Video Article

Methods to Assay *Drosophila* Behavior

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Abstract

Drosophila melanogaster, the fruit fly, has been used to study molecular mechanisms of a wide range of human diseases such as cancer, cardiovascular disease and various neurological diseases[1]. We have optimized simple and robust behavioral assays for determining larval locomotion, adult climbing ability (RING assay), and courtship behaviors of *Drosophila.* These behavioral assays are widely applicable for studying the role of genetic and environmental factors on fly behavior. Larval crawling ability can be reliably used for determining early stage changes in the crawling abilities of *Drosophila* larvae and also for examining effect of drugs or human disease genes (in transgenic flies) on their locomotion. The larval crawling assay becomes more applicable if expression or abolition of a gene causes lethality in pupal or adult stages, as these flies do not survive to adulthood where they otherwise could be assessed. This basic assay can also be used in conjunction with bright light or stress to examine additional behavioral responses in *Drosophila* larvae. Courtship behavior has been widely used to investigate genetic basis of sexual behavior, and can also be used to examine activity and coordination, as well as learning and memory. *Drosophila* courtship behavior involves the exchange of various sensory stimuli including visual, auditory, and chemosensory signals between males and females that lead to a complex series of well characterized motor behaviors culminating in successful copulation. Traditional adult climbing assays (negative geotaxis) are tedious, labor intensive, and time consuming, with significant variation between different trials[2-4]. The rapid iterative negative geotaxis (RING) assay[5] has many advantages over more widely employed protocols, providing a reproducible, sensitive, and high throughput approach to quantify adult locomotor and negative geotaxis behaviors. In the RING assay, several genotypes or drug treatments can be tested simultaneously using large number of animals, with the high-throughput approach making it more amenable for screening experiments.

Video Link

The video component of this article can be found at https://www.jove.com/video/3795/

Protocol

1. Larval Crawling Assay

1. Larvae Collection

1. Set up an 8 ounce bottle of flies (10-15 males + 10-15 females).
2. Let flies lay eggs for 24 hours, then clear bottle of flies. (Transfer the adults into a new bottle and repeat as necessary).
3. Incubate bottle for 3-4 days, or until third instar larvae are visible.
4. Add 50 - 100 ml of 20% sucrose to the bottle with larvae and let sit for 20 minutes. Larvae will float to the top.
5. Collect larvae using a 25 ml serological pipette with the tip cut off, and place into a mesh basket.
6. Wash larvae in the mesh basket two times with deionized H_2O. Larvae are now ready for the experiments.

2. To treat larvae with drug

1. Use a brush to transport the desired number of larvae to a 5 ml beaker containing a solution 5% sucrose + drug.
2. Let larvae feed for at least 15 minutes.
3. Pour drug-treated larvae into a mesh basket and rinse. They are now ready to use.

3. Locomotor Assay (measuring total distance travelled or body wall contractions)

1. Use a brush to transport individual larva to a:
    1. 15 cm Petri dish containing 2% agarose (previously poured and allowed to harden) over graph paper with a 0.2 cm^2 grid.
        1. Count number of grid lines crossed in 1 minute.
    2. Well of a glass dissection dish containing a dilute yeast paste solution.

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1. Count peristalsis contractions (full anterior to posterior movement = 1 contraction) in one minute while observing under a dissection microscope.

2. Repeat until the desired numbers of larvae have been counted.

2. Rapid Iterative Negative Geotaxis (RING) Protocol

This assay was originally described by Gargano *et al*[5].

1. Collect newly emerged adult male flies under light CO_2 anesthetization and place into a standard vial containing food (or food + drug).
2. Maintain flies at room temperature (on the bench top. ~22 °C) for 2-3 days to allow for recovery from CO_2 (and accumulation of steady-state drug levels if appropriate).
3. Transfer about 25 flies *without anesthetizing* to prepared polystyrene vials.
4. Assemble vials with flies into the RING apparatus (Figure 1).
5. Allow flies to acclimate to the environment, undisturbed, for 15-20 minutes.
6. During this time place digital camera ~1 m in front of the apparatus (on a platform if necessary to align the center of the lens at mid-height of the vials), focus and zoom the camera onto the apparatus, and set a timer to 3.0 seconds.
7. Carefully take hold of the RING apparatus with your left hand so as not to disturb the flies, and hold the timer with your right hand.
8. Sharply tap the apparatus down on the surface of the bench three times, ensuring that the tap is hard enough to knock down all the flies to the bottom of the vials.
9. Simultaneously with completion of the third tap, start the 3 second countdown timer.
10. At three seconds take a picture.
11. Reset the timer for 1 minute and start. During this time reset the camera and focus on the apparatus, and set another channel of the timer for three seconds.
12. After 1 minute, repeat steps 1.7-1.10
13. After a total of 5-6 trials, upload images onto a computer and use your favorite image viewer to open, and score the average height climbed for each vial.
14. Perform statistical analysis on your different groups comparing the mean height climbed.

3. Courtship and Mating Assay

1. First thing in the morning, clear well-producing bottles of flies to be used.
2. Over the course of the day (every 3-4 hours), collect newly emerged sexually naive males and females:
    1. Place males individually in vials or tubes with medium.
    2. Place 5-6 females together per vial/tube.
    3. Isolate collected flies at 25 °C under 12 hr light/dark for 5 days.
    4. Transfer one female into the chamber of a mating wheel.
    5. Transfer one male into the chamber of a mating wheel.
    6. Observe pair under a dissection microscope for the following behaviors:
        1. Orientation (the male orients towards the female)
        2. Tapping (the male taps the female)
        3. Wing song (the male extends and vibrates one wing)
        4. Licking (the male licks the female genitalia)
        5. Curling (the male curls its abdomen under itself)
        6. Copulation attempt (Curling activity while attempting to mount the female)

3. Observe for 10 minutes or until successful copulation, noting the time at which each behavior occurs (latency), the total time engaged in courtship behavior until copulation (to calculate the courtship index), as well as the number of pairs that successfully perform a given behavior (frequency). 100% of wild type pairs will generally mate within 5 minutes.
4. Calculate a courtship index (CI) by dividing the time spent in courtship divided by the total time until copulation. For wild type pairs this usually ranges between 0.6-0.8.

4. Representative Results

Crawling assay

Normal wild type larva will wander ~3 cm/minute, and exhibit ~40-50 body wall contractions in one minute. We have recently developed a Drosophila model of FUS/TLS-related amyotrophic lateral sclerosis that shows larval crawling defect, reduced life span and adult climbing impairment[6]. We targeted expression of wild type and mutant forms of FUS/TLS to the motor neurons (OK-371-gal4 driver) and performed a larval crawling assay. As shown below, wild type larvae crawl up to 12 cm, whereas expression of wild type FUS decreased the larval crawling ability to approximately 6 cm. Animals expressing the ALS-causing mutation R521C in FUS/TLS demonstrate a very severe impairment in their crawling movement (Figure 1), crawling only about 1 cm/minute.

Negative geotaxis RING assay

Young wild-type adult flies should have an average climbing height of ~4-5 cm in a 3-second time period (the time can be adjusted from 3 seconds to accommodate different strains or activity levels to define a given average height for a given strain/treatment). Flies that remain at the

bottom are assigned a value of 0. It is not advised to use more than 25 flies per vial because it then becomes difficult to determine the location of each individual fly to measure height. No desensitization has been observed at up to the 6 consecutive trials spaced 1 minute apart that we have employed. It is critical *NOT* to reuse the polystyrene testing vials in this assay after the initial sets of data are gathered because new flies placed into used vials will not climb to the same extent as in fresh vials.

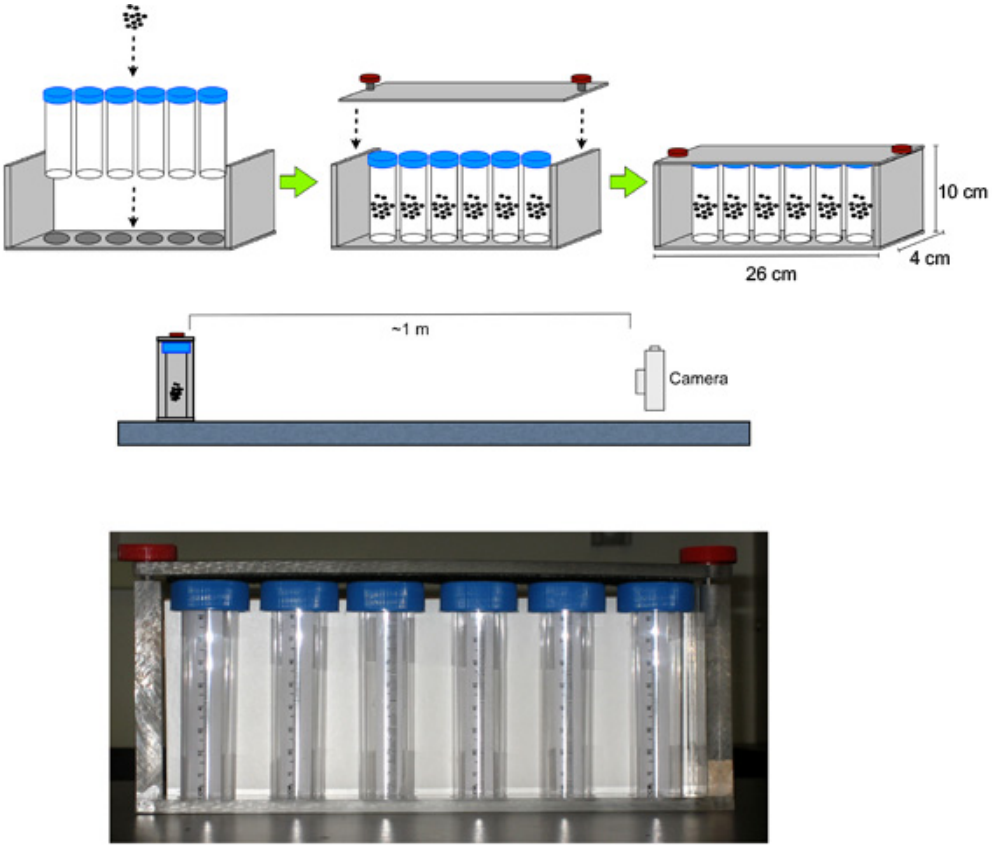

Figure 1. Setup for the RING Assay. The digital camera is placed ~1 m in front of the apparatus containing flies in the polystyrene vials; focus and zoom the camera onto the apparatus; and set a timer to 3.0 seconds.

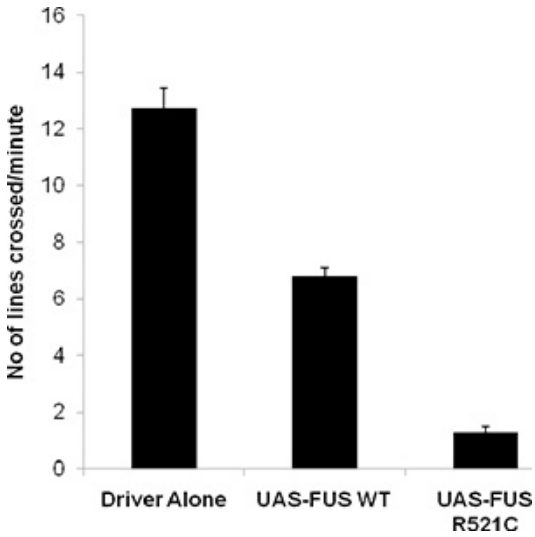

Figure 2. Representative data from the larval crawling assay using flies ectopically expressing UAS-FUS WT, and UAS-FUS R521C under the control of a motor neuron driver (OK371-gal4).

Discussion

Drosophila behavior is tightly regulated by genetic and environmental factors. We, and others, have previously used the assays described here to gather data to examine genes related to fly behaviors and to human neurodegenerative diseases modeled in *Drosophila*[5-19]. For the crawling

assay, careful selection of 3rd instar larvae is a critical step. If treating with a drug, it will take 10-15 minutes (or more depending on the type and nature of the drug) to achieve maximal effect if it has good solubility. Therefore, we routinely feed flies the drug for 15 minutes, and then wait an additional 15 minutes before testing. It is important, however, to keep drug concentrations in solution, and exposure times, between treatment groups the same for accurate comparison. CNS active drugs will usually have a maximal effect lasting ~45 minutes. The larvae should be washed well after selection (or dug feeding) to remove the fly food and allowed acclimatized for 1 minute before starting the crawling assay. The agar plate should be kept at room temperature (~22° C) for an hour, as low temperature can influence larval crawling ability. Although the larval crawling assay can provide important information regarding activity levels, it is not suitable for analysis of subtle coordination deficits. Therefore, as a screening platform it is most appropriate for a first pass examining gross activity deficits.

An adult behavior that involves fine motor coordination is courtship and mating. This behavior has been used to examine aspects of behaviors relevant to human diseases, and involves sensory processing (olfactory, visual, acoustic) in addition to fine motor control[18]. When a male notices a female, he initiates the courtship ritual that progresses in a stereotype pattern beginning with orientation behavior (turning toward and chasing the female). This is followed by wingsong, licking and tapping of the female genetalia, curling of the abdomen towards the female, and culminates in copulation, which can last several tens of minutes[20-22]. Because many of the aspects of courtship involve visual cues, dark conditions lead to a degradation of mating performance, and assays should be performed with enough light so that flies can see one another. Accordingly, white-eyed flies usually have extremely poor performance in our described assay and readers are advised against planning experiments utilizing them with this protocol. If treating the flies with a drug, put the drug into the medium the flies are isolated on (e.g. 1% agarose + 10% sucrose + drug instead of standard food when treating with drug to avoid any possible degradation of the drug by microorganisms in the food). Generally the flies are isolated into 5 ml test tubes that have 300-500 ml of food in them, plugged with cotton. Do *NOT* anesthetize the flies for transfer to the mating wheel. It is absolutely *CRITICAL* to wash the mating wheel very well between uses to remove any residual pheromones (warm water with small amount of alconox soap over night with shaking, then washed for a minimum 48 hours in deionized water with shaking, and numerous changes of water). Another consideration when performing the assay we describe is the weather. In our experience flies will not court if it is raining or looks like it will rain. They perform best on bright sunny days, regardless if the laboratory has windows or not. Our current theory is that this phenomenon is related to atmospheric pressure, but we have not investigated this.

The traditional negative geotaxis assay relies on measuring how many flies climb above a predetermined height in 10 seconds (described in http://www.jove.com/details.php?id=2504). We believe that the RING assay has certain advantages over the traditional assay. One is throughput, as six independent replicates can be measured simultaneously compared to the one of the standard assay, and the system is generally scalable. Another is sensitivity, because the average height climbed in a defined time period is quantified, rather than a pass/fail number for absolute height. Using this approach, more subtle deficits may be observed. Because of the level of throughput, the assay is more suited to screens than the more tedious traditional negative geotaxis assay. Further, Garagano *et al.* (2005) describe a computerized scoring method that if employed would further increase throughput. Primary considerations for this assay are that it is imperative that the flies are not anesthetized before testing, and that fresh vials are used after a given set of trials before a new batch of flies are tested.

Disclosures

We have nothing to disclose.

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