Video Article

An Automated Rapid Iterative Negative Geotaxis Assay for Analyzing Adult Climbing Behavior in a *Drosophila* Model of Neurodegeneration

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Abstract

Neurodegenerative diseases are frequently associated with a progressive loss of movement ability, reduced life span, and age-dependent neurodegeneration. To understand the mechanism of these cellular events, and their causal relationships with each other, *Drosophila melanogaster*, with its sophisticated genetic tools and diverse behavioral features, are used as disease models for assessing neurodegenerative phenotypes. Here we describe a high-throughput method to analyze *Drosophila* adult negative geotaxis behavior, as an indication for possible motor defects associated with neurodegeneration. An automated machine is designed and developed to drive fly synchronization using an initial electric impulse, later allowing the recording of negative geotaxis behavior over a course of secs to mins. Images from the digitally recorded video are then processed with the self-designed RflyDetection software for statistical data manipulation. Different from the manually controlled negative geotaxis assay based on single fly, this precise, fast, and high-throughput protocol allows data acquisition from more than hundreds of flies simultaneously, providing an efficient approach to advance our understanding in the underlying mechanism of locomotor deficits associated with neurodegeneration.

Video Link

The video component of this article can be found at https://www.jove.com/video/56507/

Introduction

A variety of protocols and methods have been developed for analyzing *Drosophila* adult climbing behavior. Rather laborious, the traditional analysis mostly involves putting a single fly into an individual vial and uses a manual force to tap flies down for synchronization[1,2,3,4]. It is tedious and time consuming, unsuitable for large high-throughput studies, and has potential variations of the manual force used to tap down the flies as well as other limitations. To improve the assay, a Rapid Iterative Negative Geotaxis (RING) assay was developed which allows high-throughput analysis over numerous flies at the same time[5]. However, the assay still requires a manually exerting force to synchronize fly action. Our version of the RING assay, revised upon the previous assay, includes a metal base hosting multiple fly-containing vials automatically controlled by an electric motor to drive fly synchronization[6]. Upon recording, the fly climbing immediately after synchronization is recorded then analyzed using a self-designed software. Our automated RING assay has eliminated the tedious and labor-intensive process in collecting data from a single fly, one at a time, and enabled the data acquisition process to be more efficient. In addition, the automated RING assay has been employed in a number of studies to elucidate the mechanism underlying Alzheimer's and Parkinson's Disease, validating the approach with high efficiency[7,8,9].

In this article, we demonstrate the automated RING assay using the *DDC-Gal4* driven RNAi flies. *DDC-Gal4* is a Gal4 line specifically expressing in dopaminergic (DA) and serotonergic neurons, thus representing a great tool for analyzing the target gene effects associated with locomotor deficits accompanying neurodegeneration[10]. In addition, we incorporate *UAS-Dicer2*, a fly line that enhances RNAi efficiency, to generate the *UAS-Dicer2; DDC-Gal4* tool line. The RNAi flies we choose to use is the *auxilin* (*aux*) RNAi v16182 ($auxR^{16182}$), a gene that we have previously identified to exhibit an effect on fly locomotor activity[8]. *auxGFP* flies are also prepared for analyzing effects upon *aux* overexpression. We will show how to use the automated RING assay to measure fly negative geotaxis, present the results, and discuss any implications acquired from the results.

Protocol

1. Fly Collection

1. Maintain the flies on standard fly food at 25 °C, 70% humidity, and a 12 h/12 h light/dark cycle.
2. Collect *UAS-Dicer2; DDC-GAL4* fly virginsunder carbon dioxide (CO_2) anesthesia.
3. Cross these virgins to 2 day old adult male flies carrying the following genotypes: *UAS-mCD8GFP* (control), *auxR*^{16182} (*aux* RNAi), *auxGFP* (*aux* overexpression), and *auxR*^{16182}*; auxGFP* (rescue), with a male: female ratio of 1:2.
4. Seperately collect newly eclosed males and females in 3 vials for each group per experiment, placing 10 flies in each regular fly vial with standard food at 25 °C.
5. Depending on the experiment, keep the collected flies up to 35 days and use them for automated RING analysis at day 5, 15, 25, and 35.

2. Automated RING Assay

1. Transfer 10 collected flies (unisex) per genotype into each vial, then secure the vial with a screw. Analyze control flies and flies carrying different genotypes together for each set of experiments (up to 10 vials simultaneously, 10 flies in each vial).
2. Turn on the digital camera placed in front of the apparatus, and start recording once flies are all loaded and ready.
3. After allowing 1 min for flies to settle in vials, turn on the step controller that controls the step driver; this drives the small electric motor to control the lever attached so that it consecutively rises and taps the apparatus 4 times in 2 s. See **Figure 1**.
    1. After tapping, note that the flies begin to ascend the wall. Ensure that recording continues.
4. Repeat the synchronization as described in step 2.3 in 60-s interval for 3 to 5 consecutive trials.
5. Repeat the experiment for 5, 15, 25, and 35 day old flies. Conduct at least 3 independent experiments for each group, each experiment with at least 30 collected flies (3 vials).

3. Data Analysis

1. Import the recorded video into the computer.
2. Take a snapshot of the video at 6 s after tapping, for each trial.
3. Import the snapshot image into the RflyDetection software (see **Figure 2**), using the 'File' menu.
4. Set the upper and lower baselines of the vial precisely by using the baseline icon on the toolbar and then using the cursor to mark the upper and lower baselines on the image.
5. Input the number of flies per vial (*e.g.*, 10 here) into the 'Flys in rect' field and vial length (*e.g.*, 14 cm here) in the 'Tube Height' field within the settings bar.
    1. Note that the individual fly positions are detected and labeled with dots on the screen for each vial.
    NOTE: **Figure 2** indicates the positions of all menu button clicks.
6. Note that the software automatically determines the climbing distance for each fly and displays the averaged values from 10 flies in each vial in a table on the right-hand panel. (See **Figure 2**)
7. Process the climbing number with statistical software (*e.g.*, Prism) for further statistical analysis.
8. Present the data as mean ± SEM.
9. Calculate the *p*-values of significance (indicated with asterisks, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) using one-way ANOVA with Bonferroni multiple comparison test.

Representative Results

This article demonstrates the use of an automated RING assay in assessing fly negative geotaxis behavior. Unlike the previous RING assay, our assay includes an automated apparatus that provides an electric force to synchronize fly action and analyzes up to hundreds of flies simultaneously (**Figure 1**). Analysis of *Dicer2; DDC>auxR*^{16182} flies showed an age-dependent decrease in the climbing distance in a 6-s time frame for both male and female flies, suggesting that *aux* expression in DA neurons is crucial for fly locomotor activity. Climbing ability does not seem to be affected upon *auxGFP* overexpression in DA neurons, and co-expression of *auxR*^{16182} and *auxGFP* in DA neurons rescued the RNAi phenotype (**Figure 2** and **Figure 3**). In addition to validating the automated RING assay as a high-throughput and efficient approach for assessing motor defects associated with DA neuron degeneration, these results also indicate that this automated approach is useful in identifying potential Parkinson's Disease risk factors and provides a clue on their neurodegenerative function.

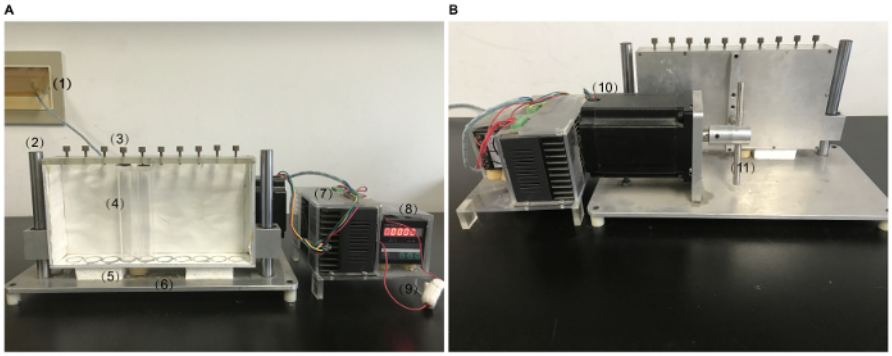

Figure 1. The automated RING apparatus. (**A** and **B**) Front and rear views of the apparatus: socket (1), vertical steel rod (2), a screw for securing the tube on the frame (3), transparent plastic vials (4), foam bar (5), metal base (6), micro-step driver (7), electronic controller (8), the switch (9), small electric motor (10), and the lever attached to the small electric motor (11). The size of the rectangular metal frame is 46 cm × 26 cm × 16 cm, holding 10 transparent plastic vials (2.1 cm in diameter, 14.5 cm in height) secured with screws. To turn on the apparatus, first click on the switch (9), which turns on (8), (7), and (10) consequently. Please click here to view a larger version of this figure.

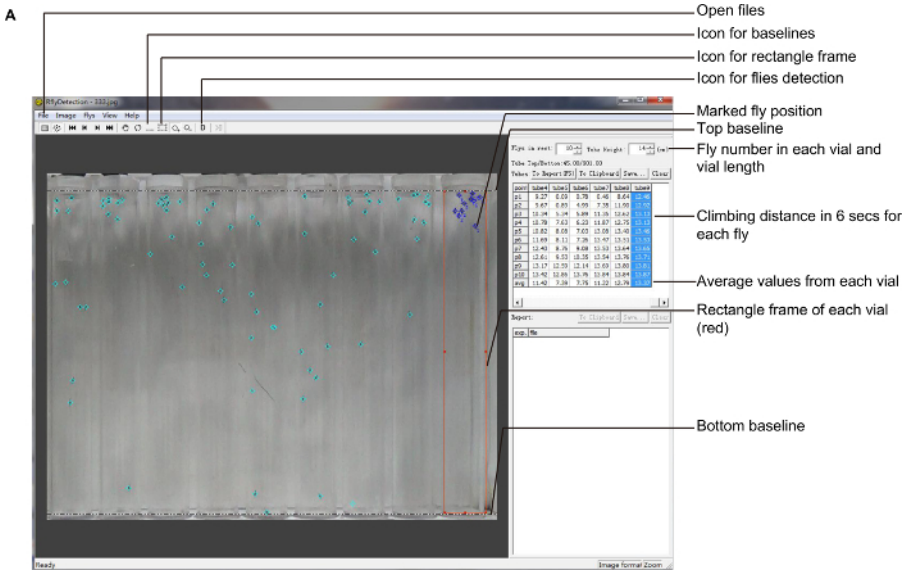

Figure 2. Analysis of a representative snapshot image by RflyDetection. Snapshot image is imported into the RflyDetection software and action for each icon at the interface is indicated with text on the right. After setting the top and bottom baselines, the software automatically detects and marks the fly position within the vial. The climbing distance (cm) in 6 s for each fly and the averaged values for each vial are displayed in a table. These numbers are then imported into the statistical software for further statistical analysis. Please click here to view a larger version of this figure.

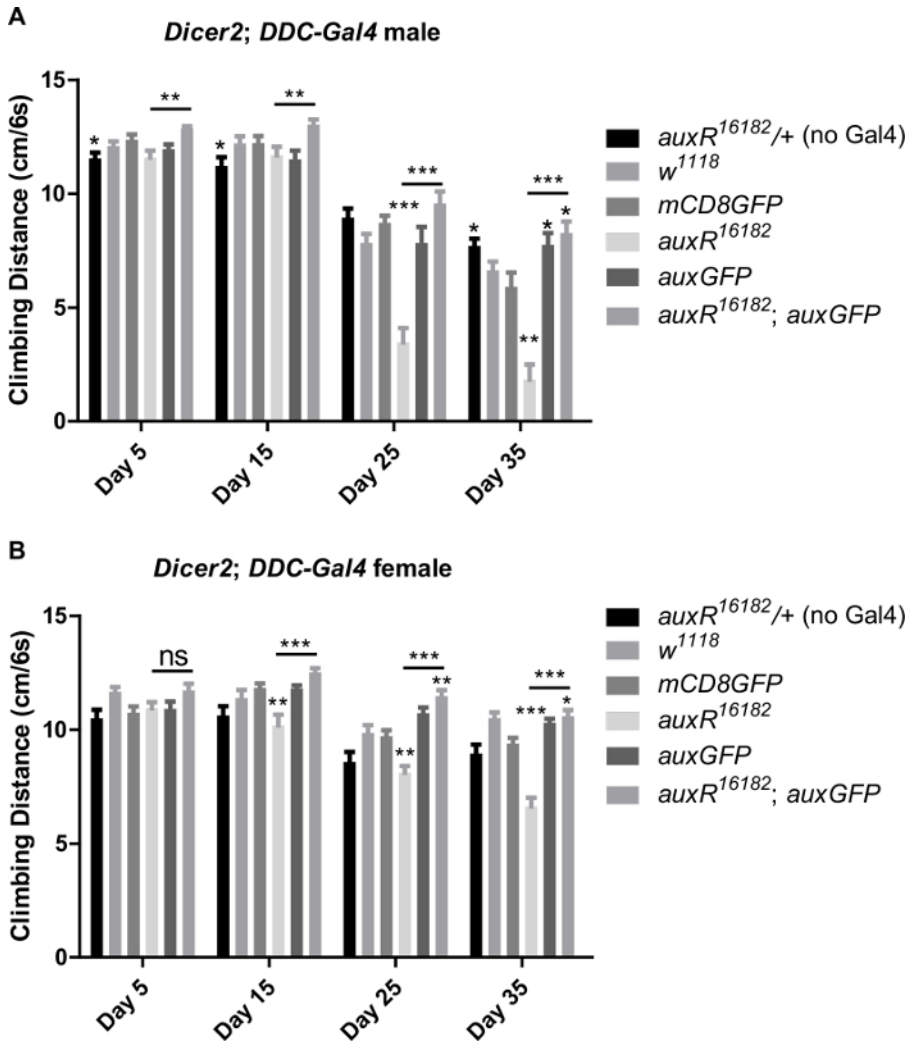

Figure 3. Statistical bar graphs showing an age-dependent decline in fly climbing ability when *aux* expression is downregulated in DA neurons. The automated RING assay was used to analyze negative geotaxis behavior for flies carrying the following genotypes: *auxR*^{16182}/+ (no Gal4), *UAS-Dicer2; DDC-Gal4* flies crossed to *w*^{1118} (control), *UAS-mCD8GFP* (control), *auxR*^{16182}, *auxGFP*, and *auxR*^{16182}; *auxGFP* (rescue). Note that both male and female flies climbed significantly slower over time when *aux* expression was reduced in DA neurons. The locomotor deficits were rescued upon reintroducing *aux* expression. Flies of 5, 15, 25, and 35 day old were assessed. Male (**A**) and female (**B**) flies are shown separately. Legends with genotype labels corresponding to different bar colors are shown on the right. Data are shown as mean ± SEM. *p*-values of significance (indicated with asterisks, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: no significance, a bar was drawn below the asterisks to indicate the objects to compare) were calculated comparing to the *mCD8GFP* control or *aux* RNAi using one-way ANOVA with Bonferroni multiple comparison test. Please click here to view a larger version of this figure.

Discussion

The automated RING assay described here enables a high-throughput analysis of fly negative geotaxis behavior for hundreds of flies simultaneously. Previously existing strategies for analyzing adult climbing involve observations of a single fly in an individual vial, and fly position is manually detected by eye. This rather tedious process might sometimes cause misreading or misinterpretation of data, as well as labor-intensive work. Our automated RING assay starts with a simple click, and the apparatus automatically synchronizes and assesses climbing ability of up to 100 flies. The electrical synchronization provides a more precise means to control time and other parameters, so the overall capturing of fly adult climbing process can be carefully measured.

Next, with our self-designed RflyDetection software, the exact fly position can be identified. The software takes into consideration the overall 2D vial area (determined by the rectangular frame), and the top and bottom baselines, before acquiring positions for all flies within the designated area and presents them as dots. In addition, the automatic calculation of the climbing distance and averaged values allows faster and easier data manipulation. Data sets over a large group of flies can be plausibly collected and analyzed within a reasonable time frame[7,8,9].

Even though an age-dependent decline in the climbing activity associated with neurodegeneration is described here, this assay is equally effective in analyzing locomotor activity relevant to developmental defects. To this end, the automated RING assay is a useful and efficient approach for analyzing *Drosophila* negative geotaxis in any adult stage, and in either the normal or pathological state of brain function.

Nonetheless, the assay has limited use in completely analyzing all flies despite the RflyDetection software's ability to manually identify any accidentally uncaptured ones.

Disclosures

The authors have nothing to disclose.

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