Video Article

A Low-cost Method for Analyzing Seizure-like Activity and Movement in *Drosophila*

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Abstract

Video tracking systems have been used widely to analyze *Drosophila melanogaster* movement and detect various abnormalities in locomotive behavior. While these systems can provide a wealth of behavioral information, the cost and complexity of these systems can be prohibitive for many labs. We have developed a low-cost assay for measuring locomotive behavior and seizure movement in *D. melanogaster*. The system uses a web-cam to capture images that can be processed using a combination of inexpensive and free software to track the distance moved, the average velocity of movement and the duration of movement during a specified time-span. To demonstrate the utility of this system, we examined a group of *D. melanogaster* mutants, the Bang-sensitive (BS) paralytics, which are 3-10 times more susceptible to seizure-like activity (SLA) than wild type flies. Using this novel system, we were able to detect that the BS mutant *bang senseless* (*bss*) exhibits lower levels of exploratory locomotion in a novel environment than wild type flies. In addition, the system was used to identify that the drug metformin, which is commonly used to treat type II diabetes, reduces the intensity of SLA in the BS mutants.

Video Link

The video component of this article can be found at http://www.jove.com/video/51460/

Introduction

Given its short lifespan and the robust genetic tools available, *Drosophila melanogaster* is an excellent model system for investigating the etiology of various diseases and the underlying physiology of various biological processes. In many cases, it is advantageous to measure the effects that behavioral, genetic or pharmacological manipulations have on locomotion in these model organisms[1,2].

There are a variety of methods that are commonly used for measuring fly movement in two-dimensions[3-7]. These systems can support the tracking of multiple flies simultaneously and can measure velocity, record path lengths and record the percentage of time a fly has spent moving. They have been used to study movement in a variety of contexts including the effects of drugs on locomotion and the sexual dimorphic nature of fly movement[6-9]. The major drawback of these systems is cost of the tracking system or the corresponding software and camera. In some cases, this can run in the thousands of dollars. Cost is a particular concern for a lab that would only need limited use of such a system, for example, quantifying locomotive patterns of a newly isolated mutant.

A simpler but less robust method is to use systems which record movement based on how many times a fly crosses an infrared light beam path that is placed in the middle of a closed tube[10,11]. While such systems can give valuable information on movement and sleep-wake cycles, they can over or under estimate movement because they fail to capture the actual path of the fly. For example, flies that exhibit considerable movement at the ends of the tube will register as low movement flies although additional high-resolution methods have been used to try and circumvent these limitations[12].

Simpler and cheaper still are climbing devices that measure geotaxis, the upward movement of flies, through a single tube or a series of tubes[2,13]. While such systems are cheap and can easily identify geotaxis defects, they fail to capture many other aspects of movement that would be of interest to investigators.

For many labs, a low-cost robust analysis system that is simple to set-up and operate would be an advantageous tool for characterizing behavioral differences in *D. melanogaster* strains. Here we describe an assay that can be set-up for less than two hundred dollars and is able to give information about the path, velocity and duration of the fly's movement. To demonstrate the efficacy of the assay, we present data showing that it can be used to identify; 1) a locomotor defect in a Bang-sensitive (BS) mutant that is susceptible to seizures and 2) the ability of the drug metformin, which is commonly used to treat type II diabetes, to reduce the intensity of seizure-like activity (SLA) in two BS mutants.

Protocol

1. Preparation of Individual Flies for Locomotion Assay

1. Transfer flies by gently tapping into individual empty vials and cap the vials with a cotton plug. Allow flies to sit undisturbed 20-30 min before observation. It is important to not anesthetize the flies within the hours prior to behavioral observation as previous studies have found anesthesia exposure can alter behavior as compared to unanesthetized flies[14].
2. Gently tap the fly out of the vial and place individual flies under a 5 cm diameter Petri dish cover (5 mm high) with small slits to allow air passage. Illuminate from below and observe the flies using a webcam mounted above the Petri dish.

2. Preparation of Individual Flies for Seizure Assay

1. Feed two-day old seizure sensitive flies either standard yeast/cornmeal/agar media or standard media mixed directly with drug. For the data presented here, flies were fed either 1 g of standard media or 1 g of standard media mixed with 25 mg of metformin.
2. After the flies have fed for two days on the media or media plus drug, transfer them by gentle tapping into individual empty vials and cap the vials with a cotton plug. Allow flies to sit undisturbed for 20 min before observation. It is important to not anesthetize the flies within the hours prior to behavioral observation as previous studies have found anesthesia exposure can alter behavior as compared to unanesthetized flies[14].
3. Vortex individual vials containing a single fly on a lab vortexer using the highest setting for 10 sec. Place the immobilized fly on a blank white sheet of paper directly below a webcam mounted above the paper.

3. Video Recording

1. Record the movement or SLA using the HandyAvi software program http://www.azcendant.com/download.htm. This program uses the webcam to capture images based upon the user settings.
2. Select the Time-Lapse images option under the Capture tab, When the window opens, select the webcam as the capture device. For most experiments the video frame size of 640 x 480 is adequate.
3. Select Intel IYUV codec for the compression and choose 0.1 sec/frame for the seizure assay or choose 0.1-0.5 sec/frame for the movement assay. Under the advanced tab, select create a .bmp image file for each capture and select a folder for the program to store the images that it takes (the image stack).
4. Place a dead fly on the sheet of paper. Click on the Video settings box and adjust the settings under the Device settings tab so that there is a bright white background and a clear contrast with the darker fly.
5. Click the start button to begin the recording. Once the recording period has finished, click the stop button. The image stack should now be in the folder selected. Note: Before recording, ensure that the folder selected for the image stack is empty.

4. Data Analysis Using ImageJ

1. Open NIH's free image processing software ImageJ, which can be downloaded at http://rsbweb.nih.gov/ij/download.html. Note: In addition to downloading the program, the Multitracker plug-in must be downloaded and installed as indicated at http://rsbweb.nih.gov/ij/plugins/index.html.
2. Import the image stack into ImageJ by clicking on the File tab and then selecting Import → Image sequence.
    1. Select the folder that contains the image stack, highlight one of the images and select open. A Sequence Options dialogue box will open. Click the Sort names numerically and then select OK and the image stack will be loaded into ImageJ.

3. Threshold all the images so that each consists of a white background with the fly being converted to a black dot. To begin this process, convert the image to an 8-bit format by selecting Type → 8 bit under the Image tab.
    1. Click on the Image tab again and select Adjust → Threshold. This will open the Threshold dialogue box in which the threshold cut off point can be adjusted.
    2. Scan through the images to see if there are extra black dots or if a slice lacks a black dot. If this is the case, adjust the threshold to alleviate the problem. Usually the threshold does not need to be adjusted as the program defaults to an adequate value.

       Note: Every slide in the stack should have just one black dot representing the position of the fly.
    3. Select apply in the Threshold dialogue box and a Convert to Mask dialogue box will open. Do not click any of the options but simply click OK to threshold the image stack.

4. After thresholding the image stack, select the Multitracker plug-in under the Plugins tab in order to measure the movement of the fly in pixels. The Object Tracker dialogue box will appear.
    1. Click all of the four options given in the dialogue box and then select OK. The Multitracker plug-in will then attach coordinates to the black dot in each slice and list these in the Results window as well as the total path length in pixels. The Paths window gives a graphical display of the path the fly took.
    2. Copy the x- and y-coordinate list from the Results window and paste the data into an Excel spreadsheet. If any slide in the stack does not have a black dot in it, the Multitracker program will halt at that slide. If this occurs, it is necessary to go back to step 4.2 and re-threshold the stack to ensure that the fly registers as a black dot in the slide in question.

5. In order to convert the path lengths from pixels to cm, take a separate image of two dots that are spaced one cm apart on a blank white piece of paper.

1. Follow the above steps (3.1-4.4.2), to determine the x, y pixel coordinates for the two dots and use this information to determine the number of pixels corresponding to a cm. This conversion factor is used in step five to generate movement data in cm. Note: Once this is done, it is important to keep the camera fixed for all future recordings. Every time the camera is moved or adjusted, one needs to repeat this step to convert the pixels to cm.
2. The path length that is generated by the Multitracker plug-in overestimates movement because slight fluctuations in body position and light intensity/reflection will affect the size and center of the black dot that is generated when thresholding the images. This noise must be removed in order to get an accurate estimation of fly movement.

5. Data Analysis Using Excel

1. Remove the noise from the recorded data by importing the series of x, y coordinates generated by the Multitracker program into Fly Analysis, an Excel Visual Basic program created by the authors. (The program is available by contacting the authors.)
2. Click the Click Me button and select a threshold value cut-off. Select a threshold value of 0.5 cm/sec for SLA analysis or select a value between 0.1- 0.3 cm/sec for movement analysis. Click OK and the program will calculate the velocity, time and distance of movement. Note: The program uses a sliding window analysis to evaluate the data in sequential 0.5-1.0 sec bins and calculates the velocity during that window. Anything above the user-defined cutoff is treated as fly movement.

Representative Results

The technique described here has been used previously to analyze differences in SLA in the *Drosophila* BS mutants[1]. The results presented here involve the BS strains *easily shocked* (*eas*), *bang senseless* (*bss*), and *technical knockout* (*tko*). The *eas* locus encodes an ethanolamine kinase involved in phosphatidyl ethanolamine synthesis[15] and the *tko* locus encodes a mitochondrial riboprotein[16]. The *bss* mutation is an allele of the *paralytic* (*para*) voltage-gated sodium channel[17]. The alleles used in this study were eas^{1}, bss^{1}, and tko^{25t}.

An illustration of the efficacy of the method to measure SLA can be seen in **Figure 1**, which depicts the path length of a *eas* seizure-sensitive control fly as compared to one that has been fed the drug metformin. Metformin is a biguanide used to treat type II diabetes. The drug inhibits mitochondrial respiratory chain complex I, which, *via* the upregulation of AMPK, triggers an increase in glycolysis and glucose uptake in mammalian cells[18]. Previous studies have found that metformin upregulates glycolytic genes, presumably to compensate for the inhibition of aerobic metabolic pathways[19]. **Figure 2** demonstrates that feeding the BS mutants *eas* and *tko* metformin for two days (25 mg/g of food) reduces the SLA path length.

In addition to reducing the path length, the data generated by this method can be used to analyze differences in SLA velocity and SLA duration. In this case, the data indicate that SLA duration was significantly reduced (**Figure 3**) while SLA velocity was not changed significantly (**Figure 4**).

The assay described above can also be used to measure locomotion over a defined period of time. **Figure 5** shows an example of a path traversed by a wild type Canton-Special (CS) fly and a path traversed by a *bss* fly over a period of 15 min. While the movement of both flies was concentrated at the edges of the circular area, the CS fly exhibited much more locomotion over the 15 min recording period. The assay is therefore useful in looking at both the amount of movement and the pattern of movement of the flies.

The average distance traversed by *bss* flies was significantly lower than that of CS flies as indicated by **Figure 6**. Not only did the *bss* flies display less movement, they tended to move with a slower velocity. Changes in the velocity of movement of two individual flies are depicted in **Figure 7**. Using this data, an average velocity can be calculated and compared as in **Figure 4** for SLA. Based on the representative flies shown in **Figure 7**, it is apparent that the CS flies had more frequent bouts of fast movement while the *bss* flies had more bouts of inactivity during the period represented in the figure.

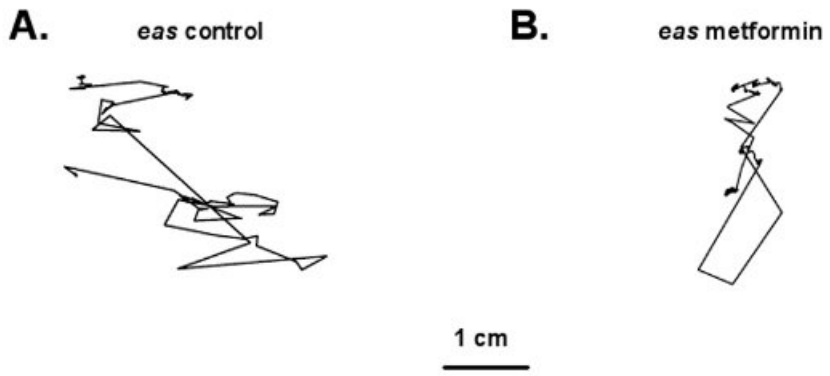

Figure 1. Representative paths of SLA in *eas* mutants and *eas* mutants fed metformin. Individual flies were mechanically shocked to induce SLA. The path of an individual fly during the SLA is represented in the figure. **A**) Path of an *eas* mutant. **B**) Path of an *eas* mutant fed metformin. (Bar represents 1 cm).

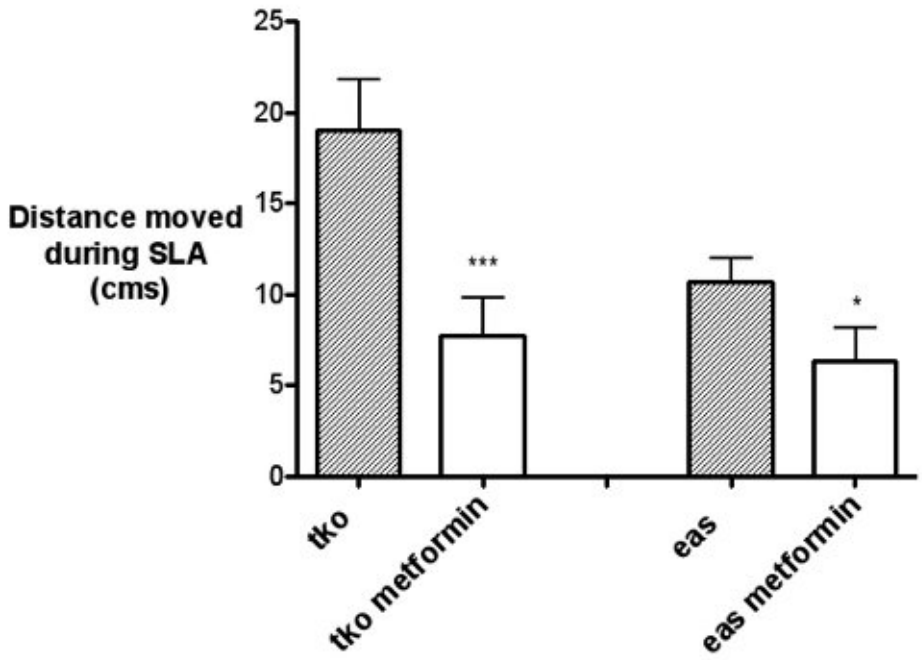

Figure 2. Individual flies were mechanically shocked to induce SLA. The distance the fly moved during the SLA was used as a measure of SLA intensity. In both BS mutants examined, metformin significantly reduced the distance moved. Data was analyzed using the Mann-Whitney U test (*$p < 0.05$; ***$p < 0.001$; $n = 15$).

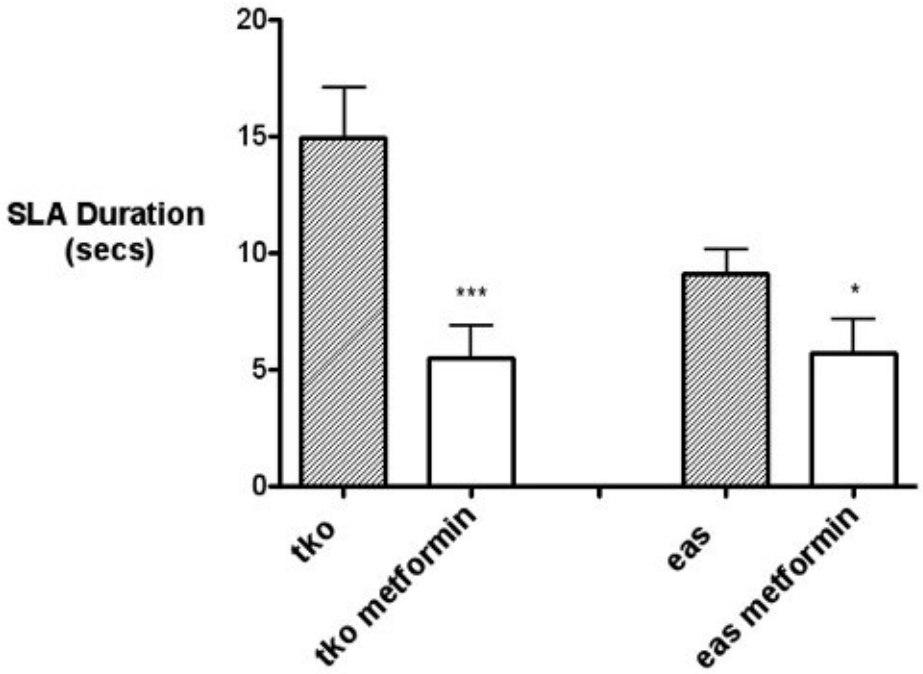

Figure 3. Individual flies were mechanically shocked to induce SLA. The time the fly spent undergoing SLA was used as a measure of SLA intensity. In both BS mutants examined, metformin significantly reduced the SLA duration. Data was analyzed using the Mann-Whitney U test (*p < 0.05; ***p < 0.001; n = 15).

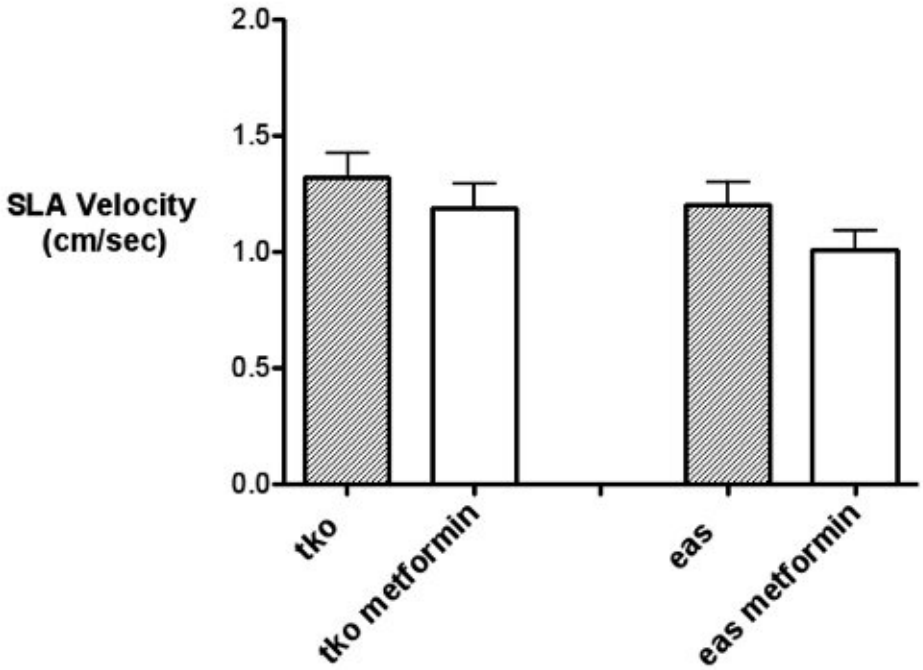

Figure 4. Individual flies were mechanically shocked to induce SLA. The average velocity of the fly during the bout of SLA was used as a measure of SLA intensity. There was no significant change in average velocity for the two BS mutants tested when fed metformin. Data was analyzed using the Mann-Whitney U test (n = 15).

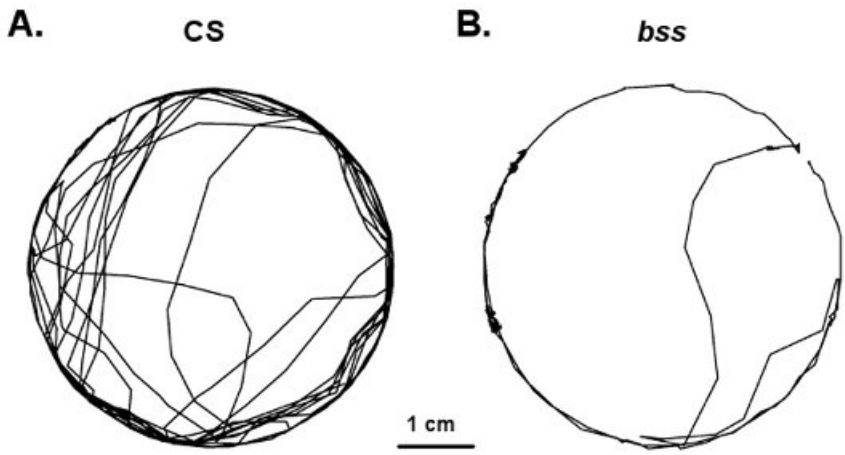

Figure 5. Paths of individual flies in the arena. Traces represent the paths individual flies traversed in the arena during the 15 min monitoring period. **A**) Path of an active CS fly with the movement concentrated around the periphery of the circular arena. **B**) Path of a typical *bss* fly. Movement was still concentrated around the periphery but there was much less movement as compared to CS flies. (Bar represents 1 cm)

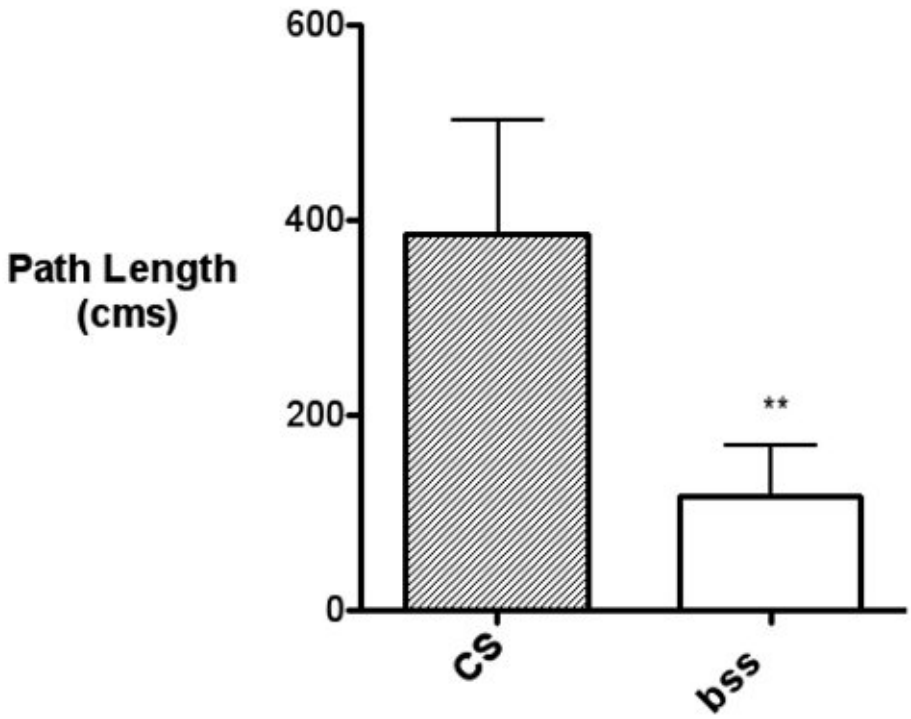

Figure 6. Fly locomotion when exposed to a novel environment. Individual flies were placed in a 5 cm diameter arena (5 mm in height) and movement was monitored for 15 min. Path lengths were calculated for each fly and the average lengths were taken for each genotype. The *bss* mutants displayed a significant reduction in path length as compared to CS flies. Data was analyzed using the Mann-Whitney U test (**$p < 0.01$; $n = 10$).

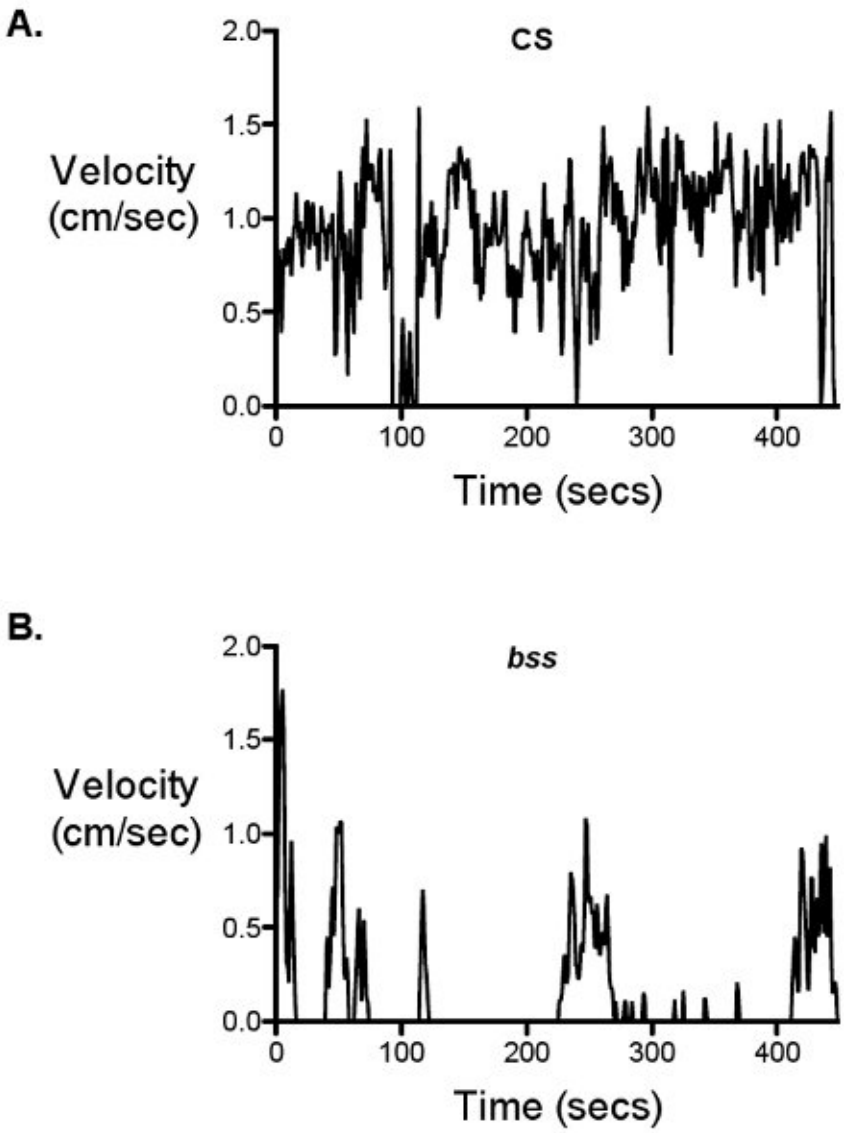

Figure 7. Velocity as a function of time during exploratory locomotion for a CS (A) and a *bss* (B) fly. An individual fly was placed in a 5 cm diameter arena (5 mm in height) and movement was monitored for 15 min. Velocity was calculated every 0.5 sec by averaging the velocity over a 1 sec sliding window (movement from the previous 0.5 sec to the subsequent 0.5 sec interval.) After a few initial bursts of activity the *bss* fly remains relatively inactive throughout the trial.

Discussion

When examining locomotor or movement patterns in *Drosophila*, it is useful to be able to extract information regarding the distance traveled, the velocity of movement and the pattern of movement. In order to extract this information, expensive equipment has traditionally been employed that is often prohibitive for smaller labs or labs that wish to use such assays sparingly[4,8,9].

The assay described here can be used to determine the distance traveled (**Figures 2** and **6**), the velocity of the movement (**Figures 4** and **7**) and the pattern of movement (**Figures 1** and **5**) using equipment that costs less than two hundred dollars. Using the Excel Visual Basic program we have developed, the series of x, y coordinates generated by the ImageJ program can be analyzed to extract all this information. This system has been used previously to identify both genetic and pharmacological suppressors of SLA in the seizure-sensitive *Drosophila* BS mutants[1].

While the system can extract valuable information, we have only analyzed one fly at a time. This can be a significant drawback as compared to the highly parallel commercial options[4]. The Multitracker plug-in for ImageJ does have the ability to track multiple objects simultaneously but we have not tried this option. For this locomotor assay, a single fly is placed under a plastic Petri dish lid. Depending on the position of the camera, one could film multiple Petri dishes to do parallel recordings. The drawback is that the resolution will decrease as the web camera is positioned

to take in a larger field. How this affects the integrity of the recording is not known, but a higher end camera would be an option if one wanted to pursue parallel recordings.

The recordings done here are limited to 15 min but it is possible to do longer recordings if one employs the 64-bit version of ImageJ. The 32-bit version has a memory limit that restricts the size of the files one can analyze. For longer recordings, it is essential to use the 64-bit version of ImageJ.

One of the biggest issues regarding this method is the need to remove the noise from the data generated by processing it in the ImageJ software. However, this is not unique to this method as similar noise issues must be resolved with other recording options[3,5,6,8]. As discussed in the methods, a stationary fly may display minor amounts of movement when processed through ImageJ because the program measures movement from the center of the object, which can vary even in a stationary object due to fluctuations in light and fly posture.

Our Visual Basic Excel program uses a sliding window analysis to set a threshold for movement. This threshold may vary depending on the assay. For seizure analysis, the sliding window looks at ten recording points, which spans 1 sec of real time. If the fly has moved 0.5 cm or more during this time it is considered to be actively seizing. If it is less, it is considered to be at rest. When looking at normal movement, a lower threshold is needed to determine the cut off and this has to be achieved *via* trial and error. A threshold of 0.1 cm/sec was used in this study. The best way to achieve this is to record a fly while it is stationary for a brief period of time and then adjust the cutoff so that it indicates no movement during the recording.

In summary, this low-cost method represents a viable assay for extracting meaningful information regarding the various parameters of fly locomotion. It is an ideal screening technique to use to identify if a behavioral, genetic or pharmacological manipulation has an effect on locomotion or seizure susceptibility.

Disclosures

The authors declare that they have no competing financial interests.

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