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Climbing assay in Drosophila as a quantitative model for discovery about the locomotor defects in neurodegenerative disorders.

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Abstract:	Locomotive defects resulting from neurodegenerative disorders can be a late onset symptom of disease, following years of subclinical degeneration, and thus current therapeutic treatment strategies are not curative. Through the use of whole exome sequencing, an increasing number of genes have been identified to play a role in human locomotion. Despite identifying these genes, it is not known how these genes are crucial to normal locomotive functioning. Therefore, a reliable assay, which utilizes model organisms to elucidate the role of these genes in order to identify novel targets of therapeutic interest, is needed more than ever. We have designed a sensitized version of the negative geotaxis assay that allows for the detection of milder defects earlier and has the ability to evaluate these defects over time. The assay is performed in a glass graduated cylinder, which is sealed with a wax barrier film. By increasing the threshold distance to be climbed to 17.5 cm and increasing the experiment duration to 2 minutes we have observed a greater sensitivity in detecting mild mobility dysfunctions. The assay is cost effective and does not require extensive training to obtain highly reproducible results. This makes it an excellent technique for screening candidate drugs in Drosophila mutants with locomotion defects.

Author Comments:	Our manuscript present a simple yet very reliable method to study locomotion in Drosophila as a model to understand the genetic basis of movement disorders such as Parkinson's disease, hereditary paraplegia and amyotrophic lateral sclerosis. As you know there is still no curative treatments for these devastating diseases. Considering the increasing number of genes associated with such conditions, the Drosophila's genetic toolbox becomes very advantageous. In addition, it provides an excellent model to test candidate drugs at the full organism level. We believe that JoVE will be a perfect venue to allow the wide distribution of this important methodology.
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TITLE:
Quantitative analysis of climbing defects in a Drosophila model of neurodegenerative disorders.

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KEYWORDS:
Drosophila melanogaster; climbing assay; negative geotaxis; Neurodegenerative disorders; locomotion; mobility dysfunction

SHORT ABSTRACT:
We present an optimized inexpensive and reliable negative geotaxis assay in *Drosophila melanogaster* as a model for neurodegenerative disorders. Being more sensitive to mild locomotor defects, this assay will help screen for potential genetic interactions and drug targets.

LONG ABSTRACT:
Locomotive defects resulting from neurodegenerative disorders can be a late onset symptom of disease, following years of subclinical degeneration, and thus current therapeutic treatment strategies are not curative. Through the use of whole exome sequencing, an increasing number of genes have been identified to play a role in human locomotion. Despite identifying these genes, it is not known how these genes are crucial to normal locomotive functioning. Therefore, a reliable assay, which utilizes model organisms to elucidate the role of these genes in order to identify novel targets of therapeutic interest, is needed more than ever. We have designed a sensitized version of the negative geotaxis assay that allows for the detection of milder defects earlier and has the ability to evaluate these defects over time. The assay is performed in a glass graduated cylinder, which is sealed with a wax barrier film. By increasing the threshold distance to be climbed to 17.5 cm and increasing the experiment duration to 2 minutes we have observed a greater sensitivity in detecting mild mobility dysfunctions. The assay is cost effective and does not require extensive training to obtain highly reproducible results. This makes it an excellent technique for screening candidate drugs in *Drosophila* mutants with locomotion defects.

INTRODUCTION:

Devastating neurodegenerative disorders such as Parkinson's disease, amyotrophic lateral sclerosis, and hereditary spastic paraplegia are increasingly recognized. Unfortunately, most of these neurodegenerative disorders are still without treatments. The widespread clinical use of genome-wide, unbiased genetic tests such as whole exome sequencing has led to an increasing number of genes being implicated in human locomotive disorders. Despite this progress, the pathological progression from early to late stages, remains elusive in these disorders. *Drosophila* provides one with the genetic tools for studying gene requirement in a controlled spatial and temporal manner. In addition, D*rosophila* has proven useful in screening drugs for neurological conditions such as Parkinson's[1], Alzheimer's[2], intellectual disability[3,4] and epilepsy[5,6] among others. Our aim was to develop a cost effective and reliable assay that would allow high throughput analysis that would still be sensitive enough to detect small changes in motor performance.

There are several assays used to quantify the effects of genetic mutation and/or environmental condition on *Drosophila* climbing behavior. Most of the assays capitalize on the natural tendency of flies to climb, known as negative geotaxis, or the climbing assay. Benzer [7] suggested in 1967 that the counter-current apparatus used for the study of phototaxis could also be used to study gravitaxis. Since then, Ganetsky[8] and many others [9] [10-12] have built on the initial assay. The principle is to place a known number of flies in a vial and tap the vial strongly against a hard surface, causing the flies to fall to the bottom of the vial. As it is an innate behavior, the flies will attempt to climb to the top of the vial, opposed to gravity. This assay is quantitative and measures how many flies have climbed past a marker on the vial during an allotted time period. Measurement of speed instead of total number of flies climbing has become a reliable parameter and shown defects in cases where the number of flies criteria was not significant[13].

The climbing assay has proven useful in the study of many neurodegenerative disorders including Parkinson's disease[14]. However, we noted that locomotive defects may not be detectable at time where neurodegeneration is already seen in pathological studies[14]. Thus, use of the traditional assay may limit the ability to study the early stages of disease pathogenesis. The appearance of locomotive defects during later stages of pathology may reflect a disease whose progression is too advanced for complete rescue.

This raises a potential issue with the sensitivity of the traditional climbing assay. The potential inability of the traditional climbing assay to detect mild locomotive defects can be attributed to the height to which the flies are required to climb. The traditional assay[15,16] measures the number of flies to successfully climb over a height of 2 to 5 cm in 10 to 20 seconds.

PROTOCOL:

Research on *Drosophila melanogaster* was in compliance with the University of Alberta's research guidelines.

1. Fly Collection

1.1) Collect 20 flies using $CO_{2\,(g)}$ anesthetisation and place in a 25 mm x 95 mm collection vial containing food.

1.2) Store vials containing flies horizontally to avoid trapping flies in any liquids that may accumulate in the bottom the vial.

1.3) Incubate flies for at least 21 hours at 22 ^{o}C at 45% humidity in an incubator for approximately for 15 hours. Set the incubator with a 12 hours light:dark cycle.

2. Climbing Assay

2.1) The following morning, transfer 20 flies from a single vial into a 250 mL glass graduated cylinder. Mark the position of the cylinder to keep it constant everyday. Use one glass cylinder per genotype to prevent cross contamination between the genotypes. Wash at the end of each experiment and rotate them between genotypes.

2.1.1) Conduct the experiments in ambient light (or red light if there is a potential defect in vision) at temperature and humidity of 22 ^{o}C and 40% respectively. To avoid circadian rhythm confound, always perform experiments at the same time of day.

2.2) Seal the top of cylinder with a barrier film (wax film) to prevent the escape of any flies **(Figure 2)**.

2.3) Set up the video camera on a tripod. Focus camera on the 190 mL line of the 250 mL graduated cylinder (17.5 cm).

2.4) Count the number of dead flies at the bottom of the cylinder and in the food vials. Record this number as the mortality.

2.5) Very lightly tap the cylinder against a closed cell foam pad repeatedly with enough force to displace the flies to the inner bottom surface. Tap 5-10 times while using the other hand to press record on the camera.

2.6) Press the “Record” button on the camera.

2.7) Start the video camera recording and tap the cylinder six times in a distinct non-rhythmic pattern.

2.8) Conduct each trial for 2 min from the time the flies are last tapped and record the number of flies crossing the height of 17.5 cm (190 mL) at each time point chosen (quantify every 10

seconds). Note: The mL marking on the cylinder will vary from one cylinder model to another depending on diameter. To avoid error, measure the height on each cylinder used.

2.9) Once the trial has ended, dispose of flies in 95% ethanol.

2.10) Repeat steps 2.1 to 2.9 until all the replicates have been tested with fresh flies every time.

Note: Although 5 replicated may be enough with mutation having strong effect on locomotion, 10 biological replicates of 20 flies (200 flies) is recommended to detect smaller differences.

2.11) Upon completion of the experiment, wash the cylinders in the lab dishwasher and dry overnight to be re-used.

3. Analysis

3.1) Analyze videos of each fly trial. Each 10 seconds, record the total number of flies that pass the target line.

3.1.1) If a fly climbs back down or falls, record that fly as -1 and count the next fly to cross the target line as the same number as the fly that climbed back down or fell. For example, if the 17^{th} fly falls below the target line, the next fly to cross the line (the 18^{th} fly) is considered the 17^{th} fly and not the 18^{th}.

3.2) Subtract the mortality from the total number of flies (20) to obtain the number of flies that remain in the trial. At each time point, obtain the fraction of flies above the target line.

3.3) Plot each percentage at each time point (see **Figure 3**).

3.4) Analyze the performance at the 120 seconds data point and perform student t-test when 2 groups are present or ANOVA and a post-test for multiple comparisons (with Bonferroni modification for planned and Tukey for unplanned comparisons). The Kolmogorov-Smirnov tests[17] is also performed to ascertain normality and equal variance but also to compare the distributions of the mutant group to the control.

3.5) To present the data over aging, plot the percentage of flies climbing at 120 seconds with flies of different ages (2 days, 1 week, 2 weeks) to see if there is a progressive deficit (**Figure 4**).

REPRESENTATIVE RESULTS:

Climbing is a strong and reproducible behavior. Indeed, one day old wild-type flies reach the target distance climbing performance rapidly (25-30 seconds). Mutant flies present a range of performance from mild (or delayed) to complete inability to climb to the target. We illustrate this here with two different mutant alleles. The first one is a severe allele of the gene spastin caused by a complete deletion of the spastin gene ($spas^{5.75}$) [18]. In this line ($spas^{5.75}$ with TM6b) one day old flies do not reach WT climbing performance even after 2 minutes. This mutant line

presents with severe defects even using the vial method (**Figure 1a,b**). The advantage of the method presented here becomes more evident when studying a mutant for the same gene but with an incomplete deletion published by the same group (spas$^{17\text{-}7}$ with TM3)[18]. In that case performance up to 8 days is normal (**Figure 1c-f**). At 8 days, those flies present a defect in climbing noted to be mild but significant in the cylinder method but not in the vial method for the same number of repetitions. This may suggest that using a greater target distance allows for detection of defects in less severe mutants. Selection of genetically appropriate controls ensures that the effect is a result of transgenic expression **(Figure 3)** or genetic interaction. For further proof, include rescue of the behavioral phenotype with the expression of a wild-type protein for the gene studied. For interaction studies, compare flies that are heterozygous for both mutations of interest with flies that only have one mutation of interest. The assay also allows one to monitor the progression of the climbing defect over time, an important aspect in modeling progressive locomotor disorders **(Figure 4).** In addition, 2 minutes allow to better see the progression of climbing in severe mutants.

Figure 1. Comparison of different climbing assays. For severe mutations different degrees of climbing defect can be seen using various methods but milder mutations may not be detected with some assays. To demonstrate this we used two published mutant lines for the gene spastin: spastin$^{5\text{-}75}$ which contains a full deletion of the spastin gene and spastin $^{17\text{-}7}$ which contains a partial deletion of the spastin gene. **A)** First, climbing is assessed by having flies climb to the top of an empty food vial. The number of flies at the top after 18 seconds is recorded. Using this protocol a significant defect is seen in Spast$^{5\text{-}75}$/TM6b when compared to wild-type controls (N=10, $p<0.001$). **B)** Next, climbing performance is assessed using the method described here. Climbing is also defective in the same genotype Spast$^{5\text{-}75}$/TM6b. The difference in performance is highly significant (N=5, $p<0.001$), but the gap in performance is larger. For mutations shown to have lesser effect thought (e.g. Spast$^{17\text{-}7}$ for instance contains a partial deletion of spastin gene), the cylinder method presented here may be more sensitive. **C)** No significant defect is observed with 3 days old spast$^{17\text{-}7}$/TM3 with the vial method (N=5). **D)** No significant defect is observed with 3 days old spast$^{17\text{-}7}$/TM3 with the cylinder method (N=5). **E)** A non-statistical trend is noted with 8 days old spast$^{17\text{-}7}$/TM3 flies (N=5). **F)** But a significance is observed for 8 days old spast$^{17\text{-}7}$/TM3 tested with the cylinder method presented for the same number of replicates (N=5, $p<0.001$).

Figure 2. Schematic representation of the experimental set up. 20 flies are inserted in a glass cylinder and then capped with a wax barrier film. The flies are then tapped to the bottom and the number of flies crossing the midline is recorded using a camera for 120 seconds.

Figure 3. Representative results of the climbing experiment. The percentage of flies having passed the threshold line is represented every 10 seconds over the duration of the assay. In this experiment, 3 genetically appropriate controls (Wild-type, UAS*spas*-RNAi/+, Elav-GAL4/+) are compared to transgenic flies containing both UAS and Gal4 components (Elav-GAL4/UAS*spas*-RNAi). The UAS spas-RNAi is from VDRC #108739. This representation allows for the assessment of the rate of climbing for each genotype.

Figure 4. Representative graph for the aging profile. Since many locomotive disorders are progressive, it is important to portray the evolution over time. In this graph, WT flies are compared to heterozygous mutants (spas/WT) and trans-heterozygous mutants (spast5-75/spast17-7) at 2 days **A)** and 8 days **B)** (N=10, $p<0.001$). Results are also depicted over time for the 120 sec. time point **C).**

DISCUSSION:

Drosophila has already proven to be an excellent model in Parkinson's disease[14] and other neurodegenerative conditions[1,2]. In addition to the genetic tools available in *Drosophila*, its genome is highly conserved for genes involved in neurological disorders[19]. The advent of genome wide genetic screening methods (including whole exome sequencing) is likely to continue to provide a larger list of candidate genes associated with human movement disorders. The development of treatments for these conditions will require animal models to increase our understanding of the pathology involved in the early stages of neurodegeneration. The use of *Drosophila* and the negative geotaxis assay provides an inexpensive and reliable method to identify genes involved in locomotive defects and subsequently screen candidate drugs for phenotype rescue. This adds to the molecular, electrophysiological, and imaging proofs that can also be obtained in the same animal model. Using the climbing assay, others have successfully reproduced motor defects in flies mutant for genes disrupting human locomotion. Nonetheless, previous research has shown that pathological changes could precede the detection of locomotive defects by several days[14]. This phenomenon is also observed in human neurodegenerative conditions where we speak of subclinical changes. We believe that by understanding and then treating these subclinical changes, disease modification would be improved greatly.

We present here a model that allows the detection of mild locomotion defects that may help with understanding the passage from "presymptomatic to symptomatic" of neurodegenerative pathology using a *Drosophila* model. Many groups have used a short climbing distance (5-10 cm) but, we increased the distance to 17.5 cm as in Palladino *et al.*[20]. Although this difference between climbing heights may seem minor, the increase in height was intended to increase the assay difficulty, thereby aiding in the identification of the comparatively minor climbing defects. Also, some methods chose to illuminate the top of the cylinder with a fiber-optic lamp, to take advantage of the phototaxic response of adult *Drosophila*. However, the light source can cause light reflection within the cylinder; thus, a diffuse overhead fluorescent light source is used instead. In addition, mutation in genes involved in neurodegeneration may affect eye function and therefore bias the results. The increase in the sample size from 10 to 20 flies increases the statistical power of each trial. Initially, we increased this number to as high as 30 flies, but it was subsequently reduced in order to minimize overcrowding and interaction effects between flies. The samples are discarded after a single use, rather than being run for four repeat trials per sample, to eliminate the possibility of learning or fatigue. Due to flies with extremely poor climbing performance, it was counterproductive to record the time required for 50% of the flies to cross the target line for it could take a considerable amount of time for this criteria to be fulfilled. Rather, flies were given a duration of 2 minutes to cross the target line. The number of

flies to cross the line was recorded and binned in increments of 10s, and the resulting value expressed as a percentage.

These conditions create are a more sensitive assessment of an adult fly's climbing capabilities. While other designs of the assay are still useful, this paradigm may be considered in cases where mild early defects are investigated. In addition, this assay may help detect smaller changes in the context of drug trials.

An important issue is that the negative geotaxis behavior is based on the flies being tapped to the bottom of the cylinder. It is therefore important to assess other forms of locomotion, such as on a flat surface or flight. Other aspects such as motivation and social interaction need to be considered as potential confound. Another caveat is that the assay presented only allows one to assess locomotion in adult flies. This limits the ability to obtain neuropathological correlates for the behavior observed which is very important in understanding the pathogenesis of a disease. Indeed, most neuroimaging work has been done at the larva neuromuscular junction so far in *Drosophila*. Obtaining locomotion behavior in larva may be an important step in order to draw direct correlation between behavior and pathological changes.

It is very important to control the temperature and humidity at which the flies are raised and tested. In addition to the effect on fly development, these factors had an important effect on the climbing ability of flies raised and stored in non-ideal conditions. In the presence of increased static electricity or humidity, flies did not perform optimally. This effect was not equal for all genotypes, mutant flies usually being more affected by such factors than controls. In addition, cylinders need to be washed and dried properly between each experiment.

DISCLOSURES:

The authors have nothing to disclose.

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REFERENCES:

1 Auluck, P. K. & Bonini, N. M. Pharmacological prevention of Parkinson disease in Drosophila. *Nature medicine* **8**, 1185-1186, doi: 10.1038/nm1102-1185 (2002).

2 Bonini, N. M. & Fortini, M. E. Human neurodegenerative disease modeling using Drosophila. *Annual review of neuroscience* **26**, 627-656, doi: 10.1146/annurev.neuro.26.041002.131425 (2003).

3 Bolduc, F. V., Bell, K., Cox, H., Broadie, K. S. & Tully, T. Excess protein synthesis in Drosophila fragile X mutants impairs long-term memory. *Nature neuroscience* **11**, 1143-1145, doi: 10.1038/nn.2175 (2008).

4 McBride, S. M. *et al.* Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a Drosophila model of fragile X syndrome. *Neuron* **45**, 753-764, doi: 10.1016/j.neuron.2005.01.038 (2005).
5 Parker, L., Padilla, M., Du, Y., Dong, K. & Tanouye, M. A. Drosophila as a model for epilepsy: bss is a gain-of-function mutation in the para sodium channel gene that leads to seizures. *Genetics* **187**, 523-534, doi: 10.1534/genetics.110.123299 (2011).
6 Marley, R. & Baines, R. A. Increased persistent Na+ current contributes to seizure in the slamdance bang-sensitive Drosophila mutant. *Journal of neurophysiology* **106**, 18-29, doi: 10.1152/jn.00808.2010 (2011).
7 Benzer, S. Behavioral Mutants Of Drosophila Isolated By Countercurrent Distribution. *Proceedings of the National Academy of Sciences of the United States of America* **58**, 1112-1119 (1967).
8 Ganetzky, B. & Flanagan, J. R. On the relationship between senescence and age-related changes in two wild-type strains of Drosophila melanogaster. *Experimental gerontology* **13**, 189-196 (1978).
9 Nichols, C. D., Becnel, J. & Pandey, U. B. Methods to assay Drosophila behavior. *Journal of visualized experiments : JoVE*, doi: 10.3791/3795 (2012).
10 Toma, D. P., White, K. P., Hirsch, J. & Greenspan, R. J. Identification of genes involved in Drosophila melanogaster geotaxis, a complex behavioral trait. *Nature genetics* **31**, 349-353, doi: 10.1038/ng893 (2002).
11 Inagaki, H. K., Kamikouchi, A. & Ito, K. Methods for quantifying simple gravity sensing in Drosophila melanogaster. *Nature protocols* **5**, 20-25, doi: 10.1038/nprot.2009.196 (2010).
12 Gargano, J. W., Martin, I., Bhandari, P. & Grotewiel, M. S. Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in Drosophila. *Experimental gerontology* **40**, 386-395, doi: 10.1016/j.exger.2005.02.005 (2005).
13 Botella, J. A. *et al.* The Drosophila carbonyl reductase sniffer prevents oxidative stress-induced neurodegeneration. *Current biology : CB* **14**, 782-786, doi: 10.1016/j.cub.2004.04.036 (2004).
14 Feany, M. B. & Bender, W. W. A Drosophila model of Parkinson's disease. *Nature* **404**, 394-398, doi: 10.1038/35006074 (2000).
15 Chakraborty, R. *et al.* Characterization of a Drosophila Alzheimer's disease model: pharmacological rescue of cognitive defects. *PLoS One* **6**, e20799, doi: 10.1371/journal.pone.0020799 (2011).
16 Orso, G. *et al.* Disease-related phenotypes in a Drosophila model of hereditary spastic paraplegia are ameliorated by treatment with vinblastine. *J Clin Invest* **115**, 3026-3034, doi: 10.1172/JCI24694 (2005).
17 Lehmann, E. L. & D'Abrera, H. J. M. *Nonparametrics : statistical methods based on ranks*. Rev. 1st edn, (Springer, 2006).
18 Sherwood, N. T., Sun, Q., Xue, M., Zhang, B. & Zinn, K. Drosophila spastin regulates synaptic microtubule networks and is required for normal motor function. *PLoS biology* **2**, e429, doi: 10.1371/journal.pbio.0020429 (2004).

19 Inlow, J. K. & Restifo, L. L. Molecular and comparative genetics of mental retardation. *Genetics* **166**, 835-881 (2004).
20 Palladino, M. J., Hadley, T. J. & Ganetzky, B. Temperature-sensitive paralytic mutants are enriched for those causing neurodegeneration in Drosophila. *Genetics* **161**, 1197-1208 (2002).

Figure

Figure 1

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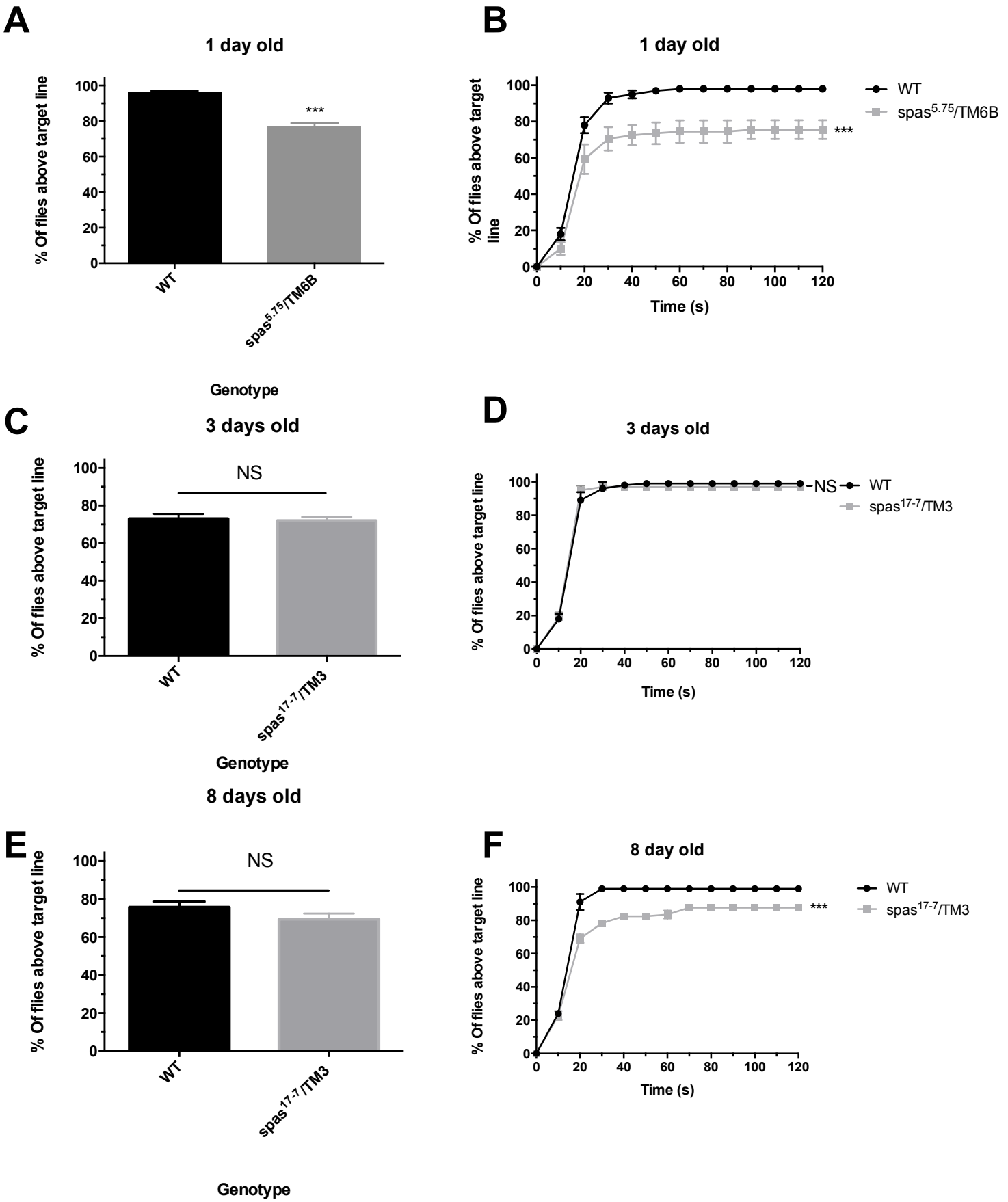

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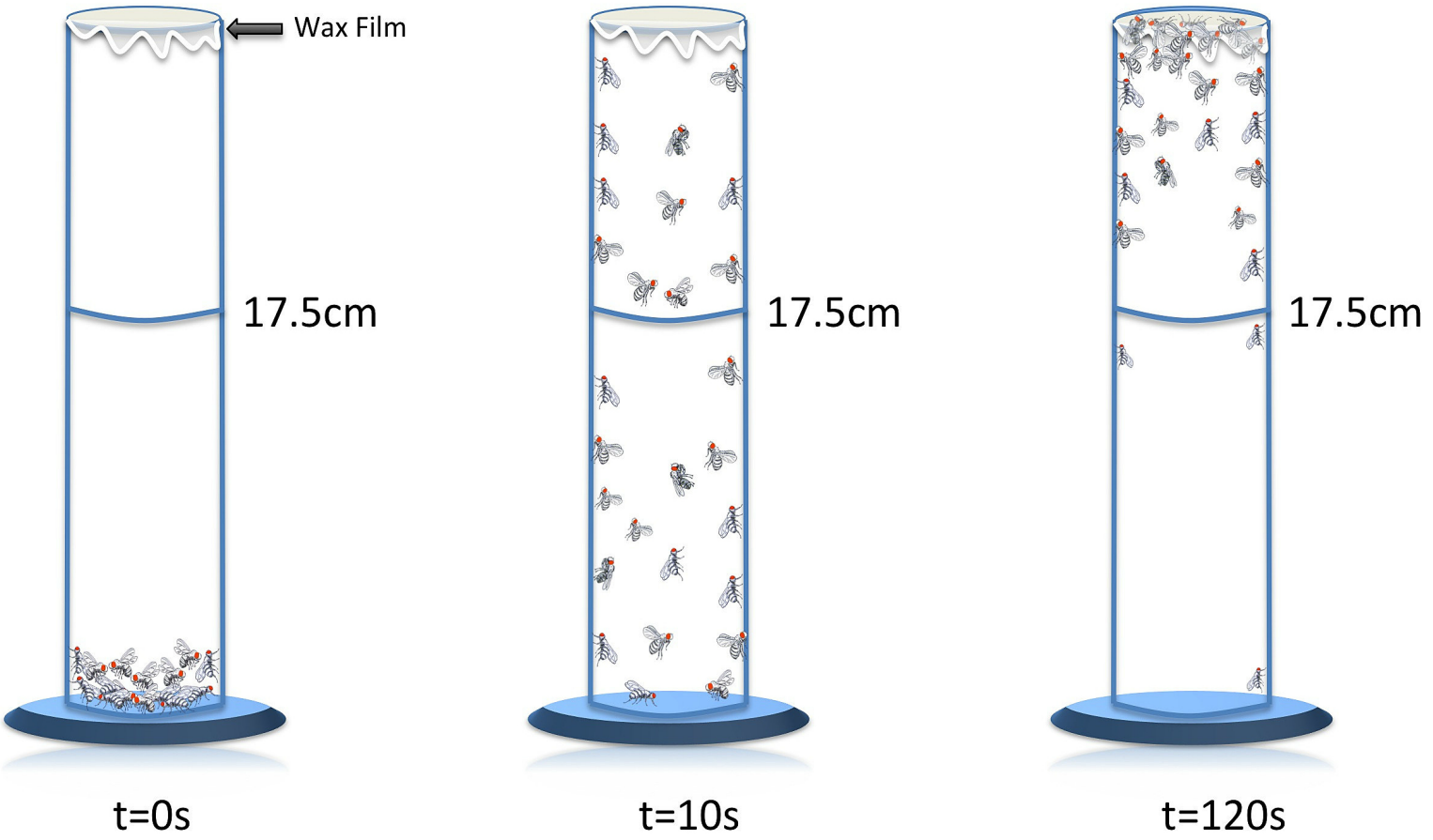
Figure 2



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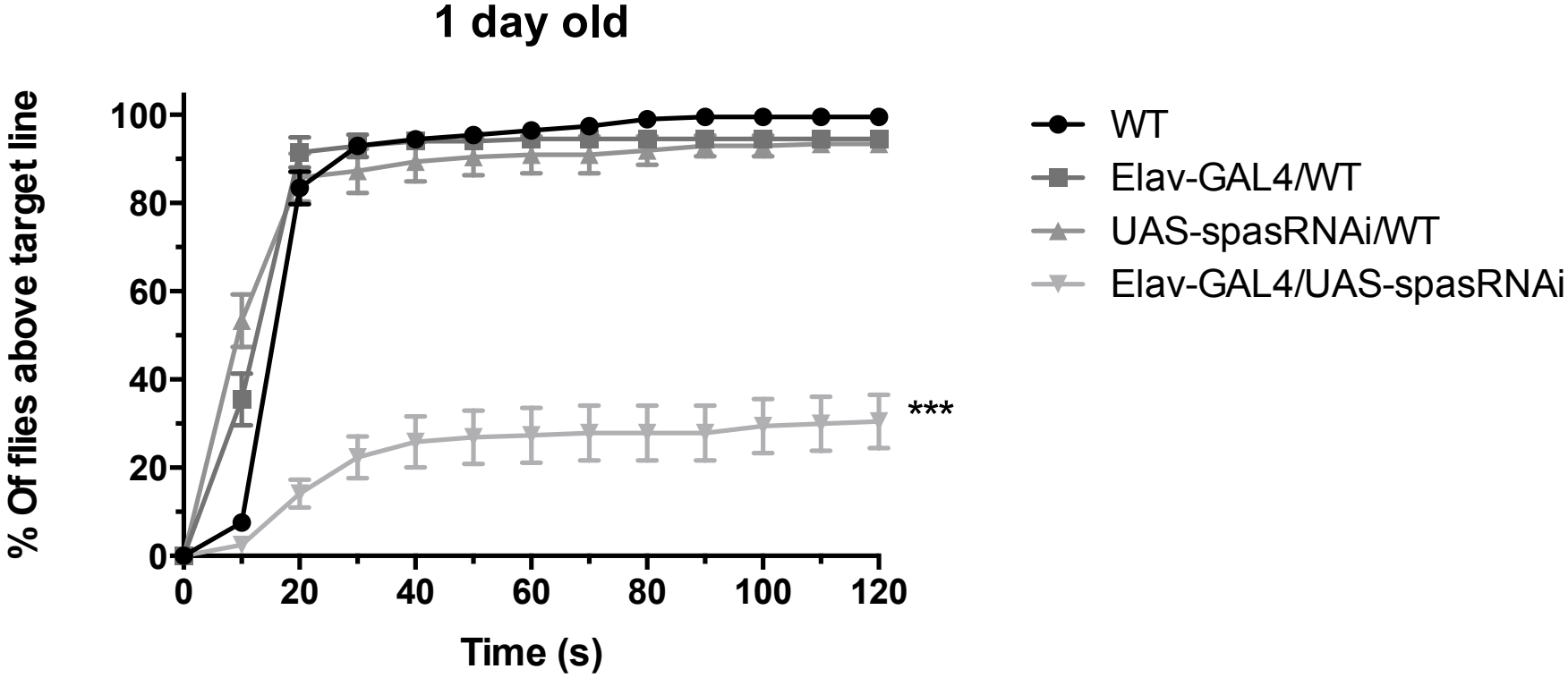

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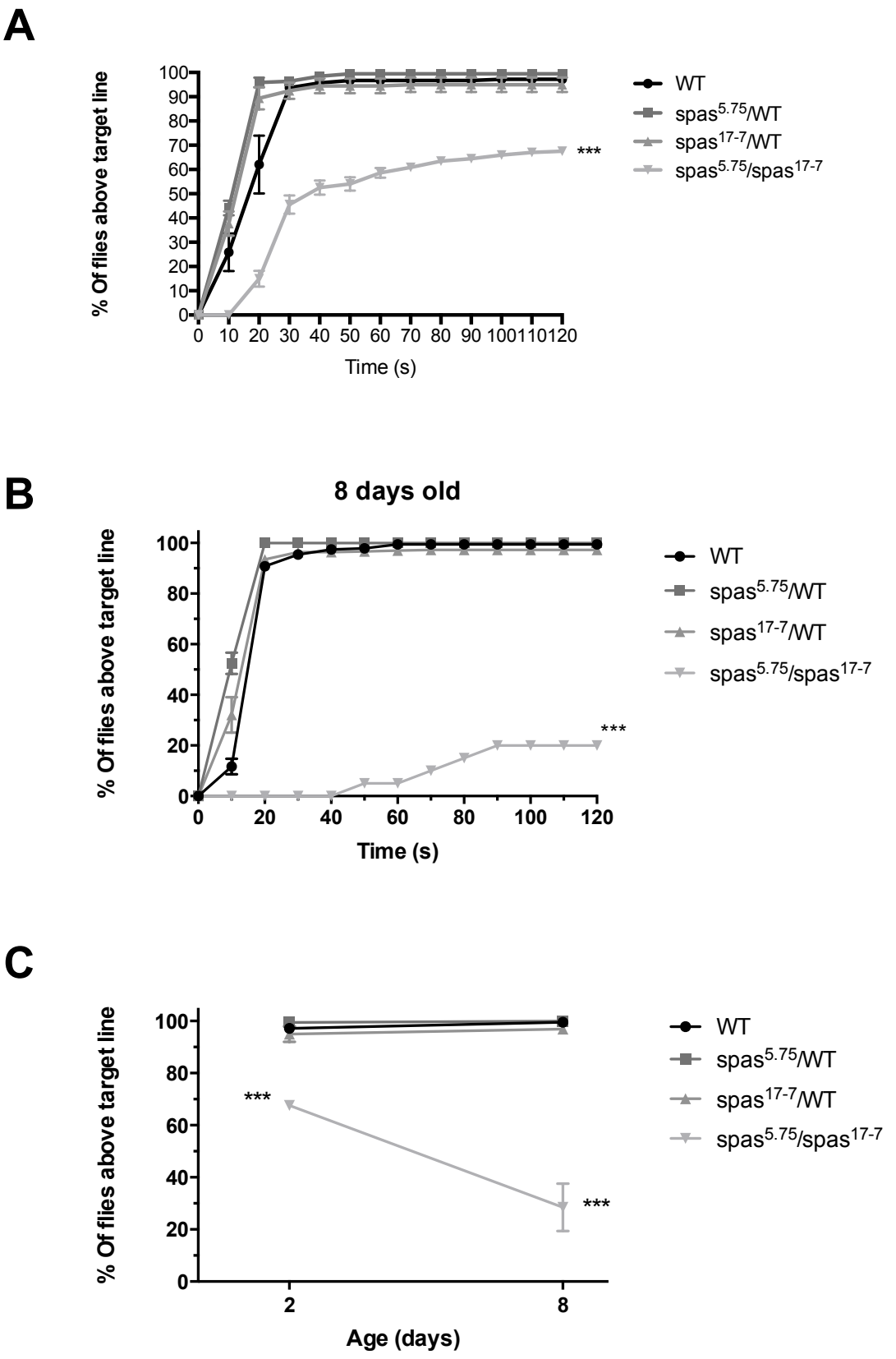

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Drosophila stocks			The stocks are selected depending on the experiments. The tempera
Video camera			Any digital camcorder will do. Make sure they can focus on close obj
Graduated cylinder	Kimble	20028W	Different models of graduated cylinder may have different diameter
computer			Any model will do. We used the computer to monitor the climbing o

ature and humidity in the room and in the incubator must be controled and consistent to avoid flies being too staticky or too wet.
ject.
. It is therefore imporant to measure the height.
f the flies and record the number of flies at each time point

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CORRESPONDING AUTHOR:

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Department: Pediatrics

Institution: University of Alberta.

Article Title: Quantitative analysis of climbing defects in a Drosophila model of neurodegenerative disorders.

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Done

3) Please minimize use of the pronoun “our” throughout the manuscript.

Minimized.

4) Please add units to the x-axis label.

Added.

5) Editor removed sub-headings from the Discussion section to comply with the JoVE format. In the Discussion, please included any modifications that can be made to the protocol that is presented in the manuscript (not modifications that were made to previous protocols) and any tips to troubleshoot problems that researchers may face when using your protocol.

Done.

6) step 3.1 is unclear as written - should the total number of flies that have passed the target line be recorded as +1?

Fixed.

Reviewers' comments:

Reviewer #1:
This technique manuscript describes an improvement in the already well-utilised climbing assay as a method of assaying locomotor defects in freely climbing Drosophila melanogaster. Using glass cylinders the authors claim that by allowing flies to climb approximately 3 times higher (17.5 cm c.f. 2-5 cm) over a longer period (120 secs c.f. 10-20 secs) they are able to detect milder defects earlier and with a greater sensitivity.

Major comments:
While an interesting and potentially useful advancement on the current assays used to detect locomotor deficits in flies carrying mutations or silenced genes its clear that the authors need to add a little more work to their paradigm to make it a truly useful approach.

We thank the reviewer for pointing this out. We think that each method has its own advantage. We think that depending on the type of mutation and gene as well as the strength of the mutation, scientists may opt for one method over the other. We have added further experimental results with milder allele to show the enhanced detection of the method presented. In addition, we thought useful of presenting our method in JoVE as we had to go through several troubleshooting steps to optimize this method and believe it will help other researcher to “see it” presented.

As the authors acknowledge in the discussion it is clear many of the components of this method has been described previously in Palladino et al. (2002) but has the added component of a temporal measurement (every 10 seconds over a 120 second period) versus the simpler measurement of the time required for 50% of flies to climb above the 17.5 cm mark on the cylinder. This is fine but it is unclear what advantage the more laborious method of collecting video footage, analyzing it and producing data for every 10 seconds has over simply using the measure of time for 50% to climb (Palladino et al., 2002) or the

numbers that climb over a shorter timeframe as used in other studies (e.g. refs 12,14 &15). The authors should comment on this explicitly.

This method of analysis came from the observation of severe mutants where there was severe defects. Indeed, in flies with extremely poor climbing performance, it was difficult to record the time required for 50% of the flies to cross the target line for it could take a considerable amount of time for this criteria to be fulfilled. Rather, flies were given a duration of 2 minutes to cross the target line. Recording the number of flies having climbed in a short amount of time is also widely used but we noted by doing recording of distance over time that the slope of the climbing curve was not the same for all genotypes which would have led to under estimation of the effect of a gene or a treatment effect.

In figure 1 the authors show that defects in a fly heterozygous for a mutation in the spastin gene can be as successfully detected after 18 seconds in an empty fly vial as it can in the much larger cylinder. In fairness the difference is much more pronounced in the cylinder system. But where does this method differ to that of Palladino et al. (2002)? What sets this technique apart?

We thank the reviewer for pointing this out. This method provides a more descriptive quantification of climbing ability. By observing climbing performance over time we can detect climbing deficits in flies that are described as good performers in other assays. For example, two lines of flies may both reach maximum climbing performance but one line may take longer than the other in doing so. This assay is more sensitive to these slight differences in climbing between the flies.

The Palladino paper presented an excellent method. We made several modifications based on repeated experimentations and felt that other groups may also benefit of those in their experiments.

It is possible that the true power of this approach lies in its potential ability to detect weak defects: a) in the case of moderate to strong mobility dysfunction, defects can be detected much earlier than current methods allow and/or b) in the case of moderate to mild mobility dysfunction to sensitively assay and show a role for candidate genes in locomotor control. Unfortunately this possibility is not supported by direct data. The article would be a lot stronger if they could show with strong examples how the approach is a major improvement over current methods.

We thank the reviewer for this suggestion. We have updated figure 1 with new experiments. We tested that directly using two different alleles of spastin previously characterized and published. A stronger allele spast5-75 contains a full deletion of the spastin gene. In that case, both method detect the impairement althought the difference is more present in the cylinder method. We have now added data from another allele containing a partial deletion only and shown to have milder locomotor defect. In that case, we observed that the defect was only significant with the cylinder method.

That being said, the details and procedures and clearly presented and in that sense, is appropriate for a JOVE article.

We thank the reviewer for their positive feedback.

Other comments:

Figures 3 and 4 should detail the RNAi and mutants used

Done.

Line 135 - CO2 (as indeed chilling) anaesthetization is known to affect fly behaviour (e.g Barron (2000) J. Insect Phys 46(4): 439) including locomotion (vanDijken et al. (1977) Experentia 33(10): 1360) and it may be a slightly confounding issue. May I suggest for at least a certain proportion of the experiments to simply flip the flies from bottles/vials into vials and approximating a number of 20 in order to satisfy the

reader that CO2 anaesthetization is not an issue? Since the data is represented as a fraction of the total number small variation in numbers will have little or no effect on behavioural outcome or scoring.

We thank the reviewer for pointing this out. As noted by the reviewer, Barron noted an effect for up to 20 hours for the copulation behaviour. We were also concerned with a potential effect so we select the flies the day before and leave them to rest for at least 21 hours. We also observe that wild type flies climb normally.

Line 135: What ages were the flies? Although detailed later it would be nice to know the age ranges at this early point.

Flies used in figures 1-3 were all 1 day old flies. Flies used in figure 4 are 2 and 8 day old flies.

Line 136+: While drug delivery is detailed, none were used in the experiments and is not necessary.

Deleted.

Lines 154 & 175: Although the experiments were conducted under ambient light it might be useful to conduct - at least some of - the experiments under red light conditions so as to eliminate the confound of phototaxis in general, but also eliminate potential differences caused by the effect of gene perturbation on the visual system. Moreover, the markings on the cylinder may also affect fly behaviour, as they provide a visual reference. In this vein how was the background colouration controlled? It is well known that background architectural features of different colour and shape can affect fly movement.

Line 158: What type of barrier film was used?

We used parafilm® wax film but considering this was a brand name we had to replace the name with something else.

Line 166: Could a number or range be given for the repeated taps as a guide for the reader?

Tap 5-10 times while using the other hand to press record on the camera.

Line 174-177: The exact type of cylinder used should be noted.

Noted.

Line 181: How many groups of flies were tested per cylinder in any given experiment? Flies quickly lay down trails containing pheromones etc that can affect the behaviour of successive groups of flies (see Tully and Quinn (1985) J Comp Physiol A. 157(2):263-77)

We thank the reviewer for bringing this ip. Usually 10 groups of flies are tested per genotype. Each genotype is tested in a different cylinder for a given day. The cylinder are washed in a commercial dishwasher daily. The cylinder are rotated between groups to prevent any possible instrument bias. The flies are usually tested in groups of 5 for each genotype each day and compounded with data obtained the next day.

Line 184: The ideal target of 10 replicates per experiment ironically was not attained for Fig1B and unknown for Fig. 3.

We thank the reviewer for pointing this out. We recommend a target of 10 replicates for fly strains that may have milder differences in climbing performance. Usually, if the deficit is severe enough, we have observed that as little as 5 replicates are sufficient to obtain significant results. This is illustrated in figure 1 for the two different alleles presented. The more severe allele of spastin 5.75 reaches a defect in cylinder and vial methods. In the other hand, the defect in the less severe allele 17-7 is seen only with the cylinder method. He we suggest the N=10 as it will include replication in different cylinder in different days.

Lines 191-199: This section is overly complicated for what is essentially a fractional measurement and should be simplified. It is also unclear if the numbers recorded for each time point were those values at the 10, 20 ,30 etc second time point or the time interval between each time point.

We clarified the text to indicate that it is the time point and not the interval.

Line 203: 'Final' needs to be removed.

Done

Line 204: Why a preference for the Tukey post hoc test?

Thank you for pointing this out. We included more details about the choice of the post test.

Line 204-5: The Kolmogorov-Smirnov should be completed first to ascertain normality and equal variance, as this will advise on the use of parametric or non-parametric testing.

We thank the reviewer for pointing this out. We have clarified this further.

Lines 207-208: these could be moved to the start of the methods to better inform the reader.

We felt that it was easier to understand this line at the end after explaining how to construct each time point.

Line 213: On instead of In

We modified that sentence for clarity

Line 249: reference needed at the end of 'neurodegenerative conditions'

Done

Line 266: Here an improved model

We modified that sentence for clarity

Line 269: Palladino needs a year

Done

Line 273-276: Further validation for the need to complete the experiments under red light.
We thank the reviewer for this suggestion. We performed the experiments for wild type and the spastin mutant and did not observe any significant defect. We added this comment to the manuscript in the method section

Line 280: acute practice = learning?

We clarified this sentence with the suggested term.

Figure 1: Error bars on the control blue line not visible for much of the data points.

corrected

Figure 2: Is wax film the same as barrier film?

yes

Reviewer #2:
* Are the title and abstract appropriate for this methods article?
The abstract is fine but the title does not reflect the content of the manuscript. The authors describe a method to easily perform climbing analysis in Drosophila melanogaster but they do not test any drug in their examples to prove that the method is powerful enough to detect the influence of such treatment. This may be reasonable for a discussion but it should be removed from the title.

We have done the modification to the title

* Are there any other potential applications for the method/protocol the authors could discuss?
No. In our opinion, the discussion covers all the critical applications.

* Are all the materials and equipment needed listed in the table? (Please note that any basic materials or equipment that a lab who might
use this protocol would already have do not need to be listed, e.g., pipettes.)
Yes, they have indicated all the special elements needed.

* Do you think the steps listed in the procedure would lead to the described outcome?
The description of the method is very exhaustive and accurate.

* Are the steps listed in the procedure clearly explained?
Yes

* Are any important steps missing from the procedure?
No

* Are appropriate controls suggested?
In the figures, the authors have included the right controls for the experiments they have presented.

* Are all the critical steps highlighted?
Yes. The authors have put enough emphasis in the most important steps.

* Is there any additional information that would be useful to include?
In the step 2.1), the authors indicate that the experiment should be performed under ambient light. Since light is known to have a strong influence in the locomotor performance of the flies (the phototaxis in the Benzer paper the authors cite!), the authors should be more precise when describing this aspect of the experiment. The discussion mentions that the light comes from above. If so, do blind flies perform worse than seeing flies (as the authors allude to)? If so, light probably will produce many false positives, i.e., blind flies. We would suggest performing the experiments in dim red light to avoid these confounds. If the

experiments have to be performed in lit conditions, the authors should show that blind flies do not perform worse or that such false positives have to be considered as a negative consequence of some more important function of the light.

We thank the reviewer for this comments. We included in the revised manuscript experiments addressing this question. Using a filtered red light, we did not observe any difference in the climbing ability of the wild type or spastin 5-75 mutant flies. Nonetheless, we added this comment to the method as it may be important for other genes involved in neurodegeneration.

* Are the anticipated results reasonable, and if so, are they useful to readers?
The results are clearly reasonable and the method proposed here might be useful in order to detect slight or moderate effects in the climbing ability.

* Are any important references missing and are the included references useful?
We have noticed at least two references that have not been included in the manuscript. In both of them, the authors use speed (cm/s) instead of number of flies to monitor locomotor performance. In Sherwood et al., authors used two different methods to describe locomotor behavior and it is already reported that speed is more sensitive than the number of flies reaching the top of the vial (85% reduction compared to 40%). This reference is especially important since it described phenotypes of the Spas5.75 allele that the authors also used in the current manuscript. The authors should discuss the advantage of their method compared to the measure of walking speed.

We apologize for this oversight. We have included those references in the revised manuscript as they are key to our discussion about the sensitivity of the method.
The references are:
- Sherwood, N.T., Sun, Q., Xue, M., Zhang, B., Zinn, K. (2004). Drosophila Spastin Regulates Synaptic Microtubule Networks and Is Required for Normal Motor Function. PLoS Biol. 2(12): e429. 2004
- Botella, J.A., Ulschmid, J.K., Gruenewald, C., Moehle, C., Kretzschmar, D., Becker, K., Schneuwly, S. The Drosophila carbonyl reductase sniffer prevents oxidative stress induced neurodegeneration. Curr. Biol. 14:782. 2004.

* General comments.
- Line 106-108 (intro): The authors write "Benzer suggested in 1967 that the counter-current apparatus used for the study of phototaxis could also be used to study gravity." Surely, Benzer did not suggest to use flies to study gravity? I thought this was done, in part, at the LHC in Geneva? Certainly, the authors mean gravitaxis, another term for negative geotaxis?

We thank the reviewer for pointing this out and made the correction.

- The method reported in this manuscript constitutes a welcome improvement over other Drosophila locomotor approaches because it seems to promote a faster detection of early climbing defects and increases the statistical power of the samples. Figure 1 reflects these facts very nicely.

We thank the reviewer for their comment. We have also included further data to support this.

However, we have serious concerns when considering this method because since 2004 the monitoring of speed has been shown to do exactly the same. Moreover, the authors never tested the efficiency of the method when applying drugs although this is the application more frequently mentioned in the text. Thus, the difference to measuring speed ought at least to be addressed and the authors either need to show an example of drug application or changing the title before this manuscript should be accepted for publication.

We thank the reviewer for pointing this out. We included the information about the usefulness of the speed in the manuscript. We modified the title to better reflect the content of the manuscript.

- In general, climbing involves more than just locomotion: motivation, the ability to move away from other flies after the bang, the decision to move with a group of other flies or independently of them, the ability to be startled by the bang to the bottom of the vial, etc. None of the above are all that related to motor coordination but will yield deficits in the climbing assay described here, when manipulated. Some of these defects might even manifest themselves as progressive, for instance if the neurons degenerate that mediate these functions. Such potential false positives (common to all climbing assays, not just this version!) need to be addressed in the discussion.

We agree and thank the reviewer for pointing this out and we included it in our discussion.

* Specific comments.
- Step 1.1) Authors indicate here the usage of sucrose for drug administration, however a starvation process may alter the locomotor ability of the flies and promotion of autophagy has been shown to have promoting effects in neurodegenerative models. Authors should comment on these possibilities or show that the locomotor index of flies kept on a sucrose diet does not differ from the performance of flies on standard food.

We have removed this as no drug treatment is demonstrated in the paper.

- Step 1.3) Here the authors indicate that the experiments are done at 22ºC. Authors should better explain why this particular temperature instead of the standard 25ºC.

This temperature is easier to maintain without special equipment. It was therefore used.

- Step 3.4) The authors analyze the results after 120 seconds. However, in the examples shown in figures 1 and 3, a sufficient difference is already present after 30-40 seconds and it does not vary significantly during the following time-points. The authors should discuss the need for increased observation time or show examples where this time is actually needed.

We observed following several other preliminary experiments that some genotypes would present a delayed climbing and have flies climb passed the 1 minute mark. We therefore empirically decided to perform the assay for 2 minutes.

- The age of the flies is missing in the figure legend of figures 1 and 3. This piece of data must be included.

We apologize for this and included it.

- In figures 3 and 4, authors present data from unidentified RNAi lines and mutations. This is not acceptable. The reader needs to know which flies were manipulated in which way.

We included the information in the revised manuscript

- Lines 258 and 291: climbing is not a reflex, it's probably not even simple: see the effects of wing manipulation on phototaxis in the Benzer paper cited in this manuscript. These same wing manipulations also affect climbing.

We agree with the reviewer and modified the manuscript to reflect that.

Reviewer #3:

Manuscript Summary:

The authors describe a protocol to quantitatively measure locomotor defects in Drosophila.
Climbing assay is one of the many ways Drosophila biologists quantify behavioral defects related to the neuromuscular system.
Alternative but complementary methods include flight assays, climbing assays, and Drosophila activity monitor (DAM) assays.

We agree with the reviewer and integrated that comment in our discussion.

Since the method also rely on the innate behavior of the fly to move against gravity, the assay will also likely pick up defects in gravity sensation and motivation/decision making.

The authors claim that the compared to the classical method, where investigators score the number of flies that climb to a certain high (which is lower) within a certain amount of time (which is shorter), the method described here is more sensitive and allows them to pick up subtle defects that are missed in previous assays.

We revised the manuscript to nuance this notion. We do not mean to diminish other assay but rather to present JoVE readers with an illustration of a assay that can be easily reproduced in their lab.

The authors recommend that the assay to be designed so that the flies are asked to climb a longer distance and given a longer time compared to classical assays to achieve higher sensitivity.

Major Concerns:

Although the authors claim that their assay is indeed more sensitive, hence more useful, using an example shown in Figure 1, I am not entirely convinced that this is indeed the case.

We thank the reviewer for pointing this out. We have added more experimental data to support our suggestion of using the cylinder method for detecting milder defects. Indeed, we show now in figure 1 the results of testing a milder allele of spastin (spas 17-7) where defects are significant in the cylinder method but not in the vial method.

What is there rational of selecting 17.5 cm as the distance that the fly needs to climb?

We used the same distance as in Palladino (2002) to have a common ground for comparison.

Is the assay even more sensitive when one selects a longer distance and a longer time?

The effect of distance is possibly important as shown in figure 1. With regard to time, we now provide in figure 4 some data showing that shorter time may not be as accurate.

If one does not see any defect in the climbing assay that the authors propose, can the person conclude that there is NO locomotor defect in the flies they are testing?

This is a good question. I think one could state what they observe which would be that there is no defect in "mutant x" in a given assay.

How does the defect in this assay relate to histological defects found in the fly's brain or the muscle?

We thank the reviewer for this question. We did not include this in the scope of our manuscript but think this is an important question and added it to our discussion.

Minor Concerns:
1) The authors do not explain what the genotype "Spas 5.75/+" is.
What kind of mutation is Spas5.75
Is it a dominant mutation? What is the lesion? What is the source?
The readers may want to use this as a positive control when setting up there assay so it is important to specify the detail of the flies that they use here.

We included the information about the nature of the mutation as well as the reference.

Similarly, the authors should explain what the "UAS-RNAi" is in Figure 3 is, and what mutant 1 and mutant 2 are in Figure 4.
These reagents must be publically available upon publication of this protocol on Jove so the authors should present data using mutants/transgenic lines that they can share.

We included that information in the revised manuscript

2) When testing mutants and RNAis, it is important that the authors perform rescue experiments with WT cDNA or genomic rescue reagents.
The UAS/GAL4 only, and heterozygous mutations over WT is a decent control, but showing rescue with a WT protein is required in mid-high profile journals.

We included this advice in the protocol to strengthen the protocol

3) The authors show the aging profile of WT fly and mutant flies at day 2 and 8 in Figure 4.
It would be really nice if they can show how the pattern changes for a commonly used wild-type fly (such as CS) to provide a reference point for the readers that wish to perform this assay.

We included that information for the wild-type line used which is derived from w1118 line.

4) In 2.1), how are the flies transfered from the single vials into the 250ml cylinder?
I assume this is done in the absence of anesthesia.
Should one wait a certain amount of time so that the flies get used to the new environment?

Additional Comments to Authors:
None.

Reviewer #4:
Manuscript Summary:
This manuscript focuses on the use of a newly adapted and more sensitive negative geotaxis assay for screening mild locomotion defects associated with neurodegenerative disorders such as Parkinson's disease. The authors have taken the well established standard negative geotaxis assay and adapted it for

more sensitivity by increasing the climbing distance from 5-10 cm to 17.5 cm and increasing experiment duration to 2 minutes. They also adapted the assay by using a diffuse fluorescent light to avoid light reflection. After adapting this assay for sensitivity, they then tested its use on fly lines that included one mutant for the locomotion gene spastin.

Major Concerns:
1. The structure of the paper is very confusing. Nowhere is it outlined exactly what mutants the researchers are using except for figure 1 (mutant types and rationale are not clearly labeled in the rest of the figures). The authors need to more clearly define the mutants that they test in the assay and why they choose to use the mutants for a given particular assay.

We apologize for this. We did several edits to enhance clarity. We also included the information about genotype in the revised manuscript.

2. Most importantly, the researchers to not logically test whether the adaptations to the assay they make are indeed more sensitive. To do this, the researchers should use several different mutant lines that cause increasing levels of severity for the locomotion defects well as several neurodegenerative fly models with varying levels of severity to definitively show that their newly adapted assay does indeed pick up more mild defects for a given mutant.

We included further experiments using a milder allele of the spastin gene that was previously characterized. We present data showing that for an equal number of trials, the method described here allows to obtain significant results when other method only show trends. We also tested the effect of light on the assay directly.

Minor Concerns:
1. While the adaptations to the assay making it more sensitive are important, they are also very minor. The negative geotaxis assay is a well-established quick and simple assay to perform. Other researchers have certainly made simple adaptations such as these to more fine tune this assay to the mutants they are testing.

We revised the manuscript to include that comment. We acknowledge that several good methods exist and may be adapted to the gene studied and the lab set up. We feel that JoVE will allow researchers new to the locomotion field will be able to start quickly addressing research questions by "seeing" the experimental set up we developed.