

## Video Article

# High-throughput Analysis of Locomotor Behavior in the *Drosophila* Island Assay

Ilse Eidhof<sup>\*1</sup>, Michaela Fenckova<sup>\*1</sup>, Dei M. Elurbe<sup>2</sup>, Bart van de Warrenburg<sup>3</sup>, Anna Castells Nobau<sup>\*1</sup>, Annette Schenck<sup>\*1</sup><sup>1</sup>Department of Human Genetics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center<sup>2</sup>Centre for Molecular and Biomolecular Informatics, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center<sup>3</sup>Department of Neurology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center<sup>\*</sup>These authors contributed equallyCorrespondence to: Anna Castells Nobau at [Annacnobau@hotmail.com](mailto:Annacnobau@hotmail.com), Annette Schenck at [Annette.Schenck@radboudumc.nl](mailto:Annette.Schenck@radboudumc.nl)URL: <https://www.jove.com/video/55892>DOI: [doi:10.3791/55892](https://doi.org/10.3791/55892)Keywords: Neuroscience, Issue 129, High throughput, automatic quantification, motor behavior, locomotion, flight response, disease models, movement disorders, island assay, *Drosophila*

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## Abstract

Advances in next-generation sequencing technologies contribute to the identification of (candidate) disease genes for movement disorders and other neurological diseases at an increasing speed. However, little is known about the molecular mechanisms that underlie these disorders. The genetic, molecular, and behavioral toolbox of *Drosophila melanogaster* makes this model organism particularly useful to characterize new disease genes and mechanisms in a high-throughput manner. Nevertheless, high-throughput screens require efficient and reliable assays that, ideally, are cost-effective and allow for the automatized quantification of traits relevant to these disorders. The island assay is a cost-effective and easily set-up method to evaluate *Drosophila* locomotor behavior. In this assay, flies are thrown onto a platform from a fixed height. This induces an innate motor response that enables the flies to escape from the platform within seconds. At present, quantitative analyses of filmed island assays are done manually, which is a laborious undertaking, particularly when performing large screens.

This manuscript describes the "*Drosophila* Island Assay" and "Island Assay Analysis" algorithms for high-throughput, automated data processing and quantification of island assay data. In the setup, a simple webcam connected to a laptop collects an image series of the platform while the assay is performed. The "*Drosophila* Island Assay" algorithm developed for the open-source software Fiji processes these image series and quantifies, for each experimental condition, the number of flies on the platform over time. The "Island Assay Analysis" script, compatible with the free software R, was developed to automatically process the obtained data and to calculate whether treatments/genotypes are statistically different. This greatly improves the efficiency of the island assay and makes it a powerful readout for basic locomotion and flight behavior. It can thus be applied to large screens investigating fly locomotor ability, *Drosophila* models of movement disorders, and drug efficacy.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/55892/>

## Introduction

In recent years, advances in next-generation sequencing technologies have greatly contributed to the identification of genes underlying degenerative movement disorders of the brain (e.g., cerebellar ataxia and Parkinson's), of peripheral neuronal origin (e.g., amyotrophic lateral sclerosis and hereditary spastic paraplegia), and of muscular origin (e.g., Duchenne muscular dystrophy and myotonic dystrophy)<sup>1,2,3,4</sup>. Despite this, little is known about the molecular mechanisms that underlie most of these disorders. A better understanding of these mechanisms is essential to developing therapies.

As in humans, movement in model organisms, such as flight and locomotion in *Drosophila*, is controlled by the central brain, peripheral nervous system, and muscles. In addition, the fast generation time and genetic toolbox of *Drosophila* make this model organism particularly suitable for the high-throughput screening of genes involved in movement disorders and for drug testing<sup>5,6</sup>. Due to the substantial number of conditions that need to be tested in such screens, reliable, cost-effective, and relative simple assays, as well as tools to quantify the output results in an automated manner, are highly desirable.

Schmidt *et al.* (2012)<sup>7</sup> described a low-cost test called the "island assay" to evaluate *Drosophila* locomotor behavior. The island assay has been used successfully in large-scale screenings to identify genes with glia-specific functions<sup>7</sup>, in the evaluation of *Drosophila* models of intellectual disability<sup>8</sup>, and for the general evaluation of fly motor behavior<sup>9</sup>. The principle design of the island assay consists of an elevated platform onto which several flies are thrown. This induces an innate motor behavior that enables healthy flies to escape from the platform within seconds. The assay measures the number of flies remaining on the platform over time<sup>7,8,9</sup>. All these features indicate that the island assay may be a powerful screening tool for genes involved in movement disorders.

Currently, the quantitative analysis of filmed island assay data is done manually<sup>7,8,9</sup>. To improve the efficiency of the assay, a low-cost method was developed to semi-automatically quantify the escape of flies from the platform. The setup uses a simple webcam connected to a laptop to collect image time series of the platform, with frames acquired every 0.1 s. Frames are then processed with the "Drosophila Island Assay" macro that quantifies the number of flies on the platform over time. The "Drosophila Island Assay" macro is divided into three independent sub-macros: (I) The "Create stack and projection," sub-macro identifies different island experiments stored in different subfolders and creates a stack and a projection of each time series. (II) The "Define Platform" sub-macro will consecutively open all "Projection\_image\_name.tif" files located in the individual experimental subfolders, at which point the user is requested to manually define the island platform as the region of interest (ROI). (III) "Analysis" automatically quantifies the number of flies remaining on the platform during the time series. The sub-macros can be run consecutively (in one run) or independently. For statistical data analysis, a script was developed to automatically process the obtained data and to apply a statistical test to determine whether treatments/genotypes behave significantly different from each other (Figure 1). Finally, it is demonstrated that this setup can be used to evaluate and quantify the aberrant locomotor ability of a *Drosophila* model for ataxia-telangiectasia (AT).

## Protocol

### 1. Construction of the Island Assay Box

1. Prepare a tray made of a robust material, such as poly methyl methacrylate (PMMA), to contain a layer of water (*i.e.* a bath). Ensure that the material is not white.  
NOTE: Dimensions of 40 x 35 x 2.5 cm<sup>3</sup> are recommended (Figure 1A).
2. Prepare a box (42 x 38 x 25 cm<sup>3</sup>) made of a robust, transparent material, such as PMMA, to be placed around the bath to prevent the flies from escaping into the laboratory space. Place a hole (20 x 30 cm<sup>2</sup>) on the lateral side that is large enough for the experimenter to easily handle the vials with flies and drop them on the island (Figure 1A).
3. Prepare an elevated platform 10 x 15 x 2.5 cm<sup>3</sup> in dimension, made of an impermeable material (PMMA or plastic) that is watertight.  
NOTE: This platform is required to have a uniform white surface to ensure good contrast for image analysis. The size is not necessarily fixed, but it should be large enough to make sure that all flies initially land on the platform and get the chance to walk on it (Figure 1A).
4. Fix the platform by either gluing it in to the bath or placing weights or other heavy objects inside the platform box to prevent positional changes of the platform during the experimental/filming session.

### 2. Software Requirements and Installation

1. **Installation of image-recording software.**
  1. Download image-recording software to record the island image series (see the **Materials Table**) and install the software on a computer.  
NOTE: The imaging-recording software described in this protocol is only supported by Windows. An alternative for other users has been added to the **Materials Table**.
2. **"Drosophila Island Assay" macro installation.**
  1. Download the "Drosophila Island Assay" macro and the compatible Fiji<sup>10</sup> version (1.4 or higher) from the following website: <https://doi.org/10.6084/m9.figshare.4309652.v1>.
  2. Move the cursor to the "Island assay" directory and click on "view."
  3. Click on "Download all." The folder content will be downloaded as a .zip file; unzip the downloaded file.
  4. Copy the "Drosophila Island Assay.ijm" file into "Fiji.app/plugins/directory."  
NOTE: When starting Fiji, the "Drosophila Island Assay" macro will appear at the bottom of the plugins dropdown menu.
3. **"Island Assay Analysis" script installation and download.**
  1. Download R<sup>11</sup> from the following website: <https://cran.rstudio.com>. Install it on a computer.
  2. Download R studio from the following website: <https://www.rstudio.com/products/rstudio/download/>. Install it on a computer.  
NOTE: The "Island test analysis" script can be run with R only. R studio, with its easier user-interface, is presented as alternative step for users inexperienced with R.
  3. Download the "Island Assay Analysis" script from the "Drosophila Island Assay" directory at the following website: <https://doi.org/10.6084/m9.figshare.4309652.v1>.  
NOTE: After performing step 2.2, the "Island Assay Analysis" script can also be found in the unzipped folder called "Island assay."

### 3. Preparation of the Flies to Be Tested in the Island Assay

1. **Collect staged flies for each experimental condition under cold or carbon dioxide (CO<sub>2</sub>) anesthesia, as previously described<sup>13</sup> (over a period of 1 - 2 days).**
  1. Prepare a minimum of 3 sample vials per experimental condition, each containing approximately 15 staged flies. Only use flies with intact wings and that are of similar age and gender.  
NOTE: For the experiments described here, 5 sample vials containing 15 male flies of the genotypes *w*<sup>-</sup>; Actin-Gal4/+ (control) and *w*<sup>-</sup>; Actin-Gal4/GD11950 (tefu RNAi) were collected on the day of eclosion, aged for 4 days, and were used to perform the island assay. RNAi, control strains, and the Actin-gal4 driver were obtained from a commercial source (see the **Materials Table**).
2. Let the flies recover to avoid effects from the anesthetics (at least 1 day when using CO<sub>2</sub>) before testing the collected flies in the island assay at the age of interest.

## 4. Experimental Setup

NOTE: See **Figure 1B**.

1. Add cold water with a small amount of soap to the bath tray and position the platform in the middle.  
NOTE: The soap decreases the surface tension of the water; flies that touch the water will drown. This prevents increasing amounts of flies flying in the box during the progression of the experimental session.
2. Place the transparent box on top of the tray and illuminate the platform from above using a lamp.  
NOTE: Illumination of the platform is mainly required to ensure proper video contrast. An ordinary 12-V LED light is appropriate for this.
3. Position the webcam directly above the platform (outside the box) and connect it to a computer.
4. **Create new folders on the computer to store the different experimental data prior to the experiments.**
  1. Follow the structure of the example illustrated in **Figure 2A** to create the folders. For example, if the experimental design requires testing two genotypes with five replicates each, first create a main folder containing the date of the experiment. Inside the main folder, create two subfolders (one per genotype). Inside the genotype folders, create five new subfolders, one per replicate.  
NOTE: For further analysis, it is essential that the image series corresponding to individual experiments are saved in folders with unique names.

## 5. Video Settings Setup in the "Capture Device" Section of the Interface

1. Open the image-recording software and, under the "Capture" tab, click on "Time-Lapse Images..." and select the appropriate webcam as the "Capture Device."
2. Place a dead fly on the island; adjust the video settings by clicking on the "Video Settings" box. Scroll through the tool bars, adjusting brightness, contrast, and color in a manner such that the dead fly appears black on a white background (**Figure 2B**). When the adjustments are complete, click "Ok."

## 6. Recording and Video Saving Settings Setup in the "Time-lapse" Section of the Interface

1. Adjust the settings in the "Time-Lapse Movie Setup" to save the experiment as an .avi file. Click on "Browse...", select the directory where the movie will be stored, define the name of the video, and press "save."  
NOTE: The video file is not used for the quantification of data; however, it might be useful to get an overall idea about the experiment.
2. **Adjust the settings in the "Time-Lapse Movie Setup" section.**
  1. In the compression box, select "Intel IYUV code" for video compression and choose "Take one frame every 0.1 seconds," with the "Play-back rate (images per second):" at 10.
  2. Save the experiment time series as .bmp frames by clicking on "Advanced..." Select "Create a .bmp image for each captured frame." Click on "Browse." Select the same directory in the "During AVI movie capture" window (as chosen in step 6.1), click "Open," and press "Ok."  
NOTE: Notice that, during the experiment, the frames are stored as .bmp files (required for data analysis). The program names the frames as "A" followed by the number corresponding to the frame capture sequence (e.g., "A\_number.bmp") (**Figure 2C**). Always save the images belonging to different experiments in a new folder to ensure that previously recorded image series will not be overwritten. Be aware that the images are not automatically saved in the same folder as the video file unless this folder is selected under "Browse..." upon clicking the "Advanced" box.

## 7. Island Assay and Data Collection

1. Press the start button in the time-lapse image interface of the image-recording software to start the recording.
2. Tap the experimental vial containing flies (step 3) 2-3 times to ensure that flies are at the bottom of the vial. Quickly remove the plug of the vial and, with a vigorous movement, tap the vial on the platform so that all flies fall onto the platform at the same time (**Figure 1C**).
3. After approximately 30 s, press the "Stop" button to stop recording.  
NOTE: If all flies vanish from the platform, the recording can be stopped earlier.
4. Remove the flies that remain for 30 s on the platform by hand after stopping the image recording.
5. Before proceeding to recording the next experiment, change the target directory for the .bmp files and movies (see section 6).

## 8. Data Processing: Running the "*Drosophila* Island Assay" Macro

NOTE: See **Figure 1D**.

1. **Run the "Create stack and Projection" sub-macro I to produce stacks and projections of the collected image series.**
  1. Start Fiji, click on "Plugins" in the toolbar, and chose "*Drosophila* Island Assay" in the dropdown menu; a new window will appear.
  2. Enter the "First image time-series identifier" setting in the macro graphical interface.  
NOTE: The recorded image frames are saved as "A-number.bmp" according to the order in which the images are obtained. In the "First frame identifier" macro interface, fill in the setting with the number given to the first frame by the program and with the file extension. Fill "First frame identifier" with "-0001.bmp," since the first frame is called "A-0001.bmp" (**Figure 2C**). In case other image formats (such as .tiff or .jpeg) are generated by the webcam software, specify the correct file extension of the first image in "First image time-series identifier."

3. Select only the "Create stack and projection" sub-macro, click "Ok," and select the main directory that contains all subfolders with individual island assay experiments.  
NOTE: The .bmp files in each individual experiment folder that contain the "First frame identifier" will subsequently be processed. Two new files will appear in each individual experiment subfolder, named by default "Stack\_image\_name.tif" and "Projection\_image\_name.tif" (**Figure 1D**). Once this step is performed, the .bmp files can be deleted. Stack and projection .tif files contain all data and are sufficient for further analysis.
  
2. **Run the "Define Platform" sub-macro II to select the exact location of the platform.**
  1. Open the graphical interface of the "*Drosophila* Island Assay," select only the "Define platform" checkbox, and press "Ok."
  2. In the "Choose a Directory" window, select the main directory where experimental subfolders are stored and press "Select."  
NOTE: The "Define Platform" sub-macro automatically searches for "Projection\_image\_name.tif" files in all subfolders stored in the main directory. The "Projection\_image\_name.tif" image stored in the first folder, together with two windows—"Define the platform" and "ROI manager"—will open.
  3. Select the "Polygon selections" tool in the toolbar to draw a selection that matches the island platform.  
NOTE: It is very important to exclude the borderline of the platform from the selection. See **Figure 2D**.
  4. After selecting the platform in the first image, click on "Add" in the "ROI Manager" window; the selected area will appear in the ROI manager window as a set of values (**Figure 2D**). Press "Ok" in the "Define the platform" window. The macro will proceed to the next projection.
  5. Click on the numbers (**Figure 2D**) stored in the "ROI manager" window; the previous selection will then automatically appear in the current image projection.  
NOTE: Since the island platform is likely to have the same size and position when experiments are performed in a row (as long as the position of the webcam and island remain unchanged), it is useful to store the ROI defining the platform in the "ROI manager." By doing so, the user will save time; in the upcoming projections, it is only necessary to click on the temporarily stored selection in the ROI manager.
  6. If the position of the island has slightly moved compared to the previous experiment, adjust the position of the selection by left-clicking in the center of the selection and dragging the selected area to the required position.
  7. If the selection does not match with the platform, left-click outside of the selection and delineate a new selection for the platform using the "Polygon selection" tool. Store the new selection in the ROI manager by clicking "Add;" a new selection will appear in the "ROI manager" window that can be used continuously.
  8. When the selection is adjusted, click "Ok" in the "Define the platform" window and repeat the procedure until all platforms are defined.  
NOTE: Notice that a binary image of the platform corresponding to the delineated ROI area in white on a black background, named "Platform\_image\_name.tif," appeared for each processed image in the same experimental subfolder as the stack and projection images generated in step 8.1.3 (**Figure 1D**).
  
3. **Define the minimum fly size.**  
NOTE: This setting defines the minimal fly size in pixels. Particles that are smaller than the specified minimal size will be excluded from the analysis to avoid false positives due to noise.
  1. Open an image stack created by the "Create stack and projection" sub-macro I.
  2. Convert the image stack to 8-bit by clicking Image>>Type>>8-bit.
  3. Go to the menu, press Image>>Adjust>>Threshold, make sure that the "Dark background" box is selected, and press "Apply;" a second window called "Threshold" will appear. Click on "Dark background" and press "Ok."  
NOTE: A binary image stack will be created in which the flies are defined in black and the platform in white. When this is not the case, apply the "Invert" function by clicking Edit>>Invert>>run.
  4. Set the scale to detect the number of pixels by clicking Analyze>>Set Scale. Apply the following settings: distance in pixels = 1, known distance = 1, pixel aspect ratio = 1, and unit of length = pixel. Press "Ok."
  5. Select the "Wand" (tracing) tool in the toolbar and click on a fly (black dot) present on the platform. Press ctrl+m (Windows users) or cmd+m (Mac users); a new "Results" window will indicate the area of the selected spot in pixels. Do this consecutively for several flies and determine the minimum fly size.  
NOTE: When running the macro, set the "Minimum fly size" setting to the smallest observed fly size minus a margin of 10%. (**Figure 2E**).
  
4. **Run the "Analysis" sub-macro III to quantify flies escaping from the platform.**
  1. Go to the toolbar, select "Plugins," and chose the "*Drosophila* Island Assay."
  2. Adjust the "Minimum fly size" setting according to the value defined in step 8.3.  
NOTE: Use the value defined in step 8.3 only if the standard setting of "Minimum fly size" causes exclusion of flies that were present on the platform or if the macro detects background signal as flies.
  3. In the "Number of flies per vial" setting, fill the maximum number of flies present in the vials during the complete experiment. For example, if one experiment has vials containing 15 flies, others containing 20 flies, and others containing 23 flies, the "Number of flies per vial" must be indicated as 23.
  4. Select the "Analyze" checkbox and press "Ok;" a new "Choose a Directory" window will appear. Select the main directory (with all subfolders and files inside) and press "select."  
NOTE: The macro will analyze all images stored in the subfolders as long as they contain the "stack\_image\_name," "Projection\_image\_name," and "Platform\_image\_name" files. The macro will process image after image. The macro output consists of a binary result image stack named "result\_stack\_subfolder\_name.tif" and a result text file called "result\_subfolder\_name.txt," which appear in each data folder. The resulting text file (.txt) contains quantitative measurements corresponding to the image stack and consists of 9 columns. The content of these columns is summarized in **Table 1**. "Result\_stack\_subfolder\_name.tif" corresponds to the image time-series of one experiment, where flies detected during the experiment are represented as black dots on a white background (**Figure 1E**).
  5. Carefully inspect the result stack to ensure that no artifacts have occurred and that the macro worked accurately (**Figure 2F**).

NOTE: Examples of artifacts can be image elements that are not flies but that are detected as such by the image segmentation algorithm (false positives). This can, for example, be due to detection of background signal because of an inaccurate selection of the ROI.

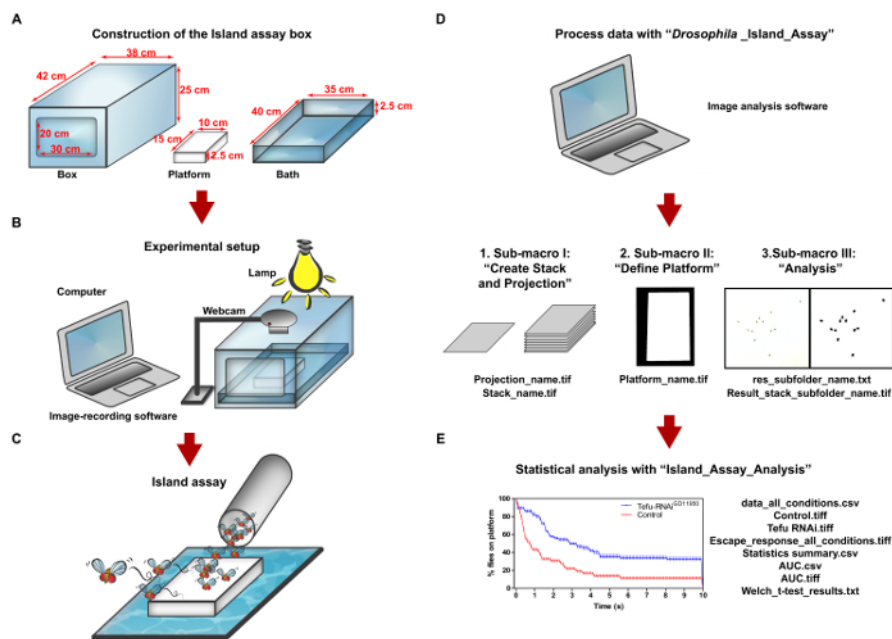
## 9. Data Analysis Using "Island Assay Analysis"

- Structure the data as indicated in **Figure 2A** to allow for analysis with the "Island Assay Analysis" script. Generate a main directory with subfolders, where each subfolder corresponds to one experimental condition to be analyzed.
- In the subfolders, generate folders that contain the independent experimental replicates (**Figure 2A**) and the results.txt files produced by the "Drosophila Island assay" macro.  
NOTE: The "Island Assay Analysis" macro will process all experimental conditions located in the main directory at once.
- Start R or R studio. Click on File>>Open File... in the toolbar and select the "Island Assay Analysis" script.**
  - Install the ggplot2 and matrixStats packages when running the "Island Assay Analysis" script for the first time. Type the following in the console window:  
> install.packages("ggplot2"), enter  
> install.packages("matrixStats"), enter.
- Specify the location of the data and analysis output files in the script. Insert in the following script rows:**
  - Row 16: Insert the path to the main directory that contains the experiments to be analyzed and compared (in **Figure 2A**, this is the path directing to the "Island Assay" folder).
  - Row 19: Insert the path to the folder where the analysis output files are stored.  
NOTE: The directory indicated in row 16 can only contain the folders to be analyzed. The script will also not work properly if the paths inserted in row 16 and row 19 are the same.
- Run the script by clicking Code>>Run Region>>Run All from the toolbar.  
NOTE: Notice that three resulting .csv files and one resulting .txt file (**Figure 1E**) appear in the directory defined in row 19. These are: (I) The "data\_all\_conditions.csv" file contains the processed data corresponding to each experimental condition and experimental replicates, organized as described in **Table 2**. (II) The "Statistics summary.csv" file summarizes the mean, the standard deviation (SD), and the standard error of the mean (SEM) of the percent of flies on the platform for each experimental condition. (III) The "AUC.csv" file contains the area under the curve for each experimental replicate. (IV) Depending on the number of conditions present in the main folder, the script will either export a "Welch\_t-test\_results.txt" file (2 conditions) or an "AUC\_anova\_results.txt" file (more than 2 conditions), where the results of the t-test or ANOVA comparing the area under the curve between experimental conditions are indicated. Notice that four different types of .tiff image files (**Figure 1E**) appear in the path defined in row 19. These are called: "Name\_Of\_Data\_Folder.tiff" (where Name\_Of\_Data\_Folder represents the folder name given by the user), "AUC.tiff," "Escape\_response\_all\_conditions.tiff," and "AUC\_anova.tiff." Detailed information about the content of these graphs can be found in the Representative Results of this manuscript and in **Figure 3**.

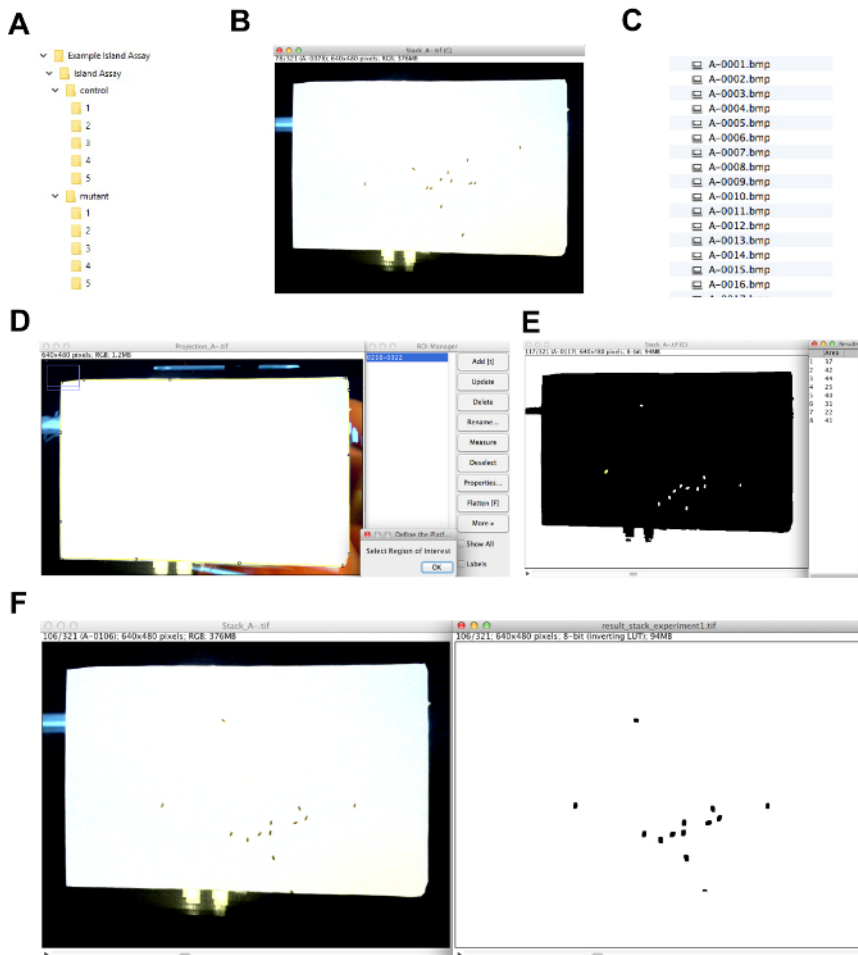
### Representative Results

In the described protocol, *Drosophila* island assay data are acquired and processed in three steps. First, the flight escape response of *Drosophila* thrown onto the island platform is recorded with a webcam and stored as individual .bmp images (protocol sections 1 - 7). Second, the "Drosophila Island Assay" macro (step 8) processes the frames, generating a "result.txt" text file (**Table 1**), in which the number of objects detected in each frame is summarized, and an image stack "Result\_stack.tiff," which shows the objects detected within the area of the platform in each frame. Third, the "Island Assay Analysis" script (protocol section 9) processes the macro data stored in the "results.txt" files of individual experiments. Several steps are incorporated in the script to filter and combine the data for statistical analysis. The first frame in which the flies are thrown on the platform is detected and considered as time point 1. Earlier frames are eliminated from the dataset. 100 frames following time point 1 (corresponding to 10 s) are selected for analysis. Experiments in which the initial number of flies detected on the platform is smaller than 5 are automatically excluded from the analysis, eliminating unreliable results from underpowered experiments. Experiments in which the initial number of flies detected on the platform exceeds the "Number of flies per vial" setting plus a tolerance of 3 are also excluded. This eliminates datasets in which noise particles were erroneously detected as flies. The script then calculates the percentage of flies detected for each time point compared to the highest number of flies detected in the series. Errors in datasets caused by flies walking in and out of the ROI (detected as a decrease followed by an increase in the percentage of flies on the platform over time) are automatically corrected by regarding them as consistently present on the platform at earlier stages. All replicate datasets for a specific experimental condition that were present in the main directory are combined and exported to the "data\_all\_conditions.csv" file. Its columns represent the variables described in **Table 2**. The script will also export a line graph for each experimental condition, named according to the folder containing the data. This graph shows the percent of flies remaining on the platform over time (flight escape response) for the experimental replicates present in the respective folder (**Figure 3A-B**). The mean, SD, and SEM for each experimental condition are calculated and summarized in the "Statistics summary.csv" file. A line graph called "Escape\_response\_all\_conditions.tiff" shows the average flight escape response for up to 12 experimental conditions present in the main folder (**Figure 3C**). Finally, the area under the curve for all experimental conditions present in the main folder is calculated and summarized in the "AUC.csv" file. Depending on the number of conditions present in the main folder, the script will either perform a two-tailed unpaired Welch t-test (2 conditions) or an ANOVA with Tukey correction for multiple testing (more than 2 conditions) to determine whether the experimental conditions differ significantly from each other. These results are summarized in "Welch\_t-test\_results.txt" or "AUC\_anova\_results.txt." When performing the ANOVA, the script will also export an "AUC\_anova.tiff" file that displays the difference in the mean AUC and the 95% confidence intervals of the experimental conditions that are compared. The values of the absolute area under the curve of the experimental replicates for all experimental conditions are displayed as individual data points with medians in "AUC.tiff" (**Figure 3D**).

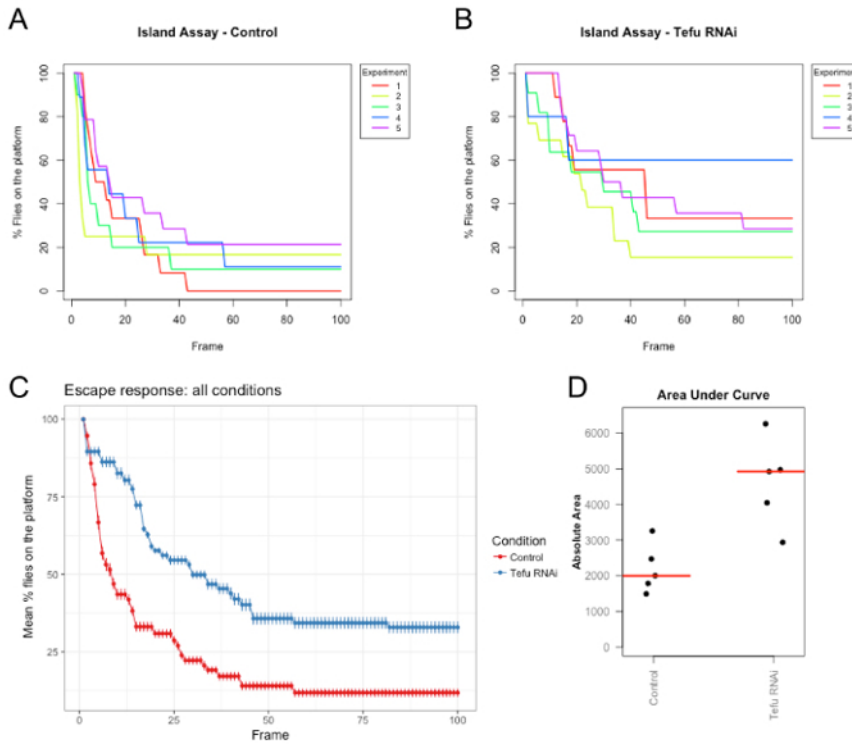
Ataxia Telangiectasia (AT) is an autosomal recessive movement disorder characterized by early onset of cerebellar ataxia due to mutations in the Ataxia Telangiectasia Mutated (*ATM*) gene<sup>14</sup>. Mutants of the *Drosophila* orthologue of *ATM*, *tefu*, display defects in mobility and longevity<sup>15</sup>. To evaluate the "*Drosophila* Island Assay" macro, a *Drosophila* model of AT was tested in the island assay, and the data output of the macro was compared to manual data counts. The results show that ubiquitous *tefu* knockdown (*w*; Actin-Gal4/GD11950) significantly decreases the ability of these flies to leave the platform compared to their genetic background controls (*w*; Actin-Gal4/+) **(Figure 4)**. After 1 s, 50% of the control flies had escaped the platform, in contrast to the <1% of *tefu*-RNAi flies. Importantly, the data obtained with the macro faithfully reproduced the data obtained by manual counting, indicating that the macro is a reliable tool that can be used for the quantification of island assay data and the evaluation of movement defects **(Figure 4 A-B)**.



**Figure 1: Flowchart outlining the requirements, experimental procedure, and analysis of the island assay.** (A) Island assay equipment. (B) Experimental setup for the island assay. (C) Island assay. (D) Processing of island assay data with the "*Drosophila* Island Assay" macro. The "*Drosophila* Island Assay" macro is composed of 3 sub-macros: 1) make stack and projection, 2) define platform, and 3) analysis. (E) Processing and statistical evaluation of data using the "Island Assay Analysis" script. [Please click here to view a larger version of this figure.](#)

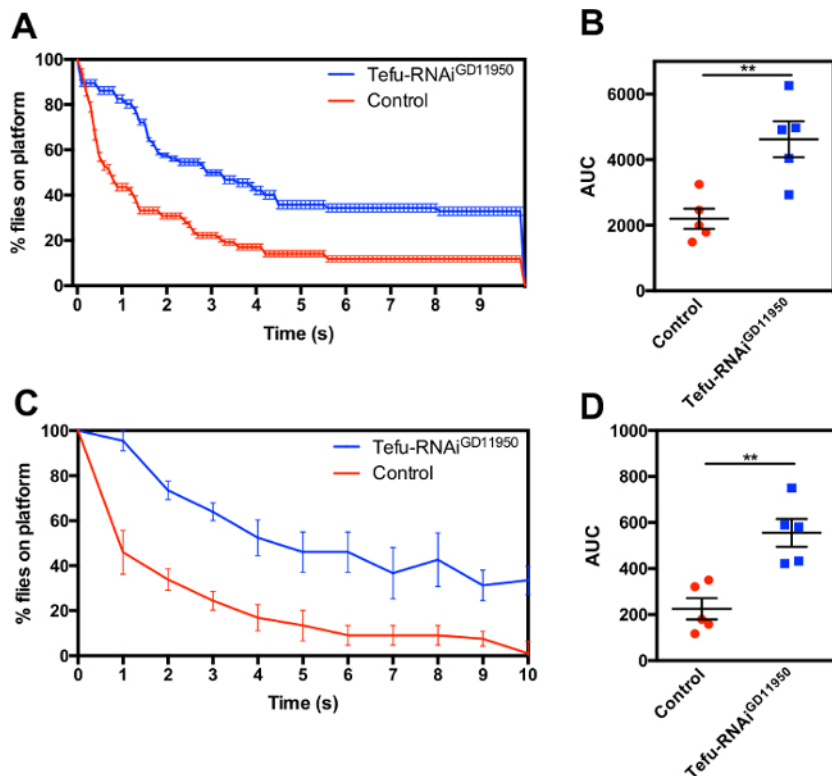


**Figure 2: Examples of different adjustments required during the protocol.** (A) The required directory structure in which island assay experiments must be stored for data processing and analysis. (B) When adjusting the video settings, flies must appear black on a white background. (C) Image frame output files as saved by the image-recording software described in this manuscript. (D) The yellow outline shows the platform selection. The stored platform selection in the "ROI Manager" is highlighted in blue. (E) Flies are represented as white dots during the adjustment of the "Minimum fly size setting." The results window shows the area of the flies in pixels. (F) Example of a single recorded image frame (on the left) and the corresponding frame in the resulting image stack, obtained with the "*Drosophila* Island Assay" macro (on the right). [Please click here to view a larger version of this figure.](#)



**Figure 3: Result images obtained after data processing with the "Island Assay Analysis" script. (A)** Line graph showing the flight escape response for each control experimental replicate. **(B)** Line graph showing the flight escape response for each tefu RNAi experimental replicate. **(C)** Average flight escape response for indicated experimental conditions; the error bars represent the SEM. **(D)** Dot plots representing the area under the curve distribution for control and mutant conditions. Experimental replicates for both tefu RNAi and control conditions are displayed as individual data points (in black) with medians (red line). [Please click here to view a larger version of this figure.](#)





**Figure 4: Ubiquitous *tefu* knockdown flies show a significantly decreased ability to leave the platform.** The data represent the percent of flies on the platform over time (s) for control (*w*<sup>+</sup>; Actin-Gal4/+ ) and *tefu* RNAi (*w*<sup>+</sup>; Actin-Gal4/GD11950) flies. (number of replicates = 5; the error bars represent the SEM). (A) Raw data obtained with the "Drosophila Island Assay" macro. (B) Dot plots representing the area under the curve distribution for control and *tefu* RNAi conditions obtained with the "Drosophila Island Assay" macro (Welch unpaired t-test, \*\*p < 0.01). (C) Raw data obtained by manually counting the number of flies present on the island per second. (D) Dot plots representing the area under the curve distribution for control and *Tefu* RNAi conditions obtained by hand-counting (Welch unpaired t-test, \*\*p < 0.01). The error bars represent the SEM. [Please click here to view a larger version of this figure.](#)

Column name	Description
Slice	Frame name.
Count	The number of objects detected in the frame within the limits of the platform (ROI).
Total Area	Total area of the objects detected in the frame within the limits of the platform (ROI) in pixels.
Average Size	Total area of the objects detected in the frame divided by the number of objects within the limits of the platform (ROI).
% Area	The percentage of area occupied by the objects with respect to the total area of the platform (ROI).
Perim.	Total perimeter of the objects detected in the frame within the limits of the platform (ROI) in pixels.
Min. Fly Size	The minimum fly size setting defined by the user in the graphical interface of the "Drosophila Island Assay" macro (in pixels).
Area ROI	The area of the platform (ROI) defined by the user during the run of the sub-macro define platform (in pixels).
Number of Flies	The number of flies used per experiment defined by the user in the graphical interface of the "Drosophila Island Assay" macro.

**Table 1: Parameters measured by the "Drosophila Island Assay" macro.** The parameters described in this table will appear in the "results.txt" file upon running the "Drosophila Island Assay" macro.

Column name	Description
Slice	Frame number.
Count	The number of flies detected in the frame within the limits of the platform (ROI).
X.Area	Total area of the flies detected in the frame within the limits of the platform (ROI) in pixels.
Min. Fly Size	The minimum fly size entry setting defined by the user in the graphical interface of the "Drosophila Island Assay" macro (in pixels).
Area ROI	The area of the platform (ROI, in pixels) defined by the user when running the sub-macro "define platform".
Number of Flies	The number of flies used per experiment defined by the user in the graphical interface of the "Drosophila Island Assay" macro.
X.Count	% flies present on the platform in the respective slice/frame relative to the highest number of flies detected on the platform during experiment.
Timepoint	Time point 1 represents the first frame to be analysed and corresponds to the frame where the flies first appear on the platform. There are a total of 100 frames per replicate analyzed (corresponding to 10 s, when using the described settings).
Experiment	Number of replicates per condition.
Condition	Indicates the name of the experimental condition (according to the user-defined name of the folder containing the data).

**Table 2: Description of the variables obtained after processing the data with the "Island Assay Analysis" script.** The parameters described in this table appear in the "data\_all\_conditions.csv" file upon processing the data with the "Island Assay Analysis" script.

## Discussion

This protocol describes the "Drosophila Island Assay" macro that quantitatively assesses *Drosophila* motor behavior during the island assay. The macro accurately counts the flies on the platform over time, making the island assay highly sensitive and suitable for quantitative high-throughput evaluation of locomotor defects. The methodology allows for the comparison of any condition, with flies grown under different genetic and/or environmental conditions, including drug exposure. This readout is thus particularly useful as discovery tool when performing large genetic or pharmaceutical screens, when studying *Drosophila* models of movement disorders and other neurological diseases, or when examining locomotion or flight behavior.

The island assay protocol presented in this manuscript provides advantages over existing/alternative methods. For example, video-tracking locomotion is much more time-consuming and less suitable for testing large sample sizes. The island assay is a high-throughput screening tool, and, in this sense, is comparable to the rapid interactive negative geotaxis (RING) assay<sup>16</sup>. The difference between the two is that the island assays allows for the detection of a broader range of locomotor problems; the inability of flies to leave the platform can be caused by defects in flight, jumping, or walking behavior caused by wing (muscle/neuronal) and/or leg (muscle/neuronal) defects. On the other hand, the RING assay assesses defects in climbing/walking behavior caused by leg (muscle/neuronal) defects. In case users are interested in multiple behavioral readouts, the island assay can also easily be combined with other assays, such as the RING assay. In addition, lasers required for optogenetics can easily be installed in the island assay box, and the setup is so simple that it can easily be moved to a room where temperature and light can be controlled.

To ensure the success and reproducibility of the island assay described here, several recommendations should be followed. Aliquot and transfer the flies to the experimental test vials at least one day before the experiment to avoid the effects of CO<sub>2</sub> or cold anesthesia. Do not overcrowd the experimental vials (use 10 - 15 flies per vial; it is best to always place the same number of flies per vial). Keep the flies on fresh food at all times. If not yet familiar with conducting the assay, practice throwing flies onto the platform to maximize the yield. Also practice quickly retracting the hand right afterwards, as it interferes with the data analysis (image analysis and fly counting start only after the hand is out of the picture). Keep the environmental and experimental conditions identical in experiments that need to be compared (e.g., controls versus mutants or a genotype tested at different ages). Always perform the experiments at the same time of the day and maintain the vials under controlled temperature and humidity conditions. For statistical power, test at least three technical replicates per biological replicate.

To ensure the successful performance of the macro described here, webcam and image settings must be adjusted to achieve maximal contrast: flies appearing as black objects on a white platform. When the number of flies is not properly counted by the macro, adjust the contrast settings, check whether the ROI is properly selected, and ensure that the size of the flies on the platform is above the specified minimum fly size setting (see step 8.3 of this protocol). The settings only need to be defined once. They are applicable to all experiments, as long as the distance between the webcam and the platform is not changed. The Circularity\_min and max settings define the circularity of the particles (particles = counted flies) that will be taken into account for the analysis (flies = counted objects). 1 represents a perfect circle, and 0 represents a line<sup>17</sup>. Since the flies always present a certain degree of circularity (a fly cannot appear as a straight line), the "Circularity\_max" setting is set at 1 and the "Circularity\_min" setting is set at 0.4. It is unlikely that the user needs to adjust these settings.

The macro occasionally makes counting mistakes when a fly is located close to the border of the platform. This can occur if the flies cannot fly but walk in and out of the user-defined ROI. In most cases, reselecting the ROI (fitting it as much as possible to the platform) can easily solve this issue. However, the "Island Assay Analysis" script is able to detect and correct improper data counts caused by flies walking in and out of the ROI relatively well. Although the resolution of the webcam presented here is high enough to discriminate flies in close proximity fairly well, we have implemented additional algorithms in the image processing procedure of the "Drosophila Island Assay" macro, such as the watershed and erode function<sup>17</sup>. These facilitate the proper delineation of flies that are in close proximity on the platform. In addition, the macro is unable to

distinguish between flies that jumped from the platform or flew away from it. Nonetheless, it is generally observed that healthy young fly strains fly away immediately when dropped onto the platform, whereas older flies and flies with locomotor deficits remain longer on the platform and will eventually jump or fall off the platform. Despite these limitations, the assay and analysis provide a very accurate measure of locomotor behavior.

To ensure the successful performance of the "Island Assay Analysis" script, the user has to make sure to enter the correct paths for the input and output files in the script rows indicated in the protocol and to provide the data in the correct folder format (as indicated in **Figure 2**). If the user finds the criteria used to filter out unreliable experimental data too stringent (row 68: the first value in the "Count" column is less than or equal to 5; row 71: the first value in the "Count" column is higher than the total number of flies thrown on the platform +3), turn off these filter settings by adding a # in front of the text in rows 68 and 71 in the "Island Assay Analysis" script. In this case, all datasets will be included in the analysis. Alternatively, filter settings can be changed by adjusting the values in rows 68 and 71 according to user needs. Possible artifacts in the count values in the "results.txt" generated by the "Drosophila Island Assay" macro can also be manually adjusted, and the script can be re-run on the adjusted data. When the user is interested in processing more than 10 fps, or more than 10 s of data, the number of frames processed by the "Island Assay Analysis" script should be adjusted. The statistical analysis can also be replaced by user-defined alternatives.

A folder called "Examples Island Assay," containing examples with image time-series obtained using the island assay, can be found at the following website: <https://doi.org/10.6084/m9.figshare.4309652.v1>. Download the "Examples Island Assay" folder and follow the steps described in this protocol to quickly become familiarized with the structure of file storage, the processing of the images with the "Drosophila Island Assay" macro, and the "Island Assay Analysis" script.

The island assay, in combination with the developed macro and analysis script, can be used to evaluate and quantify the aberrant movement behavior of a *Drosophila* model of Ataxia-Telangiectasia. Since the assay can be efficiently applied to various ages, it is well suited to analyzing the potentially progressive nature of phenotypes.

In summary, the island assay, in combination with the "Drosophila Island Assay" macro and the "Island Assay Analysis" script, is a cost-effective, reliable, and highly efficient assay to objectively analyze and quantify locomotor defects of *Drosophila* models of movement disorders in a high-throughput manner.

## Disclosures

The authors have no conflicts of interest to disclose.

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