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An adjustable high-definition imaging system for behavioral studies of Drosophila adults

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TITLE:

An Adjustable High-definition Imaging System for Behavioral Studies of *Drosophila* Adults

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KEYWORDS:

Behavior, adult *Drosophila*, observation and recording system

SUMMARY:

This protocol describes how to make a simple adult *Drosophila* behavior observation chamber, and how to take high-definition photographs/videos of the morphology or behavior of different types of fruit flies in the observation chamber through relatively simple and affordable methods.

ABSTRACT:

Drosophila melanogaster is a very powerful model in biological research, but a bad model for photography or videography. This paper describes a simple but effective method to observe and document the behavior or morphology of flies. Flies were placed in a translucent observation chamber c.a. Ø15 x 5mm (no food inside) or Ø15 x 12 mm (with an 8 mm-high piece of food inside). After covering with an ultraviolet (UV)/clear filter with high light transmittance, the chamber was placed under a 5–50x zoom stereo microscope, and mini light-emitting diode (LED) video lights were placed on both sides of the microscope to illuminate the chamber to obtain uniform, soft, bright, and nearly shadow-free light. Then, a compact digital camera with 3–5x optical zoom, which can record 1080 P high-definition or higher resolution video (at a frame rate of ≥30 fps), was connected to the eyepiece of microscope through a bracket, and photographs or videos were taken through the eyepiece. By adjusting the zoom knob of the zoom stereo microscope, it was very easy to track the flies and take panoramic or detailed close-up images as needed, while the camera recorded everything under the microscope. Because the flies can stay at any position in the chamber, they can be observed and recorded from all directions. The photographs or videos taken are

of good image quality. This method can be used both for scientific research and teaching.

Introduction

Drosophila melanogaster is an outstanding model in biological research; however, it is a bad model for photography or videography, as it is too small for a camera or a camcorder and too large for a compound microscope[1]. Despite excellent research described in the literature, most studies have only provided blurred, unclear images, rather than clear and sharp photographs with clear detail that illustrate the fly behavior being described. Moreover, although fly behaviors have been extensively studied (e.g., courtship and fighting), most of these papers have used illustrations to explain their research to readers.

This paper describes a simple and economical approach. Using this method, not only the various behaviors of *Drosophila* can be observed, but also all the details that can be observed under a stereo zoom microscope can be recorded clearly and sharply. Of course, this method can also be used to record the morphology of flies, as when they enter a sleep or semi-sleep state, the stationary model allows the user to take a photograph or a stack of photographs with different focal planes to get an extended depth of field photo. These methods can be realized without complicated technology and expensive equipment or even superb manual skills.

The video component of this article shows videos of several typical behaviors of flies. The purpose of showing these videos is twofold: one is to let audiences know what can be captured and present the image quality obtained by using this method; the other is to let new students who are interested in *Drosophila,* but thus far have not had the opportunity to actually observe the behavior of flies understand the behavior of flies (such as courtship, fighting) through these clear videos rather than illustrations or blurred images.

PROTOCOL:

1. Construction of the observing/documenting system

NOTE: The materials needed to construct the fly behavior observing/documenting system are shown in **Figure 1**, and the completed system is shown in **Figure 2**. The protocol to construct the system and how to use it are described below.

1.1. Make a ***f***ly ***b***ehavior ***o***bservation ***c***hamber (FBOC).

1.1.1. Obtain a small, **translucent (not transparent)** container to make an FBOC of size diameter (Ø) 15 mm x 20 mm. Use a translucent plastic bottle cap of size ~Ø17 mm x 22 mm to make an FBOC, or cut a section of ~17 mm from the thick end of a 5 mL pipette tip to make an FBOC.

1.1.2. Pour 1% agar into the FBOC to adjust its depth. If food needs to be placed in the FBOC (see for the preparation method of the food), pour the agar to obtain a depth of 12 mm. If

food does not need to be placed in the FBOC, pour the agar to a depth of 5 mm to track the whereabouts of fruit flies more easily.

1.1.3. If using a pipette tip to make an FBOC, place the cut-off pipette tip section in a Ø35 mm or a Ø60 mm Petri dish. Pour the 1% agar gel into the Petri dish to a thickness of ~5 mm, wait for the agar to solidify, and seal the bottom of the FBOC. Then, pour the agar gel into the FBOC to the desired thickness.

1.2. Make an FBOC base by boring a ~10 cm-deep hole in the center of a piece of a 60 mm x 60 mm x 15 mm ethylamine foam sheet with the same diameter as the FBOC. Insert the FBOC into the hole.

NOTE: The FBOC base keeps the FBOC stable and prevents it from tipping over and facilitates the movement of the FBOC to track the flies during observation and videography.

1.3. Make fly food, if necessary, with yeast medium[2], artificial diet[3], or pure sucrose/glucose (use 1% agar as gelling agent), depending on the purpose of observation.

1.3.1. To visually determine whether flies are feeding and increase the contrast between flies and their environment, add food dyes (see the **Table of Materials**) to the food to a final concentration of 12.5 mg/100mL.

NOTE: The abdomens of the flies change color immediately after feeding.

1.3.2. Pour the prepared food into a Petri dish to a height of 8 mm. After solidification, use a razor blade to cut out a piece of food of size 15 mm x 15 mm x 8 mm, and place it on a piece of plastic (such as candy wrapper).

1.3.3. Cut the food into a quadrangular pyramid or quadrangular frustum pyramid, as shown in **Figure 3**, to allow observation and recording of fly behavior from different angles as the flies land on the food randomly. Use the plastic under the food is to prevent the dye in the food from diffusing into the agar in the FBOC. Use tweezers to place the food in the center of the FBOC.

1.4. In some behavioral observations, ensure that the flies are starved in advance. Pour 1% agar gel (1 g agar/100 mL water, 600 µL of propionic acid) into a clean empty bottle to a thickness of 1–2 cm, and place at room temperature for 1–2 h. Transfer flies to the bottle and place at 25 °C for ≥36 h.

NOTE: Flies can absorb water from the agar gel, so there is no need to add water from time to time[4,5].

1.5. Transfer one or more flies into the FBOC using an aspirator. If using an aspirator is difficult, chill and inactivate the flies in crushed ice, sort them on an ice pack, and transfer them to the

FBOC as described previously[6].

NOTE: The use of freezing greatly facilitates the transfer of flies; the chilled flies can regain consciousness within 1 min, much faster than those anesthetized with CO_2. Although chilling could have detrimental effects on the behavior of flies, e.g., increased copulation latency of flies from 5 min[7] to 40 min[8], it does not change fly behavior (such as courtship behavior). Hence, the chilling method may be used to transfer flies for general observation (such as teaching experiments) and videography. However, if the observations are to be used in a scientific report, it is strongly recommended to not expose the flies to any anesthesia.

1.6. After transferring the flies to the FBOC, cover it with a 30–40 mm UV/clear filter for the camera to form an FBOC complex (**Figure 4**). Place the FBOC complex under the stereomicroscope for observation.

NOTE: To obtain clear and sharp images, it is strongly recommended to use high-quality UV/clear filter with high light transmittance (>98%) and reduced flare. Refer to some suggestions described previously[9,10]; although there is no need to buy expensive filters, avoid covering the FBOC with glass such as the lid of a Petri dish.

1.7. Illuminate the FBOC by mounting mini LED video lights to flash hot shoe mount stands on the left and right sides of the FBOC (**Figure 2**). Turn on the lights, and set the brightness to 100% and the color temperature to 5000–5600 K.

NOTE: The mini LED video lights with dimmable light, 5600 K color temperature can provide uniform, bright, nearly shadow-free illumination. Using the top light source that was provided with the stereo microscope, the LED Ring Light illuminator, or fiber optic illuminator did not yield satisfactory results. It is best to use continuous power supply (transformers) for LED video lights.

1.8. Observation and videography of fly behavior

1.8.1. Turn on the LED video lights, and adjust the stereo zoom microscope until the edge of the FBOC can be clearly seen with the naked eye. Move the FBOC to the center of the field of view.

1.8.2. Attach the clamp of the universal telescope digital camera adapter to an eyepiece of the stereo microscope, and then attach a compact digital camera to the adapter securely by alternately turning the camera mounting screw and camera fixing screw (**Figure 2**).

1.8.3. Turn on the digital camera, and turn the horizontal/vertical fine-tuning knobs until the FBOC edge clearly appears in the center of the bright circular field of view on the camera's LCD screen. Rotate the mode dial to **Auto Mode**. Move the zoom switch from the wide-angle end to the telephoto end, zoom into the circular image until its central portion fills the full LCD screen. Press the **Movie-record** button to start recording (press the button again to end

recording).

NOTE: If the image is too dark or too bright, press the side of the control dial close to the exposure compensation icon (**Figure 1**), and rotate the dial to alter the exposure value (EV) suggested by the camera to achieve the desired effect. Positive EVs make the image brighter, and negative EVs make the image darker. The image must be uniform, bright, without vignetting.

1.8.4. Turn the focus knob of the microscope until the flies in the FBOC are clearly visible. Choose the fly behavior of interest for observation or video recording. Turn the zoom knob to zoom in and out to achieve the desired magnification for observation or video recording.

NOTE: This method of taking images under the microscope through the eyepieces is applicable to any microscope with eyepieces. To take photographs of experimental results, use a camera that can shoot in RAW format, as RAW image files are preferable to JPEGs. Use the camera's LCD screen as a display to observe the behavior of fruit flies, and ensure that the stereo zoom microscope has at least 5–50x zoom.

2. Protocols for observation and videography of fly behavior

2.1. Preparing the flies

2.1.1. Culture the flies on cornmeal medium at 25 °C with 60% humidity and a 12 h light/dark cycle. Collect flies within 6 days of hatching for observation (except courtship and fighting behavior).

NOTE: Here, the medium was composed of 1000 mL of water, 105 g of cornmeal flour, 75 g of sucrose, 15 g of agar, 40 g of yeast powder, 28 mL of 10% methyl paraben (w/v in 95% ethanol), and 6.25 mL of propionic acid.

2.2. Regaining consciousness from anesthesia by chilling

2.2.1. Chill the flies as described previously.[6] Transfer the *Drosophila* from the ice box to the FBOC using tweezers. Record the fly's process from inactivity to normal posture on video.

2.3. Fly sleep, feeding, excretion, and social behavior

2.3.1. Starve flies for 36 h. Transfer 4–6 fruit flies to the FBOC with stained food. Observe and record fly behavior on video.

NOTE: Flies that remain motionless for more than 5 min display sleep behavior[11]. Drosophila can sleep on food or on a vertical FBOC wall (the body is perpendicular to the observation chamber wall). Although the body does not move when sleeping, the abdomen can be seen to be undulating. Feeding behavior is manifested when the fly stretches out its proboscis,

moves on the food while constantly sucking, and its abdomen turns blue. During group feeding or other activities, fruit flies stretch their feet to touch the bodies of other fruit flies in a friendly manner. This is a social behavior.

2.4. Fly grooming behavior

2.4.1. Chill the flies as described[6]. Throw the frozen flies into agar powder, and roll to cover them with agar powder. Transfer the flies to the FBOC. Observe and record grooming behavior.

NOTE: When the fruit fly regains consciousness from the freezing, it will quickly shake off the agar powder from its body and clean every part of its body with its legs[12,13]. Grooming behavior can also be seen during feeding, courtship, and other behaviors.

2.5. Fly courtship and fighting behavior

2.5.1. Collect female and male flies as described previously[7]. To observe the courtship behavior of flies, place a female fly and a male fly into the FBOC to observe and record 6 courtship (successful and failed) behaviors.

2.5.2. To observe the fighting behavior of flies, place two males in the FBOC. Observe and record their behavior of pushing and shoving each other.

2.6. Fly egg-laying behavior

2.6.1. Prepare female flies as described previously[5]. Transfer 4 female flies into FBOC with food.

REPRESENTATIVE RESULTS:

Shoot through a UV filter for clear and sharp images

Perform a simple experiment to observe the difference between a UV filter and ordinary glass in the laboratory. Take a fly culture vial, remove the stopper, place it under a stereo dissecting microscope, and cover it (alternately) with a UV filter and a Petri dish lid. The photographs taken in these two cases are shown in **Figure 5**. The photograph taken through the UV filter is clear and sharp, very similar to the photograph taken when the culture vial is not covered. The quality of the photo taken through the glass of the Petri dish is very poor even when the focus is accurate. Ordinary glass (or acrylic sheet) is not coated, the highest transmittance is 92%[14,15], and the clear/UV filter with multi-layer coating has a light transmittance of 98–99%.

Thus, the image shot through ordinary glass (or acrylic sheet) is not as clear as the image shot through the clear/UV filter. Another important defect of ordinary glass, such as the lid of a laboratory dish, is its uneven surface. It can be seen in **Figure 5** that due to the unevenness of the glass surface, part of the photo is clear and part blurred. Therefore, clear/UV filters should be used instead of using ordinary glass or acrylic sheets to cover the FBOC. The UV filter used in this protocol (Figure 5) was cheap (~$10), unbranded, and its light transmittance unknown.

In other words, even if it is a cheap UV filter, the image captured through it may be much clearer and sharper than that captured through ordinary glass.

Good quality without expensive equipment

Fly behavior was recorded with a JPEG-only camera with a considerably smaller sensor (1/2.3'). Video resolution is 1920 x 1080 pixels (at 30 **f**rames **p**er **s**econd, fps); the quality of the movie is satisfactory. A cheap UV filter was used to cover the FBOC, and the stereo zoom microscope was unbranded. The cost of the LED light was approximately $70 for two packs (see the **Table of Materials**). In other words, the equipment used was very economical; however, the video quality is good, clearly showing the panorama of some behaviors of flies, such as courtship and fighting, and the details of some behaviors, such as oviposition and excretion.

Figure 6 is a photograph taken from the video recording showing the details of each part of the fly's body. Obviously, the use of a camera and a stereo microscope with better image quality will yield videos or photographs with higher image quality. If the camera has a frame rate of ≥60 fps with good image quality, far more details can be captured in greater clarity in behavior with lots of action or movement. Another advantage of this system is that because the camera is connected to a zoom stereo microscope, it is very easy to shoot from panoramic to close-up shots using the zoom system.

All-round recording

Observation and videography are usually done from the top; however, as flies can stay on any part of the FBOC: the vertical FBOC wall, the inclined food surface, and even the UV filter (with the abdomen facing upwards), and their bodies are perpendicular to these surfaces, their behavior can be observed and documented from multiple viewing angles. For example, it can be clearly seen in **Figure 7** that the female fly is constantly rubbing the ovipositor with her hind legs during the process of laying eggs. This detail of egg-laying behavior cannot be seen clearly from the side[5].

FIGURE AND TABLE LEGENDS:

Figure 1: Photographic equipment and other accessories used to construct fly behavior observing and documenting system. Abbreviation: LED = light-emitting diode.

Figure 2: Drosophila behavior observation and recording system.

Figure 3: Illustration of the size and shape of food.

Figure 4: Illustration of the FBOC complex. Abbreviation: FBOC = fly behavior observation chamber.

Figure 5: Comparison between the photographs taken through the UV filter, through the lid of the laboratory Petri dish, and taken directly without any cover.

Figure 6: An image taken from the video recording.

Figure 7: An unusual perspective to observe the egg-laying behavior of fruit flies.

DISCUSSION:

Light is at the very heart of photography and videography and is the decisive factor for obtaining high-quality images[16]. Here, two LED video lights with adjustable brightness and color temperature were used as illuminators, and a translucent material was selected to make the FBOC. The LED light panels on both sides provided enough brightness, and the translucent material softened and scattered light, eventually producing uniform, soft, and bright light to illuminate the flies in the FBOC, without producing unpleasant overexposed or underexposed areas. The ideal illumination can be achieved without sophisticated and expensive lighting equipment. Here, the UV/clear filter used had very high light transmittance and low reflection to cover the FBOC, and the light loss is very small. These measures ensured clear and sharp images.

We connected a digital camera to the eyepiece of the stereo zoom microscope through a bracket and took photographs or videos through the eyepiece. All images that could be observed under the microscope could be recorded. By rotating the focusing button and lifting the microscope, it was very easy to track the flies in the narrow space of the FBOC and to zoom in or out as needed to record local details or overall dynamics, which cannot be achieved by using a video recorder or camera for direct videography of the FBOC. At the same time, the camera can be selected according to the image quality requirements. In fact, a digital camera can be connected to any microscope with an eyepiece through a bracket. The corresponding author of this paper has successfully recorded experimental results in this way for many years.

The compact digital camera used to record the behavior of fruit flies must have 3–5x optical zoom (digital zoom should not be used for video recording). The telephoto end of these cameras (~100 mm focal length) is used to enlarge the image in the center of the field of view to the entire screen, so that the final image obtained is a pleasant image with no vignetting around it. If a camera has only a wide-angle fixed focus lens, or an optical zoom lens above 7x, there will be more or less unpleasant vignetting around the captured image. Neither digital single-lens reflex cameras nor camcorders are suitable for the method described in this article. The camera must be capable of recording video with a resolution of at least 1080 P at 30 fps. If the camera cannot be powered by continuous power, more spare batteries must be purchased for replacement at any time.

The flies can stand on a plane at any angle, their bodies are perpendicular to this surface. Even when sleeping, they can stand motionless on the vertical culture bottle wall. Therefore, when shooting from top to bottom, as long as we provide them with a plane at an appropriate angle, the behavior of the fruit fly can be observed and photographed in all directions, without the need to shoot its behavior from the side of an FBOC. This is the reason for the quadrangular food pyramid design.

However, in this system, the camera cannot focus and lock the flies and shoot automatically as they move across the frame. The experimenter must always use the focus and zoom functions of the stereo microscope to track the flies for shooting. It is for this reason that the diameter of the FBOC should be small, and the depth of the FBOC should be shallow, so that the experimenter can quickly track the moving fruit flies. Some behaviors may need to be recorded in the dark[17,18]. This article does not discuss those aspects of fly behavior.

ACKNOWLEDGMENTS:

We thank Professor Li Xiangdong and photographer Mr. Cheng Jing for helpful discussions and suggestions.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 3

Click here to access/download;Figure;Figure 3 (1).psd

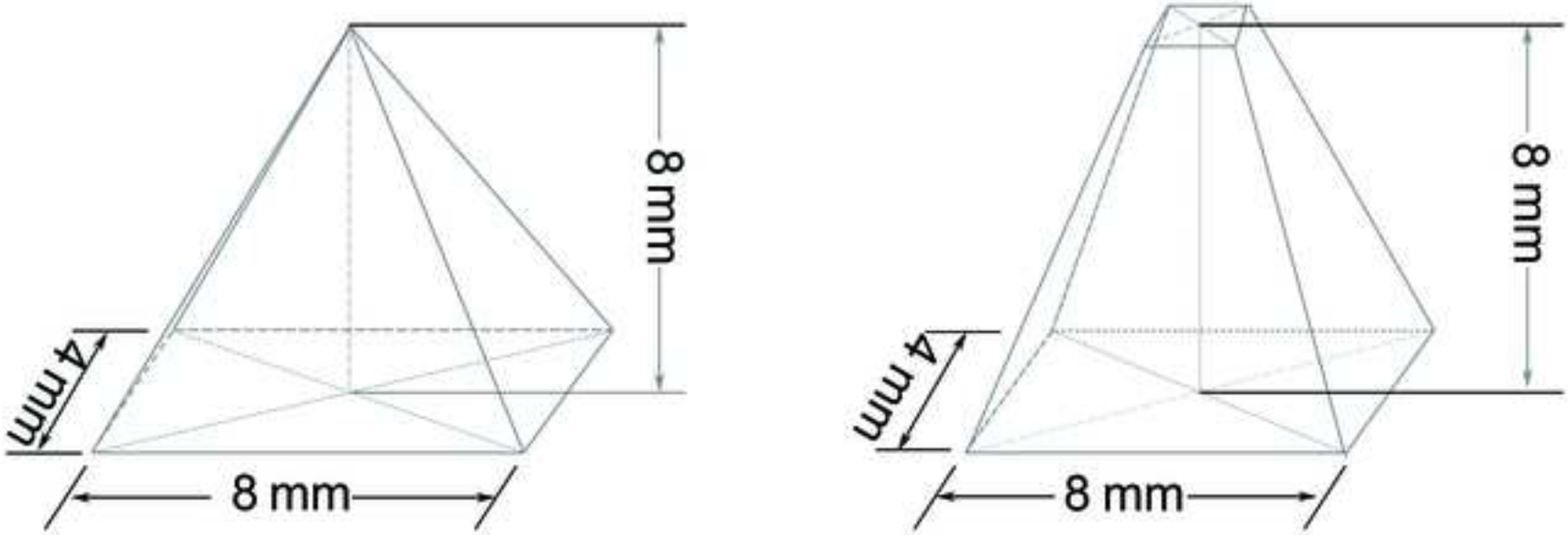

Figure 4

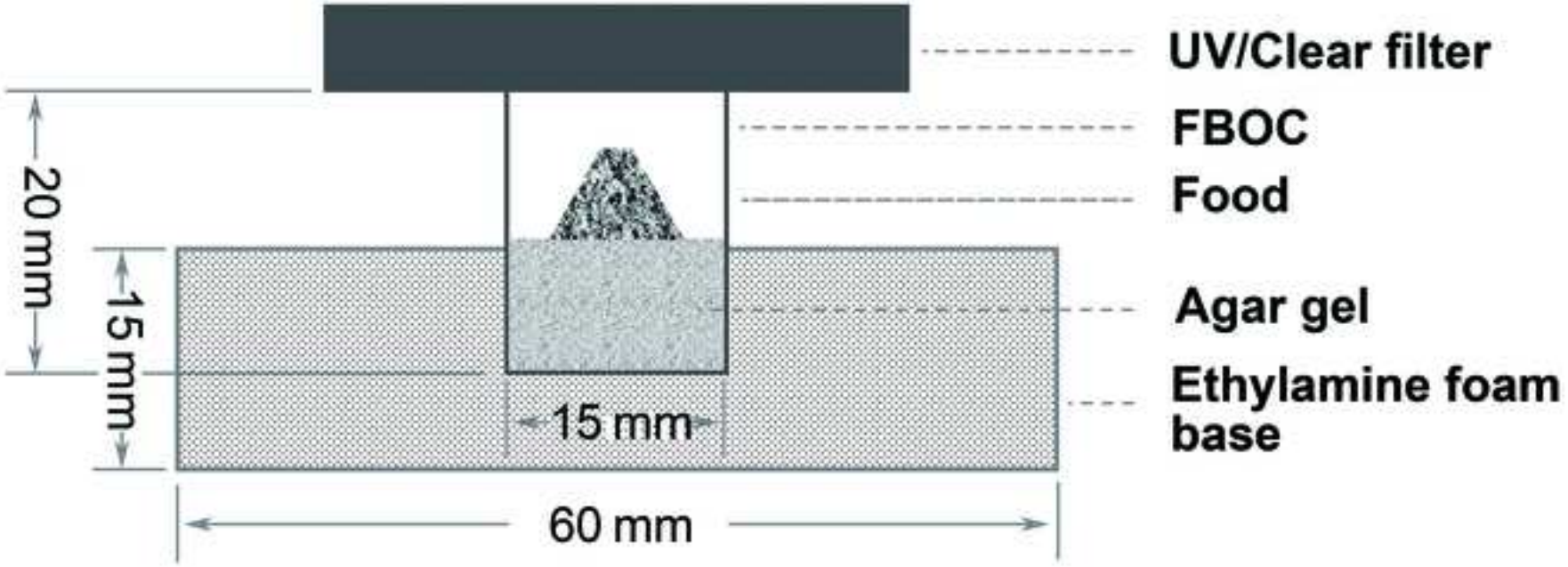

Figure 6

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Figure 7

Click here to access/download;Figure;Figure 7.psd

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
compact camera, Nikon P310	Nikon		3-5x optical zoom, cam record 1080 P HD video
ethylamine foam			60 mm x 60 mm x 15 mm
Food Blue No 1		CAS 3844-45-9	
mini LED lights and transformer	GODOX	LED-P120	have 5000-5600 K color temperature
small container (e.g. bottle cap)			about Ø 15 mm x 20 mm
UV / Clear filter			high-quality UV/Clear filter with high transmittance, 30-40 mm
zoom stereo microscope			5-50x zoom

CC: "Tong Li" 1291030706@qq.com, "Yujia Weng" 876837690@qq.com

Dear Dr. Yang,

Your manuscript, JoVE62533 "A highly adjustable system that can be used to observe the behavior of Drosophila adults and take high-definition photos and videos," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually.

Your revision is due by **Mar 18, 2021**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

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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.

OK

2. Please shorten your title; consider something like "An adjustable, high-definition imaging system for behavioral studies of Drosophila adults".

OK , The title has been modified。

3. Please provide an email address for each author.

The email addresses of the first and second authors have been provided in the paper. Since they are about to graduate and leave Capital Normal University, the domain name of their mailbox is not from Capital Normal University, but a commercial domain mailbox that can be used for a long time.

4. Please include a Summary (before the abstract) to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to ..."

A Summary has been added

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5. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

We have already described our method in more detail in the abstract, so we will not repeat these words in the introduction. I don't know if this approach meets the requirements of your journal. If it does not meet, we will modify it.

c) The advantages over alternative techniques with applicable references to previous studies

I know that in the introduction of a paper, I should systematically summarize the previous work and point out their shortcomings, to show that our work is very very important. I already thought of this question when I was writing the first draft. Obviously, the photos or videos we took are much clearer than the previous ones (e.g., Takashi Shiraiwa & John R. Carlson: Proboscis Extension Response (PER) Assay in Drosophila, J. Vis. Exp. (3), e193; Yang, et al. Drosophila egg-laying site selection as a system to study simple decision-making processes. Science. 319 (5870), 1679-1683). However, in this paper, I feel that I cannot do this. I think it is very rude and will hurt others' self-esteem if I directly criticize other people's photos or videos for being bad. Because these researchers have also made great efforts. With this in mind, I did not cite specific literature in the introduction, but said in general, "Some authors have done excellent research, unfortunately, they can only provide a blur, unclear detail, rather than clear and sharp photo with clear detail that illustrates the very fly behavior they described". This is why I didn't revise the first paragraph.

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d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

6. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have modified most of the personal pronouns, but some places introduce our own experience, so we still retain a small amount of "we".

7. 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.

OK

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to

published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to

9. 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.

OK

10. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.). Please include spaces between numbers and units in all images and throughout the text.

Ok. We made a modification to Figure 1

11. Please remove the titles and Figure Legends from the uploaded figures. The legends should appear only in the Figure and Table Legends section after the Representative Results.

I have moved the legends of the 7 Figures after the "representative results" section, but in order to facilitate editors to check the Figures and legends, I still keep the original figures and legends at the end of the article, and at the same time, I upload figures separately.

12. 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

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

OK

13. Please sort the Materials Table alphabetically by the name of the material.

OK

14. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.

We have included an Acknowledgements section.

15. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (italics). Volume (bold) (Issue),

FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references. Please do not abbreviate journal names, and use title case for journal names.

I use the EndNote template provided by your journal to import references

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Reviewers' comments:

Reviewer #1:

Dear Doctor,

Sincerely thank you for reading our paper carefully and making such kind comments.

Sincerely,

Daxiang Yang

Manuscript Summary:

Authors describe system for photographing or video-recording Drosophila behavior or morphology.

Manuscript virtually flawless. Excellent step-by-step outline for producing video.

Outstanding photographs of equipment and material.

Major Concerns:

None

Minor Concerns:

Virtually none (replace second "photography" with "videography" in Abstract).

Reviewer #2:

Dear Doctor:

Sincerely thank you for reading our manuscript carefully and making so many constructive suggestions. Many of the suggestions you made were indeed not noticed when I was writing the paper. These suggestions helped us to make the paper more perfect. I asked the editor to disclose your name and work unit, and I intend to express our gratitude to you in the "Acknowledgements". Unfortunately, the editor said that this information cannot be made public. Therefore, I can only express my sincerest thanks to you here.

Best,

Daxiang Yang

Manuscript Summary:

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This is a very interesting manuscript introducing a relatively simple and affordable method to record different types of fly behavior. The method is potentially very useful for research and university teaching, perhaps even something to consider for schools. The figure supplied are very clear and (in combination with the video) will allow the reader to rebuild the set-up. The high quality of fly images also nicely demonstrates the power of the system. The authors present an easy way to repurpose a stereo microscope for image and video acquisition of fruit flies and other small insects. The use of agar as a diffuser is a good idea and easy to adapt to different containers. More examples of materials to produce a FBOC would be desired (We have made many attempts. For example, we have tried to use a Ø40×25mm glass container, and found that it is difficult to track flies in a wide and deep container. For this reason, we come to a conclusion that FBOC should be a container with small diameter and shallow depth. Containers with small diameters are easy to find, but it is more difficult to meet the shallow depth at the same time, so we thought of using agar to adjust the depth) . The use of the UV filter as a lid is also a clever idea, as does not interfere with the image quality, but rather enhances it. As such I have no major concerns with the manuscript. There are numerous grammatical and wording errors, which I did not correct; based on the instructions I received for reviewing this manuscript.

Major Concerns:

I think the authors should emphasize more the limitations of the system. For

example, initially I thought that the system would allow for automatic video recording of fruit fly behaviors, i.e., that the system would always keep the fly in focus. But I think this is not the case, and the experimenter must always track the fly using the focus and zoom functions of the microscope. This may become clear with the video supplied, but I think this limitation (and perhaps others) should be clearly stated in the text, perhaps even in a separate section entitled 'limitations of the system'.

Thank you for your reminder. I think many readers will also ask this question, so it is very necessary to make a clear statement. We put this statement in Discussion. After stating the advantages of our system, we then state the limitation of this system, so readers will not be misunderstood.

Minor Concerns:

143: Or increasing time of exposure? Also, what exactly is the 'EV-value'? The authors do not state which settings were used to generate the various images: Aperture, speed, lens etc....This information should be supplied to give the reader an idea how to start.

Thank you for your reminder! I really didn't make this clear. I think many readers of this article are not very familiar with photography. Therefore, my main task should be to teach them simple and effective operations so that they can take acceptable photos or videos in a short period of time. I found that a novice only needs to master 3 knowledge to complete the shooting work: the first knowledge is to set the camera to automatic mode, the second

knowledge is how to change the camera focal length, zoom in or zoom out the target as needed, and the third knowledge is to increase or decrease the EV value to make the photo brighter or darker. According to this idea, I added the corresponding photos in Figure 1 and explained it, and rewrite this passage. The first two authors of this article did not know anything about photography before doing this work. They learned to shoot flies based on these knowledge.

151: Why can cameras with 7x or higher zoom not be used? Would it be possible by using other combination of lens, adaptor, etc.? This would be useful to know, in case the reader already owns such a higher zoom camera.

The image we obtain from the eyepiece of a microscope is circular. If the shot is a photo, we can cut the captured image through image editing software to obtain the desired image. But shooting a video with a circular image is very unpleasant. Although we can use some video editing software (like "live screen capture") to capture a part of the screen, this method will lose the information of the edge part. The main purpose of choosing a 3-5x optical zoom camera is to use the telephoto end of the camera to enlarge the image in the center of the field of view to obtain a pleasant image that can fill the entire screen. To get such an image, the focal length and length of the lens should be carefully selected before shooting. If it is a wide-angle fixed focus lens, or if the zoom ration is very large

(such as Nikon P1000), it is impossible to obtain images without vignetting around.

By the way, we have tried to shoot with the telephoto end (focal length 200mm) of Nikon's coolpix P7000, and we can get images without vignetting, but such images cannot be obtained with a longer focal length. This is why I say that cameras with more than 7X optical zoom are not suitable for this method.

Line 210: The UV filter is used because of surface evenness, like demonstrate in Figure 5. For the same reason mentioned above, would other types of filters also work? For example, does it work with acrylic glass or polarized filters?

I am sorry that I didn't make it clearly here. Thank you very much for pointing out the problem. The choice of materials to cover an FBOC mainly considers two things: one is the light transmittance, the other is the evenness of the surface. The high transmittance makes the final image clear. Because Clear / UV filter usually has multi-layer coating, the light transmittance can easily reach above 98%, and the highest light transmittance of ordinary glass or acrylic sheet is 92%, so the image captured through clear / UV filter is much clearer than that captured through ordinary glass. The videos and photos we demonstrated were taken through an unbranded cheap UV filter. The image obtained is much clearer than that covered with a Petri dish, and the image quality is acceptable. Therefore, the filter used for shooting do not need to be very expensive one. I have stated this in my revised paper. Some authors use petri dish lids to cover an FBOC. The glass

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petri dish lids look flat to the naked eyes but are not actually flat. This defect will cause serious consequences after being magnified under a stereo microscope.

Finally, some behaviors may need to be recorded in the dark, so have the authors tried recording with IR transmitting filters and/or IR illumination?

We have noticed the literature on recording the behavior of flies in the dark, but we have not done this kind of work, so we do not know whether our method is suitable for recording in the dark. But, thank you for your reminder, I think we should mention the work of video recording under dark conditions in the paper (in the Discussion section), and let researchers with infrared cameras or CCTV cameras try it out according to our ideas.
