Protocol

Comparing Methods for Quantifying and Analyzing *Drosophila* Aggression

Severine Trannoy,[1] Maria P. Fernandez,[2] and Sarah J. Certel[3,4]

[1]*Centre de Recherches sur la Cognition Animale (CRCA), Centre de Biologie Intégrative (CBI), Université de Toulouse; CNRS, UPS, 31062 Toulouse, France;* [2]*Department of Neuroscience and Behavior, Barnard College, New York City, New York 10027, USA;* [3]*Division of Biological Sciences, University of Montana, Missoula, Montana 59812, USA*

Here, we highlight three different assays that are used to study *Drosophila* aggression. The advantages and disadvantages of each assay are discussed, as examining different aspects of aggressive behavior presents distinct challenges to researchers. This is because aggression is not a singular behavioral unit. Rather, aggression is the result of interactions between individuals; and, as such, the initiation and frequency of these interactions are impacted by the assay parameters including the method of loading the flies into the observation chamber, the size of the chamber, and the animals' previous social experience. Thus, determining which assay to use depends on the overall question that is the subject of investigation.

MATERIALS

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

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

Reagents

Agarose (Sigma-Aldrich A4718)

Clear corn syrup (purchased from the grocery store)

Drosophila pupae in culture vials (25-mm × 95-mm [Genesee 32–113] or 28.5-mm × 95-mm [Genesee 32–121])

Eye color impacts aggression. When using transgenic lines, ensure that control and experimental adults have similar eye colors, as common mutations in the white (w) gene decrease aggression levels of single-housed males (Hoyer et al. 2008). Alternatively, transgenes can be crossed into a wild-type background.

Fly food and dietary components

Standard fly food consisting of cornmeal, molasses, sugar, yeast, and agar can be used (details on fly food recipes, amounts, and storage can be found at the Bloomington Drosophila Stock Center; bdsc.indiana.edu/information/recipes/index.html). Ready-made alternatives include Archon Scientific W10101 or Genesee Scientific Nutri-Fly BF 66-112; follow the storage and preparation information provided by each vendor.

Milli-Q-purified H_2O

[4]Corresponding author: sarah.certel@umontana.edu

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Nipagin/Tegosept (Genesee 20-258, 20-259, 20-266)
Sucrose (Sigma-Aldrich S7903, 57-50-1)
Yeast (Sigma-Aldrich YSC2)
Yeast paste for fly aggression assays <R>

Equipment

Aspirator
Basler video-acquisition software (www.baslerweb.com/en/products/software/basler-video-recording-software/)
Block (acrylonitrile butadiene styrene [ABS], 36.83-mm × 123.19-mm) with wells (22.86-mm-diameter; 17-mm-high), holes on the side (15.88-mm-diameter), and a lid

This is the sliding chamber. Sliding chambers were designed with standard freely available CAD software (www.tinkercad.com) and three-dimensionally (3D) printed with ABS material. Files for 3D printing (.stl) can be directly requested from Séverine Trannoy.

Cameras (high-resolution recording, e.g., Basler acA1920-155 µm or Basler acA2000-165 µm near-infrared [NIR] cameras [Graftek Imaging]; standard recording under light conditions, Sony HDRCX405 or similar models)
CO_2 pad
Computer
Cotton (Fisherbrand 22-456-881)
Dividers (plastic) for sliding chamber
Equipment needed for the divider chamber assay only (see Steps 28–39)
Box cover of pipette tips (inverted, 10-mL, clear; see Step 28)

This is used as a base plate to hold the 3D printed divider.

Fighting chamber (3D printed with ABS material at 100-µm thickness) with individual square arenas (13-mm wide × 4.5-mm high)

The divider assay behavioral chambers were designed with standard freely available CAD software (www.tinkercad.com) and 3D printed from an online marketplace (www.3dhubs.com) in ABS material.

Flask (microwave-safe, varying sizes [500- to 1000-mL], used for boiling 1% agarose to fill the base plate)
Glass slides (3″ × 1″ × 1-mm, placed side by side; Electron Microscopy Sciences 70329-40)

These are used for the top glass cover.

X-ray films cut with a Silhouette printer

These are used for dividers.

Flask (50-mL; optional, see Step 6)
FlyTracker software
Food cups (cap of a 1.5-mL microcentrifuge tube)
iMovie or suitable software
Incubator set at 25°C with 45%–55% relative humidity
Isolation vials (borosilicate, glass; VWR International 47729-576 or Sigma-Aldrich CLS7082010, 16-mm × 100-mm)
Janelia Automated Animal Behavior Analysis (JAABA) software
Light source (LED Artcraft Tracing Light Pad Light Box, e.g., Amazon)

This provides illumination from below.

Microscope slides (3″ × 1″ × 1 mm, glass)
Microwave

Alternatively, a hot plate may be used for Steps 6 and 17.

Paintbrush
Paint pen (optional, see Step 4)

Paper (white; optional, see Step 11)
Pasteur pipettes (VWR International 53300-567 or Fisherbrand 13-678-20A)
Plates (12-well, polystyrene, 10-mm-diameter × 5-mm-deep; Falcon 353043)

These are the multiwell assay chambers.

Pylon Viewer 5 software
Stereomicroscope
Tape

METHOD

Carry out all steps at room temperature unless otherwise noted.

Isolation of Pupae

1. Melt standard fly food in the microwave. Using a Pasteur pipette, transfer 1.5 mL of the melted food into each empty vial to prepare isolation vials. Allow the food to solidify.
2. Using a paintbrush, take a late-stage pupa from the culture vial, and gently place it on the side of an isolation vial filled with freshly added standard fly food. Plug with cotton. Repeat this process to isolate as many males as needed for the aggression assay pairings.

   Handle individual flies with care. Flies with obvious locomotor or physical defects should not be used. The late stage is distinguished when the male sex combs are visible and pigmentation has occurred.
3. Keep the isolation vials containing pupae in an environmentally controlled incubator (standard conditions, 12-h–12-h light–dark cycle at 25°C with 45%–55% relative humidity).

   Group-housed males show lower levels of aggression than isolated males regardless of the procedure used or age. Both housing conditions can be used in these assays; however, group-housed males will display lower lunge numbers and lower fight intensities.
4. (Optional) Briefly anesthetize flies with a CO_2 pad under a stereomicroscope 24–48 h before fights. Using a paint pen, apply a small dot of white (or other color) acrylic paint to the thorax, pairing opponents of the same size. Let the paint dry for 1–2 min while the fly remains on the CO_2 pad and then return the male to its original isolation vial.

   Paint both animals as the treatment could affect their behavior. To accurately identify each fly during assays, use different paint colors (avoid combinations such as yellow and white or blue and black). Perform this step 24–48 h before fights to avoid negative effects of CO_2 anesthesia. When pairing opponents, careful visual inspection is sufficient.
5. Age socially naive, isolated flies until they are 4–7 d old.

   Age is an important factor. Males younger than 3 d old display an immature pheromonal profile, often provoking courtship by other males. If the chamber includes an immobilized female, males will display aggression to each other as well as courtship toward the female. Male aggression intensity levels may be higher when fighting over a female than over a territory or food source.

   Prepare to test flies shortly after lights-on (1–2 h after lights on, Zeitgeber Time 1–2) in the multiwell chamber (Steps 6–16) or the sliding chamber (Steps 17–27) assay. The divider chamber (Steps 28–39) assay (Fig. 1) is a separate procedure with a separate analysis (Steps 42–47). For a comparison of the three assays, see Table 1.

Multiwell Chamber Assay

6. Liquefy fly food from a standard culture vial in the vial in a microwave or in a small flask (50-mL) on a hot plate. Do not boil.
7. Using a Pasteur pipette, fill food cups (lids of a 1.5-mL microcentrifuge tube) to the top (keep level rather than rounded) with melted fly food. Add a very small drop of fresh yeast paste to the center to facilitate aggressive encounters.

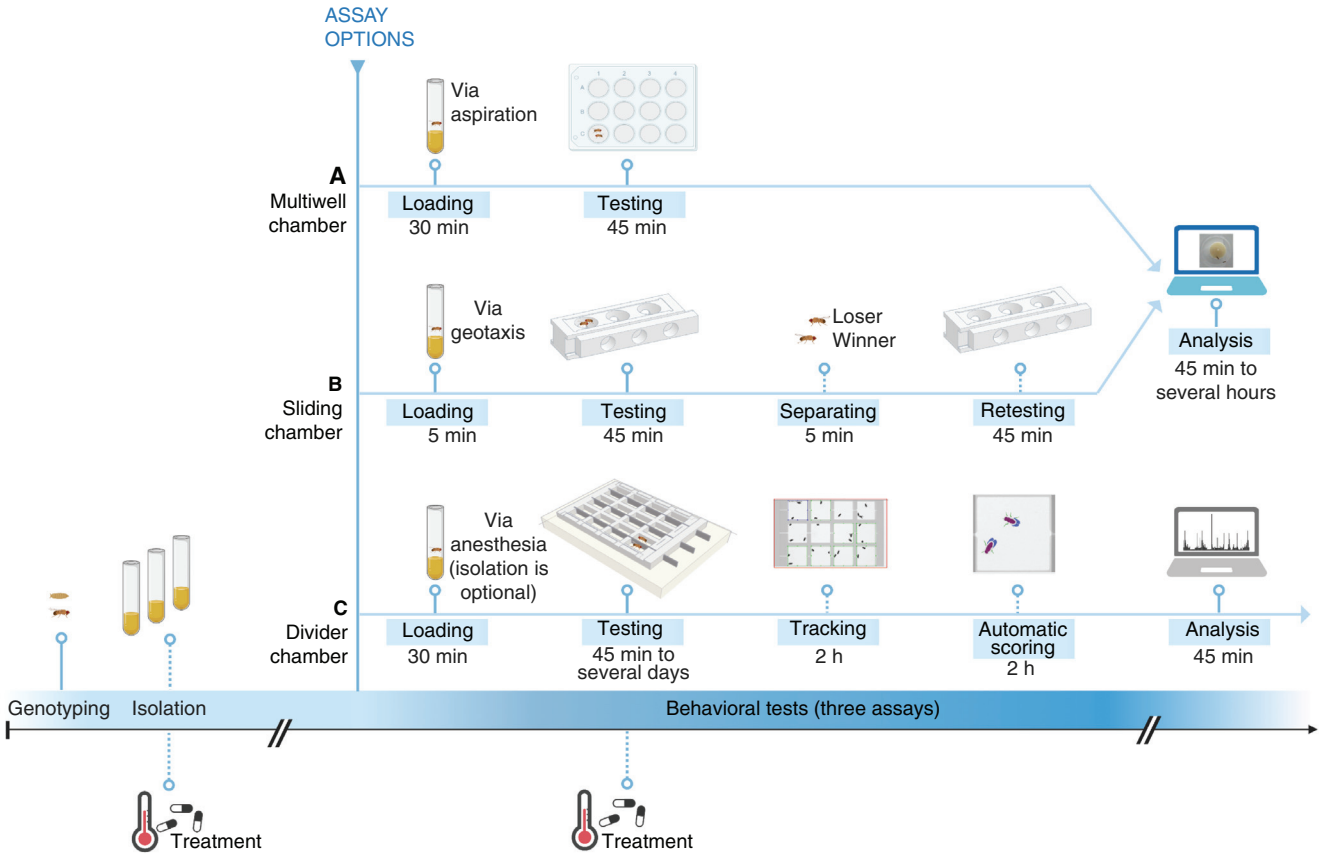

FIGURE 1. Schematic of the assays described in this protocol. (*A*) The multiwell chamber uses a standard polystyrene 12-well cell culture plate and standard microscope slides. (*B*) For the sliding chamber assay, a three-dimensionally (3D) printed custom-made chamber is required to allow flies to be introduced into the chamber via geotaxis, without need of CO_2. The sliding chamber also permits the separation of individual flies after the fight and retesting. Currently, the multiwell and sliding chamber assays require manual behavioral analysis due to the difficulty of tracking lunges in chambers in which animals are not always presented from a dorsal view. (*C*) The divider chamber also uses a 3D printed custom chamber. This chamber allows isolation of the pair by loading each fly directly into the chamber, which is separated by a divider that is removed to start the fight. This chamber is used with automatic tracking methods, as its reduced height restricts flies to a horizontal plane, allowing an automated classifier algorithm to accurately identify the lunge pattern. (This figure was created with www.biorender.com.)

TABLE 1. Assay comparison

Features	Multiwell chamber	Sliding chamber	Divider chamber
Handling adult flies	Aspiration	Negative geotaxis	Anesthesia
Separation of flies before, during, and after fights	X	✓	✓
Retrieving flies after the assay	X	✓	X
Territory	✓ (food cup)	✓ (food cup)	✓ (whole arena)
Space to retreat	✓	✓	X
Number of arenas/apparatuses	12	3	12
Automated annotation	N/A	N/A	✓
Long-term experience	X	X	✓
Commercially available chamber	✓	X (3D printer)	X (3D printer)
Individual arena volume	~22.475-mm-diameter × 19.5-mm-high	23-mm-diameter × 17-mm-high	14-mm × 14-mm × 4.5-mm high
Suitable for use inside incubators	✓	✓	X

The advantages and limitations of each assay, as well as the situations in which it may be advantageous to use one over the other alternatives are summarized. (N/A) Not applicable.

Aggression is used to defend or obtain resources; therefore, adding a territory with a resource provides an ethologically relevant environment.

8. Place one food cup in the center of each arena of a 12-well cell culture plate.

   Some or all of the 12 wells can be used as arenas.

9. Cover the wells with glass microscope slides.
10. Place each chamber under the video camera.
11. (Optional) Place a sheet of white paper under the chamber to provide contrast.
12. Illuminate the chamber and focus the camera to record from chambers.
13. Using an aspirator, collect two isolated males from each isolation vial and load them simultaneously through the gap between the slide and the chamber into one of the chambers.
14. Start recording immediately after the first pair enters the first chamber. While recording, load flies into the remaining chambers.
15. (Optional) When a row is full (three pairs of flies), tape the microscope slide in place.
16. Record for at least 45 min from the time when the last pair entered the chamber.

    Proceed to Step 40.

Sliding Chamber Assay

Preparing the Sliding Chamber

17. Melt fly food as described in Step 6.
18. Prepare food cups as described in Step 7. Add a very small drop of fresh yeast paste to the center to facilitate aggressive encounters.
19. Place one food cup in the center of each arena.
20. Cover the chambers with a lid.
21. Insert plastic dividers from the top of each arena to separate chambers into spaces of equal size.

Performing the Assay

22. Place the sliding chamber in a humidity- and temperature-controlled environment. Adjust the light source and the focus of the camera.
23. Remove the cotton plug from the isolation vial and insert the vial into the side hole of the apparatus. Let the flies enter the experimental chamber and close the side hole.
24. Repeat Step 23 to insert the second fly in the other side of the experimental chamber.
25. Begin the video recording and remove the divider to allow flies to interact for 45 min.
26. (Optional) If desired, insert the divider into the experimental chamber to separate flies after the aggression assay.
27. (Optional) To retrieve a fly if desired, return the isolation vial into the appropriate side of the apparatus, let the fly enter and close the side hold.

    Proceed to Step 40.

Divider Chamber Assay

Assembling the Divider Chamber

28. Prepare food and add it to the chamber.

Option 1

- Mix 7 mL of clear corn syrup, 4 g of sugar, 2 g of agarose, and 220 mL of H_2O in a microwave-safe flask to make the food base plate for one divider assay chamber (12 pairs of concurrent fights).
- Microwave until the food solution runs clear. Let the hot food rest on the bench until the temperature falls below 65°C (typically 30 min or so at room temperature).
- Add 2 mL of Nipagin/Tegosept. Pour the mixture into the base plate and let it solidify at room temperature before assembling the fighting chamber on top of it (recommended wait time, ~2 h).

Option 2

- Prepare ~100 mL of 1% agarose in Milli-Q-purified H_2O. Microwave 1–3 min until the agarose is completely dissolved, cool briefly and pour into the chamber. Allow it to cool completely.
- Mix 0.5 g of agarose, 1.64 g of yeast, and 7.13 g of sugar in 50 mL of H_2O. Microwave the mixture until boiling to use as a top nutritive layer.

  If raising flies on a high sugar–containing mixture is a concern, in this option, males fight on a nutritive layer (H. Dierick, personal communication; S. Certel, unpubl. results).

- After boiling, pour over the set agarose just below the brim.

  If the nutritive layer is too high, the chamber will not fit correctly; if the nutritive layer is too low, the food will dry, allowing flies to escape.

- Allow the nutritive layer to cool until solidified. The base and nutritive layer should be used the same day of preparation. Wipe off any condensation on the nutritive layer before adding the flies.

29. Assemble the divider assay chambers on top of the food.
30. Place three dividers (X-ray films cut with a Silhouette printer) into the slits between the chambers while holding the chamber upside down, supported by the inverted glass cover.
31. Gently move the inverted and assembled fighting chamber up toward the solidified food.
32. Once the chamber comes in contact with the food surface, invert the entire assembly to have a right-side-up fighting chamber resting on food.

Performing the Assay

33. Briefly anesthetize 24 flies with a CO_2 pad to allow them to be transferred as pairs into each well in the divider assay chamber.
34. Using a paintbrush or fly aspirator, gently place two flies in a single fighting well on each side of the divider separating the well.
35. Once 12 pairs of flies have been loaded, place the top glass cover on top and secure it with a thin strip of clear tape (while not obstructing the view of the fighting arenas).

    Adults may be kept isolated in the chamber for several days or loaded 24 h before the start of the fight.

36. Place chambers under the video camera in a humidity- and light/dark-controlled incubator or room.
37. Once the flies have reached the desired age for the experiments designed by the individual researcher, place the chamber assembly under the camera view and on top of the LED light source for acquisition of high-contrast aggression movies.
38. Start recording at 20 Hz. Gently remove the dividers by pulling them out in a linear motion while not disrupting the isolated flies.

- For high-resolution recording, use Basler acA1920-155 µm or Basler acA2000-165 µm NIR cameras (Graftek Imaging) with the Basler video-acquisition software (www.baslerweb.com/en/products/software/basler-video-recording-software/).
- For standard resolution, Sony HDRCX405 camcorder or similar models are sufficient.

39. Capture frames using Pylon Viewer 5 software at 20 Hz.

Proceed to Step 42.

Scoring and Quantifying Aggression

Manual Analysis

40. To score encounters, transfer the recording from the SD card to a computer, and open it in the iMovie program or another suitable software.
41. Record the genotypes of the paired animals, the calendar date, numbers of encounters in the fight, the patterns observed, and whether or not a hierarchical relationship was established.

When analyzing fights manually, the scorer should remain blind to the genotype or treatment. As in other species, fly fights consist of a series of encounters during which animals approach each other, interact, and separate repeatedly within the observation period. The end of an encounter is defined by pauses in activity and/or distance between flies. For additional information on manual scoring, see Protocol: ***Scoring and Analyzing Aggression in* Drosophila** *(Certel and Kravitz 2012) and Protocol:* ***Fighting Flies: Quantifying and Analyzing* Drosophila *Agression***. *(Fernandez et al. 2023).*

Automated Analysis

42. Convert frames to movies (.avi format) using Pylon Viewer.

This software automatically records and generates .avi movies at specified frame rates.

43. Track movies using FlyTracker version 1.0.5 software and analyze them with JAABA software, both run in MATLAB.
44. Use the lunge classifier (Lunge.jab), designed in JAABA, to identify lunging behaviors (single frames) displayed by individual flies, while maintaining classification efficiency during reciprocal lunging between two boxing males.
45. Process annotated frames in JAABA with the internal post-processing filter set to 0.04.

This value provides the best signal-to-noise ratio for lunge classification.

46. Use an additional post-processing filter in MATLAB to eliminate remaining misclassified lunges based on an inter-fly distance of two or more fly body lengths (Chowdhury et al. 2021).
47. Export raw lunge number data per fly as Excel (version 16.16.13, .csv) files.

DISCUSSION

As new equipment and technologies emerge, behavioral assays in any model organism are often modified and improved in ways that make an assay more precise, easier to implement, and potentially more automated. In this protocol, we outline three established methods that provide investigators choices to use the most suitable aggression assay for the question under investigation. Through decades of aggression research, we know that the initiation and frequency of aggressive interactions are impacted by assay parameters including the method of loading the flies into the observation chamber, the size of the chamber, and the animals' previous social experience. Regarding loading flies, the learning and memory that occurs with fighting experience is sensitive to pretest handling of animals. The sliding chamber assay eliminates the handling of flies, while the divider assay moves fly handling to 1–5 d before a fight, a window outside of possible memory effects. Although each assay

can be used to score sexually dimorphic aggressive patterns, the establishment of dominance has been measured in the multiwell and sliding well chambers in which a raised territory is present within the arena. The larger chambers in the multiwell and sliding well assays provide the opportunity for retreat and/or for the two opponents to not interact with each other, leading to levels of aggression that are not directly comparable to those in assays with smaller chambers (i.e., the divider assay). Finally, the automated behavioral analysis used in the divider assay and others provides the opportunity for high-throughput screens to continue to move the field forward by identifying genes, neurons, circuits, and microbes that promote and constrain aggression.

RECIPE

Yeast Paste for Fly Aggression Assays

Mix 1 g of dry yeast (Sigma-Aldrich YSC2) and 1.3 mL of H_2O to yield a paste.

Store for up to 2–3 d at 4°C.

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