assays are highly robust and are widely used to examine the taste preference and food intake in flies. We have now added a comparison of these two assays, using them both to examine sweet, bitter, and salty food preferences. Our results demonstrate that both methods yield the same results (**Figures 3A-C**). One big advantage of the Petri dish over the multiwell plate is that it is much faster to prepare and load the food into the feeding device (**Figure 3D**). Therefore, our Petri-dish-based assay significantly reduces the labor and expedites the entire feeding procedure.

Based on our own experience, we have observed that the food in the Petri dish tends to retain moisture for a longer time than the multiwell plate. Since this may vary between labs depending upon the humidity and temperature of the room, we have removed this claim.

Minor concern 3: Rather than persuade others to use their particular technique, my preference is for the paper to help readers make an informed decision on the best assay to use for their experiments. Hence, in addition to a more balanced comparison with other iterations of the dye-based preference technique, it would be useful to, briefly, mention the pros and cons of the dye-based assay in general, and perhaps point out the alternative approaches. Some disadvantages to the assay that I can think of include: 1) scoring of the red and blue fly bellies may be subjective, or at the very least it is not a quantitative assay (i.e. there's no quantification of consumption; 2) the assay can only be run for brief periods (<90 min); and 3) to get flies to eat, they must be starved (at least in rodent studies, this has proven to add significant artifacts to behavioral studies). Alternatives that I can think of include FLIC/FlyPAD approaches with food choices, CAFE with different foods in different capillaries, and a recent study showed quantitative food intake preference using a radioactive tracer (Absolute ethanol intake... DOI: 10.1242/jeb.224121).

Answer 3: We added further discussions regarding the pros and cons of dye-based assays as a whole (Pages 13-14). Also, we added discussions on other feeding methods such as FLIC and CAFE assays that can complement the dye-based assay to resolve the temporal feeding dynamics (Pages 14-15).

Reviewer 2:

Concern 1: For the starvation treatment, authors use a damp kimwipe inside of a fly vial. We have trialed this before in the lab and have found that the kimwipe tend to dry out rather quickly and flies get stuck (this was discussed in the paper). An alternative that we are currently using is to starve the flies in fly vials that have ~5mL of 1% agar solution. This provides a good surface for the fly, whilst preventing desiccation.

Answer 1: With regards to the suggestion about starvation using a vial with 1% agar instead of soaked paper, this is a great suggestion and something we have explored. Both methods of starvation yield equivalent results, so we have included the 1% agarose option in the protocol.

Concern 2: I was wondering why the assays were performed in the dark? Could authors explain in the manuscript?

Answer 2: The assays should be conducted in the dark to minimize any potential external stimuli such as shadows passing over the dish or colors and shapes outside the dish. Thus, it prevents visual stimulus from affecting feeding behavior.

Concern 3: Figure 1B shows what a fly should look like following the experiment, but I would suggest that authors show what flies with "minor spots" look like as well. This will avoid confusion on which flies to exclude/include in dataset.

Answer 3: We have added images to demonstrate what is acceptable vs minor food consumption for each fly (**new Figures 2B and 2C**).

Concern 4: It would be good to include some data, maybe a plot on dye bias. I think it would give readers a better sense on the quantities of dye to add to the agar mixture, and it will also provide some clarity on the boundaries of the dye bias.

Answer 4: We have added data that provides an example for what dye optimization should look like (**new Figure 1B**). Our dosage curve shows that the optimal concentration for red dye is 210 μM when paired with 50 μM blue dye.

Reviewer 3:

Major Concerns: No data are provided to show that the current protocol gives the same results than Tanimura et al (1982) nor that it is improved as respect to the original test.

Answers: We show that the Petri-dish 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 (**Figures 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 per plate, 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 prepare and load food for the Petri dish than the multiwell plate, since the Petri dish contains only two separate compartments (**Figure 3D**). Thus, our Petri-dish 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.

Minor Concerns

Minor Concern 1: Several more established methods need to be cited.

Answer 1: We have added these citations to our revised manuscript.

Minor Concern 2: "Petri" dish (with capital) - Petri is the name of a researcher.

Answer 2: We have changed to “Petri” throughout the manuscript.

Minor Concern 3: "Because the food volume loaded in each well is quite small, the food tends to dry during testing causing changes in food texture, such as hardness and viscosity. This problem may affect food preference and consumption. Moreover, it is laborious to distribute melted agarose food into 54 or more wells at a time." - show data for this. None of these remarks have been made previously by other researchers.

Answer 3: Compared with the multiwell plate, the Petri dish significantly reduces the time and effort spent on discharging agarose food into multiwell plate (see **new Figure 3D**). Regarding the food drying problem, this largely depends on lab conditions such as humidity and temperature. In our hands, we feel that our data are less affected while using the outlined protocol, though of course this will depend upon lab setting. Thus, we removed this point on food drying from this protocol.

Minor Concern 4: 1 Petri dish = 70 flies; how many repetitions (dishes) are usually needed to get statistically significant results?

Answer 4: At least 4 dishes or 4 trials with ~ 70 flies in each trial are needed.

Minor Concern 5: "the flies should be photoentrained in a 12/12h day/night/cycle": is there a reference supporting this claim? does it really affect the results of such experiments?

Answer 5: This is not an important part of the protocol, and we meant to present it as a suggestion instead of a strict guideline. We have not seen significant differences between photoentrained and arrhythmic flies, and we have removed this point from the protocol to avoid confusion.

Minor Concern 6: "concentrations should be refined in 0.5 mg/mL increments" - usually, pharmacological effects are described using Moles/l (not weight) and the effects usually follow a log10 scale: 1 - 1.333 -10 - 13.333 - 100 etc.

Answer 6: We changed the dye concentration unit to micromolar (μM), and noted that the concentrations should be refined in 1 μM increments.

Minor Concern 7: "Prolonged exposure to cold can affect the feeding" - how strong is this effect?

Answer 7: Keeping flies on ice too long can lead to lethality. We have clarified the time period beyond which cold shock is a concern in the protocol.

In summary, we thank you again for taking the time to review our submission and helping us improve our manuscript. We hope you are satisfied with our revision. We would greatly appreciate your accepting our manuscript for publication.

Sincerely

Yali V. Zhang, Ph.D.