


Aggression in *Drosophila melanogaster*

Budhaditya Chowdhury

 0000-0003-3836-0426

bc036@bucknell.edu

1 Experimental Protocol:

1.1 Materials:

- 2% Agarose
 - *This should be prepared by mixing agarose with water and microwaved till fully dissolved.*
- Clear Corn Syrup
 - *Bought from grocery stores*
- Sugar
- Nipagin / Tegosept
 - *Stock solution made by dissolving 20 grams of tegosept in 100 ml in 95% Ethanol*
- Adult flies (1 day old)
 - *All bottles containing fly genotypes of interest should be kept in an environmentally stable 12hr light/12hr dark conditions. For best results parental flies should not be housed in a crowded condition and transferred to new bottles every 3-5 days. (For best results parental crosses should be set up with 20 virgin females and 20 young males).*
- CO₂ or ice for anesthesia
- Standard fly food (see appendix)

1.2 Equipment:

- 3d printed fighting chamber
 - *For best results the chambers should be printed with ABS material at 100-micron thickness.*
- Dividers
 - *For best results X-ray films should be cut with Silhouette printer for accuracy of dimensions.*
- Top glass cover
 - *Standard large glass slides, or two regular glass slides placed side by side should be used.*
- Base plate for food
 - *Inverted 10 ml clear pipette tip covers can be used as a base plate for food.*
- Computer & Camera setup:
 - *Computer for video acquisition and data analysis: We use a standard Windows PC (Windows 10, 16 GB RAM, 2.6 ghz 4 core processor)*

- *Video acquisition camera body: acA1920-155um - Basler Ace*
- *Video acquisition camera lens: Edmund Industrial Optics 86572 (25mm focal length)*
- *1/4-20" Camera Mounting Plate for Ace Series (Basler 2000029679)*
- *USB 3.0 cable (Basler 2000033239)*
 - *Camera stand (Kaiser Repro Kid Copy Stand Kit (Consists of 23.25" Calibrated Column, 15 x 12.5" Baseboard)*
 - *LED board for bottom illumination.*

1.3 Assembling the Fighting Chamber on food

- Making the food
 - *To make food for one divider assay chamber (12 pairs of concurrent fights) the following ingredients and recipe is recommended.*
 - *Clear corn syrup: 7 ml*
 - *Sugar: 4 g*
 - *Agarose: 2 g*
 - *water: 220 ml*
 - *Mix all the ingredients in a microwave safe flask and microwave till the food solution runs clear.*
 - *Let the hot food rest on bench till the temperature falls below 65 C (typically 30 mins or so in room temperature)*
 - *Add 2 ml Nipagin/Tegosept*
 - *Pour in food boxes and let it solidify before assembling fighting chamber on top of it (recommended wait time ~2 hours)*
- Assembling divider assay chambers on top of food (*Consult Supplementary movie to see the assembly of chamber*)
 - *Place three dividers through the slits between the chambers while holding the chamber upside down, supported by the inverted glass cover.*
 - *Gently move the inverted and assembled fighting chamber up towards the solidified food and once the chamber comes in contact with food surface*

invert the entire assembly to have a right side up fighting chamber resting on food.

1.4 Loading 1-day old flies on Fighting Chamber

Newly eclosed flies (less than a day old) should be anesthetized on CO₂ or ice. Two male flies should be gently placed in a single fighting arena on two sides of the divider. Once 12 pairs have been loaded the top glass cover should be placed on top and secured with a thin strip of clear tape (while not obstructing view of fighting arenas)

1.5 Video acquisition of behavioral experiments

- The divider assay chamber with isolated future fighting pairs should be housed in a temperature and humidity-controlled environment with a 12hr light/12 hr dark cycle.
- Once the flies have reached appropriate age to carry out experiments (5 days) the chamber assembly should be placed under the camera view and on top of LED lightsource for acquisition of high contrast aggression movies.
- The dividers should be gently removed by pulling them out in a linear motion while not disrupting the isolated flies.
- The Basler video acquisition software should be run to acquire movie file of the 12 well chamber for the duration of assay (we recommend recording at 20 Hz, and for assay duration of 20 minutes).

1.6 How to Design behavioral Classifiers

1. The first step in designing a classifier is ascertaining if the behavior is discrete (Lunging), or continuous (boxing). The output of automated action-classifying algorithms (like JAABA) should return results that match conventional behavioral scoring (manual quantification).
 - **Critical Step** Discrete behavior classifiers can start off by labeling multiple frames, but rigorous subsequent training should be done till classified frames are a match with manual scoring. For classifiers that labels 5 or more frames when only one should be picked, it might be prudent to start over.
2. The framerate of the movie that records behavior should be decided a priori. It will also depend on behaviors of interest, contrast and resolution of camera, and how many pixels constitute a single animal of interest. For Lunging, a high-resolution movie should

be at least 20 fps, and a regular handheld camera 25 and above. For regular cameras we recommend the chambers are bottom lit to increase contrast.

- **Critical Step** Once a classifier is fully trained, there is some leeway with differing frame rates, but every time a movie in a new recording setup is classified, manual scoring and classified movie should be matched for accuracy, before engaging in large scale annotation. Generally, a properly trained classifier requires only minor tweaks and re-training in a new setup.
3. The first step in any automated annotation is picking a tracking algorithm that tracks flies and generates perframe features that can be imported into JAABA. Proper tracking depends on the contrast of animals on a well-lit background and a movie that is devoid of any camera shakes. A Proper tracker also should make little mis-categorization of fly Identities. We recommend FlyTracker 1.05.
 4. When training behavioral classifiers for discrete behaviors, the sequence of actions that constitutes a behavior should be kept in mind and the most salient feature should be trained. For instance, aggression constitutes a series of steps that culminate in the hallmark action of a Lunge (orientation, head-on or perpendicular alignment, rearing up on hind legs, and snapping down with front legs). In our movies, the rearing up, as well as the snap constitutes a single frame – both of which are amenable for training a lunge classifier (one frame – one count). However, the rearing up can also be seen when a fly moves from the floor to the side. This left the snap as the action pattern of choice and the lunge classifier is trained to identify this single frame snap.
 - **Critical Step** When using the lunge classifier with regular handycam acquired movies, a slowed down version should be first checked where the snap doesn't lose pixel integrity. If a freeze frame of a lunge looks like the fly pixels has broken into multiple component pieces, the single frame pre-lunge rearing up should be classified. By changing the chamber design where the arena edges are at an angle (like the flyBowl Assay for instance), misclassifications of crawling up on the side can be reduced.
 5. We recommend using at least 300+ instances of lunging frames to train a classifier, along with negative frames. 300 single lunges will for instance give 200 instances of hits, at least 500 –1000 instances of no-lunge frames should be included in the training. In

our experience a single lunge-frame bookended by multiple frames of no-lunge classifications returns the fastest results.

6. Ground-truthing helps increase the efficiency of classifiers. But in our opinion a meticulous step of quantifying multiple aggression movies manually, and then comparing them with results returned by automated annotation should be performed. This solves multiple problems:
 - a. If the classifier is labeling multiple frames as a single lunge, the average difference between manual scoring, and classified movie will be readily apparent (if two frames are being labeled as a lunge, the automated classification will return double the number of lunges as manually scored!)
 - b. A subsequent frame by frame analysis will clear up which behaviors are getting mislabeled, making it easier to train them out.
 - c. Setting post processing filters will help retain correct IDs while removing unwanted ones, making the total count get as close to the gold standard of manual scoring as one wants.