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TITLE:

Comparative Analysis of Experimental Methods to Quantify Animal Activity in *Caenorhabditis elegans* Models of Mitochondrial Disease

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worms, *C. elegans*, locomotor activity, pharyngeal pumping, chemotaxis, thrashing, ZebraLab, WormScan, high-throughput screening capacity, gas-1(fc21)

SUMMARY:

This study presents protocols for two semi-automated locomotor activity analysis approaches in *C. elegans* complex I disease gas-1(fc21) worms, namely, ZebraLab (a medium-throughput assay) and WormScan (a high-throughput assay) and provide comparative analysis among a wide array of research methods to quantify nematode behavior and integrated neuromuscular function.

ABSTRACT:

Caenorhabditis elegans is widely recognized for its central utility as a translational animal model to efficiently interrogate mechanisms and therapies of diverse human diseases. Worms are particularly well-suited for high-throughput genetic and drug screens to gain deeper insight into therapeutic targets and therapies by exploiting their fast development cycle, large brood size, short lifespan, microscopic transparency, low maintenance costs, robust suite of genomic tools, mutant repositories, and experimental methodologies to interrogate both in vivo and ex vivo physiology. Worm locomotor activity represents a particularly relevant phenotype that is frequently impaired in mitochondrial disease, which is highly heterogeneous in causes and

manifestations but collectively shares an impaired capacity to produce cellular energy. While a suite of different methodologies may be used to interrogate worm behavior, these vary greatly in experimental costs, complexity, and utility for genomic or drug high-throughput screens. Here, the relative throughput, advantages, and limitations of 16 different activity analysis methodologies were compared that quantify nematode locomotion, thrashing, pharyngeal pumping, and/or chemotaxis in single worms or worm populations of *C. elegans* at different stages, ages, and experimental durations. Detailed protocols were demonstrated for two semi-automated methods to quantify nematode locomotor activity that represent novel applications of available software tools, namely, ZebraLab (a medium-throughput approach) and WormScan (a high-throughput approach). Data from applying these methods demonstrated similar degrees of reduced animal activity occurred at the L4 larval stage, and progressed in day 1 adults, in mitochondrial complex I disease (gas-1(fc21)) mutant worms relative to wild-type (N2 Bristol) *C. elegans*. This data validates the utility for these novel applications of using the ZebraLab or WormScan software tools to quantify worm locomotor activity efficiently and objectively, with variable capacity to support high-throughput drug screening on worm behavior in preclinical animal models of mitochondrial disease.

INTRODUCTION:

Caenorhabiditis elegans is widely recognized as an outstanding model in neuroscience based on it having 302 neurons that coordinate all worm behaviors, including mating, feeding, egg-laying, defecation, swimming, and locomotion on solid media[1]. These hermaphroditic nematodes are also widely used to understand a wide array of human disease mechanisms, made possible by its well-characterized genome and high homology of ~80% genes between *C. elegans* and humans[2,3,4]. *C. elegans* have long been used to interrogate human mitochondrial disease[5–10], which is a highly genetically and phenotypically heterogeneous group of inherited metabolic disorders that share impaired capacity to generate cellular energy and often clinically present with substantially impaired neuromuscular function, exercise intolerance, and fatigue[11–14].To this end, the use of *C. elegans* models enable preclinical modeling of quantitative aspects of animal activity and neuromuscular function in different genetic subtypes of mitochondrial disease, as well as their response to candidate therapies that may improve their neuromuscular function and overall activity.

Neuromuscular activity in *C. elegans* is objectively measurable by a range of experimental methodologies, including both manual and semi-automated approaches that allow functional analyses in either solid or liquid media (**Table 1**)[1,15]. Accurate quantitation of *C. elegans* activity has proven important to enable discoveries related to function and development of the muscular and nervous system[16–18]. This study summarizes and compares experimental requirements, advantages, and limitations of 17 different assays that can be performed in research laboratories to evaluate neuromuscular function and activity on four key outcomes in *C. elegans* diseases models, both at the baseline at a range of developmental stages and ages as well as in response to candidate therapies (**Table 1**). Indeed, the study provides a detailed overview of the range of available experimental approaches to characterize rates of *C. elegans* thrashing (body bends per minute), locomotor activity, pharyngeal pumping, and chemotaxis—in each case specifying the experimental and analytic methodology used, the advantages and limitations of each method,

the equipment and software needed to perform and analyze each assay, and the throughput capacity of each method to support its use for high-throughput genetic or drug screening purposes. The throughput capacity of each assay is described as low, medium, or high based on the experimental protocol complexity, including worm maintenance, processing time, the use of single or multi-well plates, and/or experimenter time needed to complete the experimental setting and data analyses.

Manual analyses of thrashing[19], locomotor activity[20], pharyngeal pumping[17,21], and chemotaxis[22,23] are well-established methodologies to evaluate worm activity that require a stereomicroscope[24]. While measuring thrashing activity of worms requires analysis in liquid media to determine the frequency of body bends per minute, worm locomotor activity may be measured either on solid media or in liquid media. However, manual analyses of individual worm activity are inherently time-consuming and involves unavoidable user-generated bias. Automation of worm activity analyses minimizes user-generated bias and can greatly increase experimental throughput[25]. Video recordings of worm thrashing activity in liquid media can be analyzed using wrMTrck, an ImageJ plugin[26]. However, the original experimental settings that were developed for wrMTrck limited its utility, since too many worms in a single liquid drop led to overlapping of worms that made accurate tracking difficult. While this experimental limitation has been resolved[27], the wrMTrck method is not able to support high-throughput screening.

A range of methods exist to quantify worm locomotor activity at baseline and in response to candidate therapies in *C. elegans* mitochondrial disease models. These include ZebraLab (ViewPoint Life Sciences), Tierpsy Tracker[28], wide field-of-view nematode tracking platform (WF-NTP)[29], WormMotel, WormWatcher[30], WormLab[31], Infinity Chip[32], and WMicrotracker One[33] (**Table 1**). These methods enable concurrent analysis of locomotion in multiple worm strains or conditions, typically on multi-well plates, thereby supporting higher-throughput drug screening applications. Some of these methods have unique considerations that may limit or enhance their general utility, such as the need for expensive equipment versus open-access software, and varying ease of performing experimental protocols. Overall, no single experimental system or protocol is ideally suited to all *C. elegans* locomotor activity experiments. Rather, it is important to carefully choose which method is best suited to the specific investigator's experimental goals and requirements.

Pharyngeal pumping represents another important outcome to assess neuromuscular activity in *C. elegans*. The *C. elegans* pharynx is composed of 20 muscle cells, 20 neurons, and 20 other cells that enable ingestion of *Escherichia coli* (*E. coli*) at the anterior end of the worm's alimentary tract[34–36]. Several manual methods have been established to determine pharyngeal pumping rates[17,21,37,38]. Most methods are based on the use of a stereomicroscope and camera to visualize and record pharyngeal pumping frequency with direct counting by the experimental observer[21]. Automated pharyngeal pumping rate analysis is possible by performing an extracellular recording termed electropharyngeogram (EPG), which provides additional information on the duration of each pump[39]. Pharyngeal pumping rate analysis is also possible in a microfluidic system, WormSpa, where individual worms are confined in chambers[40,41]. A commercial method available to facilitate analysis of the pharyngeal pump rate is the ScreenChip System (InVivo Biosystems),

which measures, visualizes, and analyzes the neuromuscular aspects of feeding behavior in a single worm that is immobilized in a custom chip. This pharyngeal pumping quantitation approach can be used to assess both neuronal and physiological responses to drugs, aging, and other factors[42–45].

Chemotaxis describes the movement of *C. elegans* in response to an odorant placed away from the worms in a defined area of the nematode growth media (NGM) plate. Assessing the chemotaxis response provides an integrated measure of worm neuronal and neuromuscular activity that is quantifiable by observing and measuring the physical distance traveled by worms toward the odorant in a defined time period[46]. The Multi-Worm Tracker is an automatic method that can be used to improve the experimental efficiency of quantifying the distance traveled by worms toward an attractant or from a repellant[47].

Here, the detailed protocol for two novel, semi-automated methods established for quantifying worm activity is described. The first approach utilizes ZebraLab a commercial software that was originally developed to study swimming activity of Danio rerio (zebrafish), for a novel medium-throughput application to quantify overall locomotor activity in liquid media of *C. elegans* based on pixel changes during movement (**Table 1, Figure 1**). Data output is quickly obtained from a large number of concurrent conditions and samples analyzed on a glass slide, although this method is not suitable to a multi-well plate format. The second approach is a novel adaptation of the WormScan methodology[48,49] (**Figure 2**), which uses a flatbed scanner to create a differential image of two sequential scans that can variably be used with open-source software to enable semi-automated quantitative analysis of integrated physiologic outcomes such as fecundity and survival. Here, a novel high-throughput adaptation of the WormScan methodology to quantify worm locomotor activity in liquid media in populations of fifteen larval-stage 4 (L4) worms per well of a 96-well, flat-bottom plate was developed. This semi-automated and low-cost WormScan methodology can be readily adapted to high-throughput drug screens, as well as to analyses of various animal stages and ages[48,49].

Here, the protocol and efficacy of analyzing *C. elegans* locomotor activity using both ZebraLab and WormScan semi-automated methods is demonstrated in a well-established *C. elegans* model for mitochondrial complex I disease, gas-1(fc21). gas-1 (K09A9.5 gene) is an ortholog of human NDUFS2 (NADH: ubiquinone oxidoreductase core (iron-sulfur protein) subunit 2) (**Figure 3**). The *C. elegans* gas-1(fc21) mutant strain carries a homozygous p.R290K missense mutation in the human ortholog of NDUFS2[50], causing significantly decreased fecundity and lifespan, impaired respiratory chain oxidative phosphorylation (OXPHOS) capacity[51], as well as decreased mitochondrial mass and membrane potential with increased oxidative stress[5,8]. Despite its well-established use over the past two decades to study mitochondrial disease, locomotor activity of gas-1(fc21) mutants was not previously reported. Here, ZebraLab and WormScan methods were applied to independently quantify the locomotor activity of gas-1(fc21) as compared to wild-type (WT, N2 Bristol) worms, both as a way to validate the methods as well as to demonstrate their comparative utility and efficiency of the experimental protocols and informatics analyses. ZebraLab software allowed rapid quantitation of several concurrent conditions of worm locomotor activity in *C. elegans* mitochondrial disease models, with potential application for

targeted drug screening or validation studies. WormScan analysis, in particular, is well-suited to readily enable high-throughput drug screens of compound libraries and prioritize leads that improve the animal neuromuscular function and the locomotor activity in preclinical *C. elegans* models of primary mitochondrial disease.

PROTOCOL:

1. Worm locomotor activity analysis in liquid media on glass slides using ZebraLab software

1.1. Nematode growth and handling

1.1.1. Grow *C. elegans* on Petri plates containing nematode growth media (NGM) and spread with *Escherichia coli* OP50 as food source. Maintain worm culture at 20 °C, as previously described[8].

1.1.2. Synchronize worms performing a timed egg lay[52] and study worms at the desired stage. In this protocol, L4 stage worms were analyzed.

1.1.3. Grow control and mutant worm strains on NGM plates with and without drug treatments to be tested or buffer control. To evaluate the drug treatment effects, prepare the desired drug stock concentration in S. basal solution; spread the calculated specific volume onto the NGM plates and allow it to dry. Transfer the worms at a specific larval or adult stage and maintain on the drug treatment plate for the desired duration before analysis.

1.2. Experimental set-up of worms for locomotor activity video recording and ZebraLab analysis

1.2.1. Pick 5 synchronized L4 worms per strain and condition using a worm pick. Pipette a single 20 µL drop of S. basal solution onto a glass slide located under a stereomicroscope connected to a camera and transfer 5 worms into it (**Figure 1A,B**). Transfer the 5 worms from the Petri dish containing NGM and *E. coli* OP50 to the liquid drop only at the moment preceding the recording.

NOTE: Continue to maintain the other worms on the Petri dish until the prior video is recorded. This will avoid the damage caused on the other worms due to the drying up of the 20 µl drops during the procedure (dry time ~15–20 min).

1.2.2. Pipette multiple drops on one slide to obtain multiple technical replicates (**Figure 1A**). Select worms from different NGM plates (biological replicate). Do not use cover slip.

1.2.3. Adjust the microscope's working distance to visualize the complete area of a single drop. Set and maintain a low video resolution (<1024 x 768) to upload the files in the software.

1.2.4. Allow worms to acclimate on the slide at room temperature for 1 min before recording.

1.2.5. Record the worm swim activity in 1 drop for 1 min at 15 frames per second (fps). Repeat the imaging for each additional drop on the plate.

1.3. *C. elegans* locomotor activity recording analysis in ZebraLab software

1.3.1. Use the option **ZEBRALAB AVI** to upload videos to the software. Click on the option **Quantization with AVI Files** (**Figure 1C**).

1.3.2. To create a new protocol, select **File** > **Generate Protocol**, and then add the number of areas selected for the analysis. Choose **Location Count: 1**.

1.3.3. Open **Protocol Parameters** and select **1 min** in the **Experiment Duration** window. Select a different experimental duration for different experimental durations. Select or deselect the window **No Time Bin** and choose **Integration Period**, depending on the data output desired. In this study **No Time Bin** was selected (**Figure 1D**).

NOTE: Time bin is the time over which the activity will be averaged.

1.3.4. If a protocol was already created, select **Open Protocol** and select the saved protocol (in .vte format).

1.3.5. Select **File** and **Open a Movie** to upload each individual video file that was previously recorded.

1.3.6. Select the **Arena** icon indicated in **Figure 1E** (black arrow) to build a single area of detection and create an area around the whole liquid drop where worms are located. Click on **Select,** then the green circle icon (gray arrow) under **Areas** > **Build** > **Clear marks**.

NOTE: The activity of all worms in the defined drop will be detected in the selected area (**Figure 1E,F**).

1.3.7. Go to **Calibration** > **Draw scale** (**Figure 1E**) and draw a horizontal line from the left to the right of the video area. Indicate the real distance to calibrate. Then select **Apply to Group**.

1.3.8. Unselect the icon selected to build the arena (arrow in **Figure 1E**) and select or deselect **Transparent**.

NOTE: In this study, **Transparent** was selected and gave better results.

1.3.9. Adjust **Detection Sensitivity** and **Activity Threshold** to allow the detection of all the different *C. elegans* worm strains analyzed.

NOTE: In this experiment, the **Detection Sensitivity** was set at **8** with **Burst** and **Freezing** values of **15** and **2**, respectively (**Figure 1F**).

1.3.10. Set **Display Scale** at **70** to visualize the track made by the animal while activity analysis is underway. Then select **Apply to Group** (**Figure 1F**).

1.3.11. Click on **Experiment** > **Execute** > **Save as**, and then on **Start**. A window opens. Choose **Do you want to process the video media at maximum computer speed?** to analyze the video quickly (e.g., a 1 min video recording is analyzed by the in ZebraLab software in 5 s).

1.3.12. Another window opens: **Running Experiment**; click on **Start** to proceed with the experiment.

1.3.13. After the video recording is complete, the analysis stops. Click on **Experiment** > **Stop**. This saves the activity analyzed from a single drop in a spreadsheet.

1.3.14. Repeat the analysis for each video of individual drop. Each drop is one technical replicate.

1.4. **Output and analysis of ZebraLab data**

NOTE: After the experiment, data from each video is individually saved as separate spreadsheets in the chosen folder. In the data output file, the integrated activity level of all worms moving in an individual drop is recorded as pixel changes under **actinteg**.

1.4.1. Open each spreadsheet obtained from the analysis of each video. Compile them manually into a single file.

Normalize the mutant and wild-type data to a percent of control. Here, statistical analyses were performed to compare mean activity levels between groups.

2. Worm locomotor activity analysis in liquid media in 96-well plate format by WormScan software analysis

2.1. Nematode growth and handling

2.1.1. Grow *C. elegans* as described in section 1.1.1.

2.1.2. Synchronize worms as described in section 1.1.2.

2.1.3. Grow worms on specific media as described in section 1.1.3 until L4 stage or day 1 adult.

2.2. Experimental set-up of worms in 96-well plate for WormScan activity analysis

2.2.1. Add 50 µL of 2% weight per volume of *E. coli* OP50 in liquid suspension in S. basal medium to each well of a 96-well, clear, flat-bottom, microplate, as previously described[49,53].

2.2.2. Under a stereomicroscope, manually pick 15 synchronized worms at L4 stage or day 1 adult from their NGM plates into liquid media within each experimental well of the 96-well microplate. Allow the worms to acclimate to the liquid media for 20 min before scanning.

NOTE: Other animal stages and ages can be readily substituted for study.

2.3. WormScan activity analysis in 96-well plate and data export to spreadsheet.

2.3.1. Scan each 96-well, clear, flat-bottom, microplate twice sequentially using a standard flatbed scanner, with less than 10 s between scans.

NOTE: Here, the photo scanner with resolution of 1,200 dots/in and 16-bit grayscale was used to produce jpeg images. Time required to scan four 96-well plates using the photo scanner is less than 10 min.

2.3.2. Align the two sequential scans (**Figure 2A**) using open source software[49].

NOTE: The software generates a difference image to evaluate pixel changes between the two sequential images for a region of interest (**Figure 2B**) and a relative **WormScan Score**. This WormScan Score is equivalent to changes in locomotor response based on the light intensity produced by the scanner when set to a pixel threshold of 5 (**Figure 2C**).

2.3.3. Export the data from WormScan as a spreadsheet. Save the spreadsheet containing the data to the local computer. Normalize the data as percentage of control (POC) and compare across biological replicate experiments for diverse mutant or treatment conditions. Perform statistical analysis to compare mutant and control means using student *t*-test.

REPRESENTATIVE RESULTS:

Analysis of *C. elegans* locomotor activity in the liquid media could easily capture an integrated phenotype of mitochondrial disease worm models that may not be easily quantifiable on solid media. ZebraLab was used to quantify locomotor activity of the well-established mitochondrial complex I disease gas-1(fc21) strain relative to WTworms in liquid media at the L4 larval stage. The activity of 5 worms in a single liquid drop was recorded over 1 min, with a total of 19 videos (technical replicates) recorded for each strain, resulting in total the analysis of 95 worms per strain. Four biological replicate experiments were obtained per strain. Worm activity is displayed as pixel change (**Figure 3A**), and as percent of control (POC) when normalized to N2 Bristol WT control (**Figure 3B**). The gas-1(fc21*)* worms (62% ± 16% pixel change, mean ± SD, n = 19) had a significant 38% decrease ($p < 0.001$, *t*-test) in their locomotor activity at L4 stage as compared to WT worms (100% ± 11.35%, mean ± SD, n = 95 worms per condition in 19 technical replicates over 4 biological replicates).

WormScan analysis was also performed to quantify the locomotor activity of L4 stage gas-1(fc21) and WT worms in liquid media. Data was collected for three biological replicate experiments, where each biological replicate plate was evaluated by two sequential images scanned using a standard flatbed scanner. Worm activity of the differential images was compared as pixel change and normalized to concurrent N2 Bristol WT control. Similarly, as was seen by the Zebrafish behavior screening method, WormScan based analysis demonstrated that the gas-1(fc21) worms (65.9 ± 6.1, mean ± SD, n = 13 wells) had a significant decrease in locomotor activity by 34% ($p < 0.001$, *t*-test) compared to N2 Bristol wild-type worms (100% ± 4.8%, mean ± SEM, n = 12 wells) (**Figure 3C**). Analysis using WormScan on day 1 adult gas-1(fc21) worms (50.1% ± 10.7%, mean ± SD, n = 7 wells) demonstrated a decrease in locomotor activity by 49% ($p < 0.001$, *t*-test) compared to WT worms (100% ± 16.2%, mean ± SD, n = 6 wells) (**Figure 3D**).

FIGURE AND TABLE LEGENDS:

Table 1: Comparative overview of experimental assays available to evaluate *C. elegans* neuromuscular activity. A detailed overview is provided of a wide array of 16 different experimental techniques that can be used to quantify worm neuromuscular activity on the phenotypic outcomes of thrashing, locomotion, pharyngeal pumping, and/or chemotaxis in *C. elegans*. Read format, methodology, experimental throughput capacity, software and/or equipment requirements, as well as advantages and limitations of each assay are detailed. References and relevant websites for each assay and software tool are also provided. The throughput capacity of each assay is described as low, medium, or high, as based on the experimental complexity, the use of single or multi-well plates, and/or experimenter time needed to complete the experimental setting and data analyses. * Indicates that the methodologies can also be used for evaluation of locomotion.

Figure 1: *C. elegans* locomotor activity analysis using ZebraLab software. (**A,B**) Experimental protocol for worm video recordings. Five worms were introduced per drop (20 µL) of S. basal solution, with four drops placed on a single glass slide under a stereomicroscope. Each drop of 5 worms represented a technical replicate experiment and was recorded for 1 min in a separate movie using a charged-coupled device (CCD) camera. (**C–F**) Experimental settings in ZebraLab as adapted to evaluation of locomotor activity in *C. elegans*. (**C**) Selection of **Quantization with AVI** files to quantify worm locomotor activity of each recorded video. (**D**) Protocol parameter settings, with 1 min selected as experiment duration. (**E**) Build arena to select the area of interest. The arena was selected and built around 1 drop of solution in which 5 worms were placed. (**F**) Detection was determined based on gray-scale thresholding to detect the whole body of each worm (red). In the threshold section, burst and freezing values were selected to analyze worm activity as pixel changes.

Figure 2: *C. elegans* locomotor activity analysis using WormScan methodology. (**A**) Using an Epson v800 flatbed scanner, two immediately sequential scans of a 96-well plate were captured with a resolution of 1,200 dots/in and 16-bit grayscale to produce jpeg images. (**B**) These two sequential images of a 96-well plate were then aligned to a reference region of interest (ROI), of WT worms. (**C**) Image analysis is based on a difference image score calculated for each ROI with

15 worms/well for N2 Bristol. The difference image was normalized and reported as percentage of control (POC).

Figure 3: Comparative analysis of locomotor activity by ZebraLab and WormScan software assays in gas-1(fc-21) mitochondrial disease worms relative to N2 Bristol wild-type worms. (**A,B**) WT and gas-1(fc21) worm activity in liquid drops (5 worms/drop) was video recorded for 1 min and quantified as (**A**) pixels change or (**B**) percentage of wild-type control using the ZebraLab software. Overall, ZebraLab-based worm activity analysis demonstrated a significant decrease by 38% in gas-1(fc-21) L4 stage worms as compared to wild-type controls (*** $p < 0.001$). The graph displays mean ± SD of all data, where each dot conveys the overall activity of five worms per S. basal drop. Each drop represents a technical replicate, with a total of four biological replicates studied per condition. A total of 19 videos were recorded (one video for each drop of 5 worms), across a total of 95 individual worms studied per condition. Statistical analysis was performed using the student *t*-test in Prism -GraphPad v6. (**C**) WT and gas-1(fc21) worms at L4 stage were analyzed by flatbed scanning to produce two sequential images that were analyzed in WormScan software to yield a difference image. Three biological replicate experiments were performed with 15 worms per well in a 96 well-plate. The activity of WT worms was used as the baseline to normalize percentage of control (POC). gas-1(fc-21) activity was decreased by 34% as compared to wild-type control (*** $p < 0.001$). Bar graphs convey mean and standard deviation across three biological replicate experiments. (**D**) N2 and gas-1(fc21) worms at adult day 1 stage were analyzed similarly as detailed for panel C. gas-1(fc-21) activity in day 1 adults was decreased by 49.1% relative to wild-type control worms (*** $p < 0.001$). Bar graphs convey mean and standard deviation of pixel changes in one biological replicate comparing N2 (n = 6 wells of 15 worms/well) and gas-1(fc21) (n = 7 wells of 15 worms/well).

DISCUSSION:

Here, the study summarized detailed information and rationales for studying *C. elegans* neuromuscular activity at the level of diverse outcomes, including worm thrashing, locomotion, pharyngeal pumping, and chemotaxis. The comparison of 16 different activity analysis methodologies was performed in terms of the relative throughput, advantages, and limitations of quantify nematode activities in a single worm or worm populations at different ages and experimental durations. Among these, two novel adaptations and applications of semi-automated analyses were highlighted to demonstrate significant reduction in locomotor activity in larval-stage worms at the L4 larval developmental stage and in day 1 young adults of a well-established mitochondrial complex I disease *C. elegans* strain, gas-1(fc21) relative to WT controls.

In particular, *C. elegans* neuromuscular function and locomotor activity has been extensively studied on solid media since WT worm movement is very regular in sinusoidal wave patterns. Abnormalities of their regular movement path and the speed can be microscopically detected and manually scored by the experimental observer, in assays that are often low-throughput and tedious. To increase experimental throughput, automated and high-throughput methods should be selected. Impaired activity of worms can be quantifiable in liquid media, where overall locomotor activity of worms in droplets on glass slides or in multi-well plates can be video recorded and quantified in semi-automated or automated fashion with different software tools.

Indeed, our data highlight the utility of objectively and efficiently measuring nematode locomotor activity both by a novel application of ZebraLab software to quantify locomotor activity in videos of worms in liquid drops on glass slides (a medium-throughput screening capacity approach), as well as by utilizing WormScan software to quantify worm locomotor activity in differential flatbed scanning images of worms in a 96-well plate liquid media approach[54–56] (a high-throughput screening capacity approach). The ZebraLab software approach is considered as a medium-throughput assay since it requires that single plates be used for each condition studied, without current developed protocol for multi-well plate formats. While using the ZebraLab software approach requires minimal time when analyzing *C. elegans* activity in a few conditions, the experimental time increases when applied to multiple conditions. Here, the experimental time was approximately 2 h to transfer worms into liquid droplets and record videos of their activity, considering 18 technical replicates for each condition. The time spent for the analysis of these videos using the ZebraLab software was approximately 1 h. By comparison, the WormScan method is high-throughput because it incorporates a multi-well plate format that permits concurrent analysis of four 96-well plates in less than 10 min and setting up a 96-well plate with a COPAS Bisoter is also less than 10 min.

Both methodologies showed a similarly reduced activity in L4 larval stage gas-1(fc21) mitochondrial disease mutant worms relative to WT worms, thus validating both distinct approaches for quantifying differences in worm behavior. Further, WormScan analysis was used to readily demonstrate that progressive reduction in animal locomotor activity occurred with age in the gas-1(fc21) worms as was evident by the day 1 adult stage.

The main advantages of our adapting the ZebraLab software that was developed for zebrafish swimming analysis to *C. elegans* activity analysis is that it is experimentally simple and inexpensive to objectively capture worm movement in videos, with semi-automated quantitative analysis in movie files uploaded to ZebraLab software requiring only seconds per technical replicate and removes investigator-based bias that is present in manual quantitation methodologies. Further, having this one software tool in the research laboratory is useful to quantify locomotor activity in two animal model species, namely, zebrafish and *C. elegans*. The disadvantage is that this is a commercial software that requires purchase and worm videos need to be manually uploaded into the software, although the upload process is straightforward and software analysis time is relatively quick. Overall, the novel application described here of using ZebraLab software to quantify *C. elegans* locomotor activity holds direct potential to evaluate drug effects on worm behavior, although its throughput remains low-to-medium given its high-resolution requirements necessitate that movies be captured of worms moving in media drops placed on glass slides.

We also adapted WormScan software to efficiently quantify worm locomotor capacity of worms in liquid media in a 96-well plate. This approach offers a high-throughput and low-cost experimental method that uses a standard flatbed scanner to objectively quantify animal fecundity and survival and has previously been used for high-throughput screens in *C. elegans*[49]. The main advantages of this technique are that it is very amenable to high-throughput screening, enabling parallel comparisons of a large number of conditions at any stage or age, with ease of

use, low setup cost, and rapid analysis in an objective fashion by the WormScan software that is free and publicly available[49]. The disadvantage of the WormScan is that it can only interrogate the change that occurs between the sequential scans, which in some mutations or conditions may not be sufficiently sensitive to detect small degrees of phenotypic change. In addition, as both ZebraLab and WormScan methods exclusively rely on image pixel changes to assay animal activity, substantial differences in worm size that may occur between strains or in response to a specific therapy over time may need to be considered and/or used as a normalization factor for both methods, to more specifically enable evaluation and comparison of mutation and/or treatment effects on animal locomotor activity.

Overall, a wide array of experimental methods can be used to assess nematode neuromuscular activity on integrated phenotypic outcomes of thrashing, locomotion, pharyngeal pumping, and/or chemotaxis. We compared 16 of these methods (**Table 1**), highlighting their specific experimental and analytic requirements, advantages, limitations, and throughput capacity. Among these, we provided detailed experimental protocols for two novel applications of existing software tools, ZebraLab (a medium-throughput approach) and WormScan (a high-throughput approach), which are particularly useful to semi-automatically, objectively, and quickly quantify worm locomotion activity in liquid media. Both experimental approaches revealed a similarly reduced degree of locomotion activity occurred in mitochondrial disease (gas-1(fc21)) relative to WT *C. elegans* strains at the L4 stage, with progressive decline in locomotor activity by the young adult stage in gas-1(fc21) worms. This data demonstrates the validity of these experimental approaches that yield internally consistent data. Furthermore, this array of methods is highly versatile, enabling a wide-range of worm locomotor activity metrics in diverse disease etiologies, animal stages and ages, and in response to candidate therapeutic modeling or high-throughput drug screens that are useful for preclinical evaluation of lead targets for human disease.

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DISCLOSURES:

M.L., N.D.M., N.S., and E.N.-O. have no relevant financial disclosures. M.J.F. is a co-founder of MitoCUREia, Inc., scientific advisory board member with equity interest in RiboNova, Inc., and scientific board member as paid consultant with Khondrion, and Larimar Therapeutics. M.J.F. has previously been or is currently engaged with several companies involved in mitochondrial disease therapeutic preclinical and/or clinical stage development as a paid consultant (Astellas [formerly Mitobridge] Pharma Inc., Cyclerion Therapeutics, Epirium Bio, Imel Therapeutics, Minovia Therapeutics, NeuroVive, Reneo Therapeutics, Stealth BioTherapeutics, Zogenix, Inc.) and/or a sponsored research collaborator (AADI Therapeutics, Cardero Therapeutics, Cyclerion

Therapeutics, Imel Therapeutics, Minovia Therapeutics Inc., Mission Therapeutics, NeuroVive, Raptor Therapeutics, REATA Inc., RiboNova Inc., Standigm Therapeutics, and Stealth BioTherapeutics).

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Figure 1

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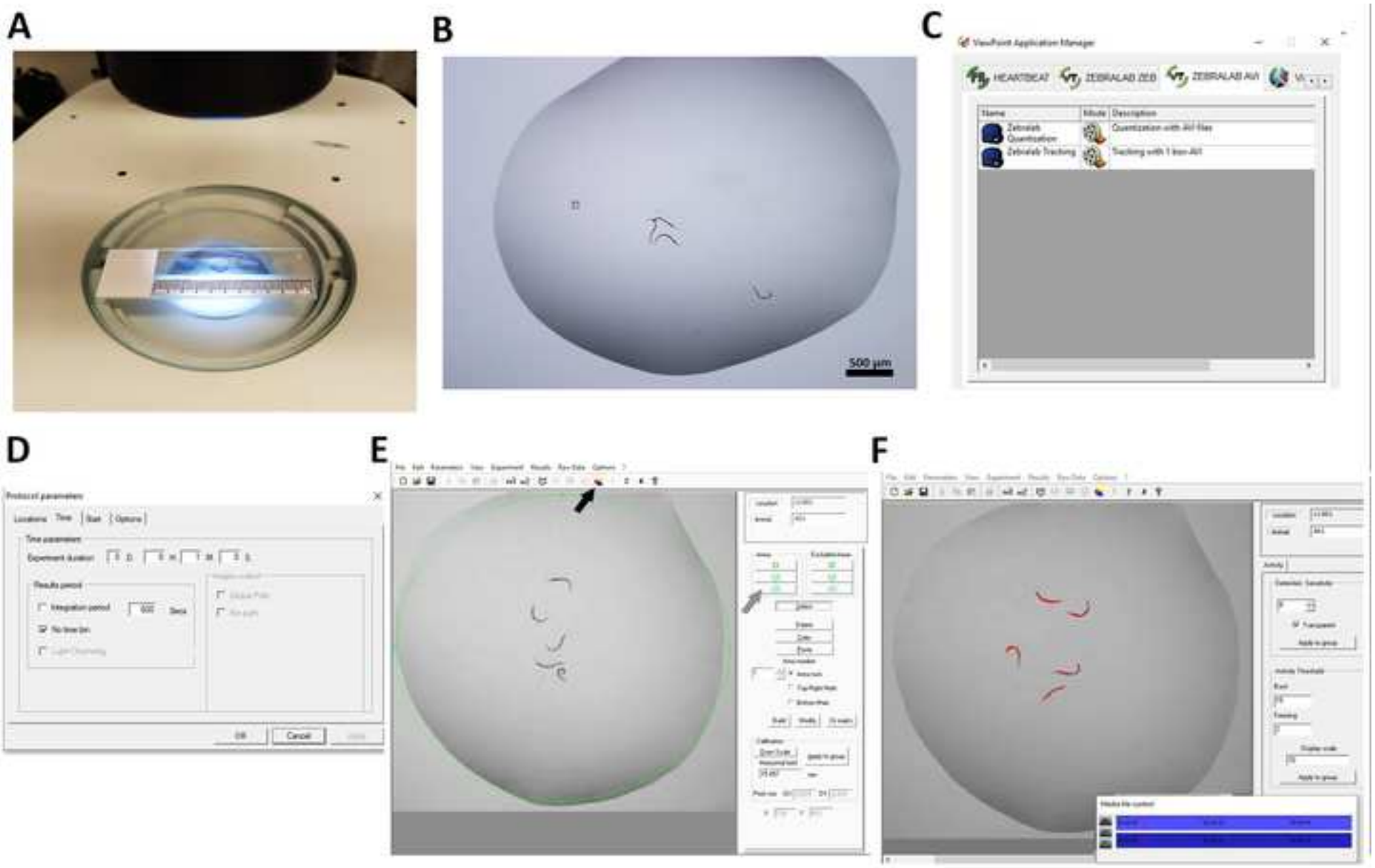

Figure 2

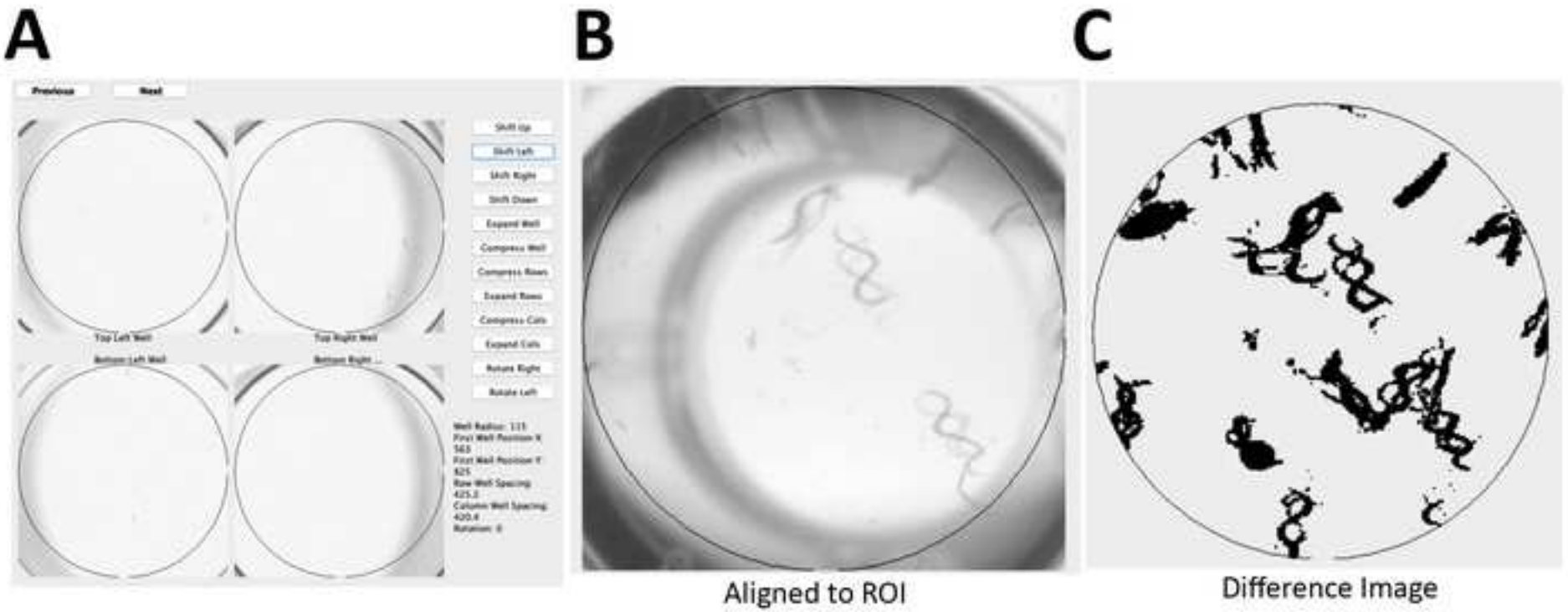

Figure 3

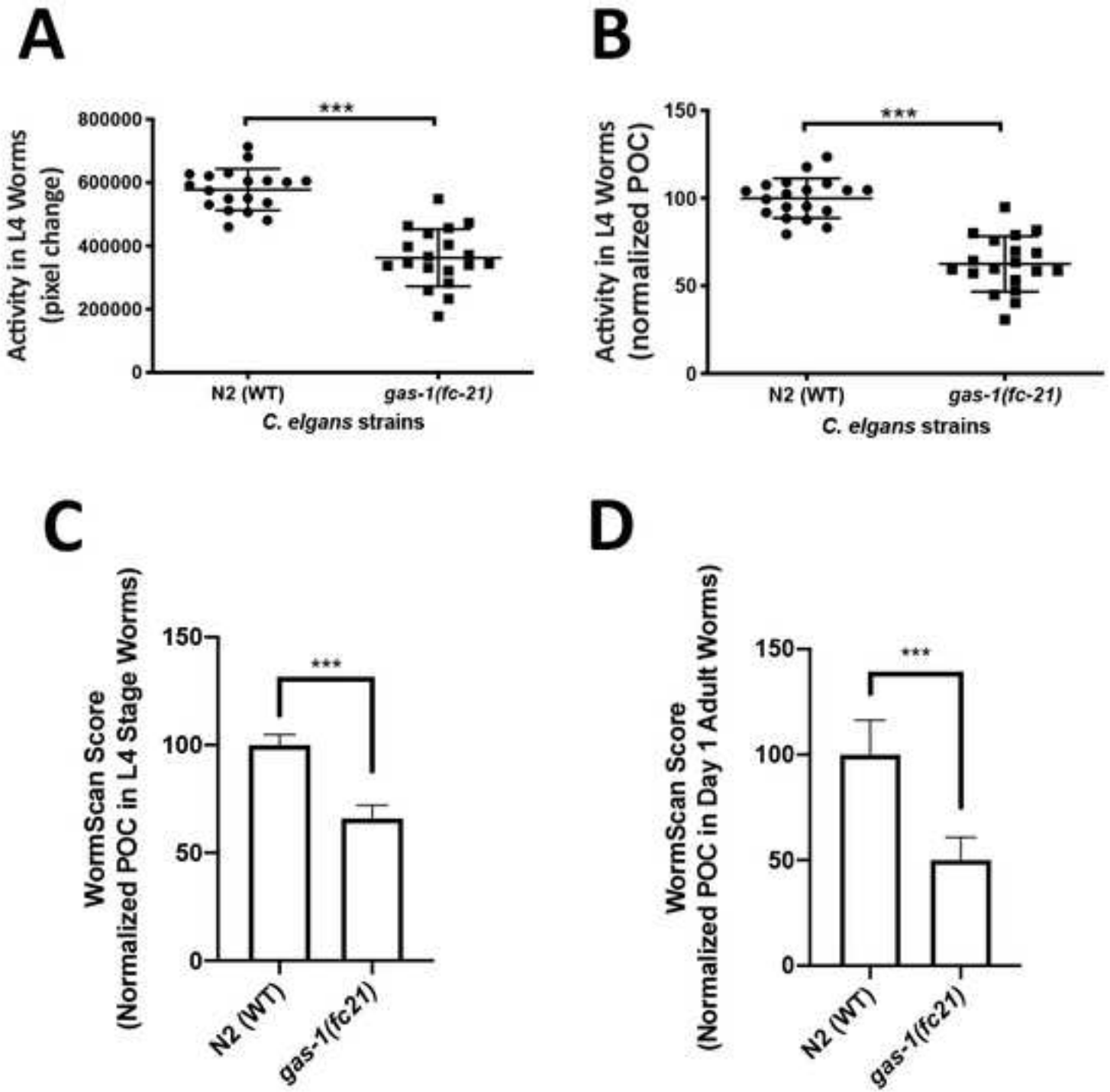

Table 1

ACTIVITY OUTCOME	ASSAY NAME	READ FORMAT			METHODOLOGY		THROUGHPUT			EQUIPMENT	ADVANTAGES	LIMITATIONS	REFERENCES
		NGM plate	Liquid culture	Multi-well plate	Imaging	Results	Low	Medium	High				
THRASHING	Manual		✓		Microscopy	• Manual analysis • Thrashing	✓			• Stereomicroscope	• Inexpensive	• User bias • Time consuming	19
	WrMTrcK		✓		Microscopy and video recording	• Body bends per minute • Length & Area	✓			• Stereomicroscope • Camera	• ImageJ plugin • up to 120 worms / 9 cm plate	• Issue with worms overlapping • One condition per plate	26
LOCOMOTION	Manual	✓			Microscopy and video recording	• Manual analysis • Velocity	✓			• Stereomicroscope • Camera	•Activity of individual worms	• User Bias • Time consuming • Tracks manual traced onto acetate sheets	20
	ZebraLab		✓		Microscopy and video recording	• Pixel change average		✓		• Stereomicroscope • Camera • Zebralab software (Viewpoint) • Microscope Slide	• Precise and quick analysis • Observe 5 worms / dropplet	•Software developed for zebrafish analysis	Novel application of software for *C. elegans* ; Software info at: http://www.viewpoint.fr/en/p/software/zebralab-zebrafish-behavior-screening
	Tierpsy Tracker	✓			Microscopy and video recording	• Velocity • Length & Area		✓		• Stereomicroscope • CCD Camera	• Observation of multiple objects at once • Locally calculated thershold • up to 300 worms / 9 cm plate	• One condition per plate • Processing time	28
	WF-NTP	✓			Microscopy and video recording	• Velocity • Length & Area		✓		•CCD Camera •Stand and LED Light	• Observe up to 5,000 worms / 9 cm plate	• One condition per plate	27
	WorMotel			✓	Video recording and pixel changes	• Pixel change average		✓		• CMOS camera and lens • Stand and LED Light • Polydimethylsiloxane (PDMS) 240-well plate	•Each worm is isolated in an individual well • Activity of a worm can be tracked over a period of days	• Due to the nature of PDMS plates cannot be reused for compound screening due to diffusion between wells	29
	WormWatcher			✓	Video recording and pixel changes	• Pixel change average per well			✓	• WormWatcher device	• Observe up to 80 24-well plates a day • Activity of 50 worms / well	• Activity is per well not indivudal worm	In Press
	WormLab	✓			Microscopy and video recording	• Velocity • Length & Area • Body bending angles • Reversal direction		✓		• Stereomicroscope • Camera • WormLab Software	• Observe up to 120 worms / 9 cm plate • Software can work with current system	• Expensive software and equipment	31
	Infinity chip		✓		Microfluidic and video recording	• Velocity • Length & Area		✓		•Infinity chip •Infinity processor	• Observe up to 70 worms / chip • Activity over time without progeny blocking drugs	• One condition per chip • Requires custom microfluidic chips	32
	WormScan			✓	Sequental scans and pixel change	• Pixel change average			✓	•Flatbed Scanner • Stereomicroscope	• 15 worms / well in 96-well plate • Previously used for High-Throughput Drug screening	• Activity only measurable between scans	48 and 49
	WMicrotracker ONE			✓	Infrared light (IR) microbeam	• IR light average change			✓	• Wmitotracker ONE	• up to 70 worms / well in 96-well plate • Previously used for High-Throughput Drug screening	• Only measures changes in infrared light	33
PHARNGEAL PUMPING	Manual	✓			Microscopy	Manual analysis	✓			• Stereomicroscope	• Inexpensive • Well-established	• User bias • Time consuming	17 and 21
	WormSpa		✓		Microfluidic and video recording	MATLAB scripts		✓		• Microfluidic device • Steromicroscope • Camera	• Inexpensive • high sensitivity	• Due to the nature of PDMS plates cannot be reused for compound screening due to diffusion	40 and 41
	ScreenChip		✓		Microfludic EPGs	• EPG average	✓			• The ScreenChip System (In vivo Biosystems)	• High sensitivity	• Single worm analysis at a time	45
CHEMOTAXIS	Manual	✓			Microscopy	Manual analysis	✓			• Stereomicroscope	• Well-characterized method	• User bias	46
	Multi-Worm Tracker*	✓			Microscopy and video recording	• Velocity • Length & Area • Distance from atractant or replant		✓		• Stereomicroscope • CCD Camera	• up to 120 worms per 9 cm plate	• No software support	47

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
C. elegans wild isolate	Caenorhabditis Genetics Center (CGC)	N2 Bristol	
Camera	Olympus	DP73	
gas-1(fc-21)	CGC	CW152	
Microscope slides	ThermoFisher	4951PLUS	
Nematode Growth Medium (NGM)	Research Products Internationa	N81800-1000.0	
OP50 *Escherichia coli*	CGC		Uracil auxotroph *E. coli* strain
Petri dishes (60 mm)	VWR international	25373-085	
S. Basal	VWR 5.85 g NaCl, 1 g K2 HPO4, 6 g KH2PO4, and 5 mg cholesterol, in 1 l H2O	VWR 101175-162, 103467-156, EM1.09828.1000, 97061-660	
Scanner	EPSON	V800	
Stereomicroscope	Olympus	MVX10 microscope	
96-well flat bottom	VWR international	29442-056	
WormScan software	Mathew et al. [45]	S1 Standalone Java platform	Software for automation of difference image of scanned plates
ZebraLab software	ViewPoint		Software for automated quantization and tracking of zebrafish behavior, designed by ViewPoint (http://www.viewpoint.fr/en/p/software/zebralab-zebrafish-behavior-screening) and here applied to *C. elegans* . This system is applicable for high-throughput behavioral analysis

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Author Response to Reviewer Critiques

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- **We have carefully proofread the manuscript and corrected the spelling and grammar throughout.**

2. The summary is exceeding the limit of 50 words. Hence, please rephrase the summary to clearly describe the protocol and its applications in complete sentences between 10-50 words. E.g. “Here, we present a protocol to …”

- **The summary has now been rephrased to fit within the 50 word limit.**

3. Please define all abbreviations before use. E.g. OXPHOS? (line 158).

- **This has been corrected in the manuscript, with the OXPHOS definition of ‘oxidative phosphorylation’ now clarified.**

4. Line 181: Pick the worms with?

- **This has been corrected to read, ‘using a worm pick’.**

5. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

- **We have now highlighted the essential steps of the protocol in the manuscript**

6. Please do not use “&” preceding the name of the last author in the references. The Jove reference style is: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).].

- **We made these corrections in the References.**

7. Please remove the color formatting in Table 1 and change the font sizes to improve its readability.

- **These changes have been made.**

8. Please sort the Materials Table alphabetically by the name of the material.

- **These changes have been made.**

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript provides comprehensive comparison of 16 different activity analysis methods for C. elegans. Although it needs a little more elaboration on the details, I think it is appropriate for JOVE.

- **We appreciate the Reviewer's recognition of the value of our work and appropriateness for JOVE.**

Major Concerns:
It is concerning that the waiting time for the worms in each drop (ZebraLab) or each well (WormScan) could be quite different. In ZebraLab experiments, the worms in the first drop will not wait too long until they are imaged, but the worms in the last (the 19th) drop will wait at least 18 minutes (1 minute recording per drop x 18 drops) plus the time for picking worms. Also, S basal drops would evaporate as time goes by and the volume might not be 20 uL at the time of imaging. In WormScan experiments, the 15 worms in the first well should wait in the liquid medium for a very long time until the experimenter pick 15 worms for each well x 95 wells. It should be proved that the experiments are not affected by these issues and the results from the first well and the last (96th) well are identical.

- **Using the Zebralab assay, each worm is maintained in a petri dish containing nematode growth media (NGM) and spread with *Escherichia coli* OP50 right up until the time it needs to be transferred to the liquid drop. Worms do not sit in liquid drops while analyzing other worm replicates. Reviewer #1 is correct this would otherwise stress and desiccate them. This description has now been clarified in the manuscript protocol in line 403 follows: "Five worms are transferred from the petri dish containing NGM and *E. coli* OP50 to the liquid drop immediately before beginning recording of worms in that droplet, while the other worms are kept in the petri dish until the previous video has been completed. This will avoid worm damage that may occur from drying of the 20 µl drops (dry time ~15-20 minutes)."**

  To minimize variation between analysis of animals in the first and last wells of a 96-well plate when manually picking 15 worms per well, it is best to wait 20 minutes before scanning to allow all worms to acclimate to liquid NGM. This step has now been added to the manuscript in line 336.

It would be nice if you could be more specific in what you mean by low/medium/high throughput. Criteria for low/medium/high throughput would be helpful. The total times for setting up and performing the ZebraLab and WormScan experiments should be provided. Then, it would be easier to understand why ZebraLab is medium-throughput and WormScan is high-throughput.

- **The general meaning of low/medium/high throughput is explained in the Table 1 legend and also now specifically addressed in the Introduction: "The throughput capacity of each assay is described as low, medium, or high based on the experimental protocol complexity including worm maintenance, processing time, the use of single or multi-well plates, and/or experimenter time needed to complete the experimental setting and data analyses." See at line 111.**

  Additional discussion of the meaning of medium vs high-throughput delineation for the Zebralab method and the WormScan method was also added to the Discussion: "The ZebraLab software approach is considered as a medium-throughput assay since it requires that single plates will be used for each condition studied, without current developed protocol for multi-well plate formats. While using the ZebraLab software approach requires minimal time when analyzing *C. elegans* activity in a few conditions, the experimental time increases when applied to multiple conditions. Here, the experimental time was approximately 2 hours to transfer worms into liquid droplets and record videos of their activity, considering 18 technical replicates for each condition. The time spent for the analysis of these videos using the ZebraLab software was approximately one hour. By comparison, the WormScan method is high-throughput because it incorporates a multi-well plate format that permits concurrent analysis of four 96-well plates in less than 10 minutes and setting up a 96-well plate with a COPAS Bisoter is also less than 10 minutes.

For locomotion analysis, it would be nice to include Yemini EI et al., Nat. Meth. (2013). Also, for pharyngeal

pumping, there is an automated pharyngeal pumping analysis method based on image analysis only. It captures the details of single pumping events, even without EPG (Lee KS et al., Nat. Comm. (2017).

- **Yemini EI et al., Nat. Meth. (2013) was added in the Introduction.**

We added the suggested reference and the original source used by Lee et al, 2017: Kopito RB, Levine E. Durable spatiotemporal surveillance of Caenorhabditis elegans response to environmental cues. Lab Chip. 2014 Feb 21;14(4):764-70. doi: 10.1039/c3lc51061a. PMID: 24336777. The reference was added in Table 1 and in the Introduction, together with a sentence regarding the WormSpa.

The figure quality needs to be significantly improved.

- **Figure quality has now been improved.**

Minor Concerns:

1. The alignments in Table 1 needs to be fixed.

- **This issue has now been corrected.**

2. In Table 1, it was confusing why imaging "less than" 120 worms / plate is an advantage.

- **"Less than" has been replaced with "up to".**

3. In Table of Materials, cell alignments needs some work to improve the readability. Ex) S Basal

- **We modified the cell alignments in the table.**

4. ln 97 - ImageJ plugin or plug-in?

- **'Plug in' was corrected to 'plugin'.**

5. ln 138 and onward - referring appropriate figures earlier would help the readers.

- **We have referenced the figures earlier, as suggested.**

6. ln 157 - the protein is not NDUFS2, but its Ce homologue. It could be confusing.

- **This point has been clarified.**

7. ln 164 - How about commenting on the merit of ZebraLab, as you did for WormScan?

- **We have now explicitly discussed the merit of ZebraLab, as follows: "ZebraLab software allowed rapid quantitation of several concurrent conditions of worm locomotor activity in *C. elegans* mitochondrial disease models, with potential application for targeted drug screening or validation studies"**

8. ln 182 - More information on how to perform drug treatments would be useful.

- **The following sentence was added: "When used to evaluate drug treatment effects, the desired drug stock concentration is prepared in S. basal solution and calculated to spread a specific volume onto the NGM plates and allowed to dry. Worms can then be transferred at a specific larval or adult stage, and maintained on the drug treatment plate for the desired duration before analysis"**

9. ln 187 - How long does it take for a single 20 uL drop of S basal evaporate? How quickly should one complete picking 5 worms?

- **These points have been clarified, as follows: "Five worms are transferred from the petri dish containing NGM and *E. coli* OP50 to the liquid drop only at the moment of the recording, while the other worms will be still maintained on the petri dish until the previous video has been taken. This will avoid that the 20 µl drops will dry during the procedure (dry time ~15-20 minutes) causing worms' damage."**

10. ln 209 - "Experiment duration" field?

- **This field was changed to "window".**

11. ln 210 - Select or deselect "No time bin": What does it do?

- **Time bin is the time over which the activity will be averaged. This information was added in the protocol section.**

12. ln 218 - the green circle icon under "Areas" - adding an arrow for this icon in the figure would be more specific. It was hard to find this icon, maybe because of the low resolution of the figures.

- **A grey arrow has now been added, as suggested.**

13. ln 250 - t-test performed with Prism?

- **This has been corrected.**

14. ln 320 - websites? . .one be picking worms?

- **'Web sites' has been replaced with 'websites'. However, we were not able to locate 'one be picking worms' phrase in the text.**.

15. ln 369 - in "a" single worm: "a" is italic.

- **This correction has been made.**

Reviewer #2:

Manuscript Summary:

Lavorato present a review on current research methods that quantify nematode behaviour, and evaluates the advantages, limitations and technical requirements of each method. Additionally, the work focuses on two semi-automated analysis programs and validates these two systems (Zebralab and WormScan) in an experiment that showed that worms with the gas-1(fc21) mutation have reduced locomotion. This reduced locomotion worsens with age. Overall, the work provides good description for replicating worm locomotion experiments with Zebralab and WormScan - the methodology was described very well.

- **We appreciate the Reviewer's recognition of the value and high quality of our work and appropriateness for JOVE.**

Major Concerns:

Table 1 - There are several errors in this table for a tracker I am familiar with- the Multi-Worm Tracker is incorrectly portrayed- it assesses many aspects of basal locomotion well and should be included in the top category as well as being used for chemotaxis and responses to stimuli (see McDiarmid et al 2020 PNAS). In addition, in Swierczek et al 2011 it states "we verified that the system robustly tracks swimming worms and fruit fly larvae in addition to crawling worms", thus the Multi-Worm tracker can score swimming behavior. The Multi-Worm Tracker does not require a dissecting microscope. The large number of errors for one technique suggest the authors might want to re-review the literature and the WormBook chapter on the other techniques to ensure there are not additional errors in the descriptions of other trackers as well.

- **We appreciate this careful review and have now amended the table to make these corrections.**

Minor Concerns:

1. As the two methods described here quantify predominantly worm locomotion some parts of the intro, especially on other modalities of worm behaviour or muscular function (such as pharyngeal pumping) appear superfluous. Instead more elaboration on tracking systems that can be used to quantify locomotion, and how it has been applied throughout the landscape of research is suggested.

- **Our intention was to describe the strengths and weakness of a wide range of activity methodologies commonly used to evaluate neuromuscular activity. This is the reason why pharyngal pumping is included as a relevant outcome in the paper.**

2. L66: "…high homology of most genes between C. elegans and humans." - perhaps specify with a rough estimate of proportion? When you say most, do you mean 80% of all human genes have C. elegans orthologs? 50%? Also, although the original Ortholist work is cited, the updated Ortholist 2 should also be referenced (PMID: 30120140).

- **The additional reference have now been added, as suggested. Clarification in the Introduction has been made that all human genes have *C. elegans* have ~80% orthologs**

3. The Wormbook chapter on worm trackers (PMID: 23436808) would be very useful and should be referenced!

- **We regret our original error in this citation and this has been corrected to include the reference by Husson et al. 2013.**

4. L77: "…aspects of animal activity" - Here and elsewhere throughout the manuscript, the word "activity" is used, but "worm activity" is not very descriptive. Perhaps narrowing down on a more appropriate descriptor will help readers understand more the scope of the methodologies you cover. Worm behaviour? Worm locomotion? Worm metabolic activity?

- **'Worm activity' has been replaced with more specific terms of 'worm behavior' and 'worm locomotor activity'.**

5. Table1/L105-106: the MultiWorm Tracker is mentioned later as a method with capacity for quantifying chemotaxis, but it is not mentioned as a method for quantifying locomotor activity at baseline.

- **This has now been addressed in Table 1 and its Table Legend, as follows: "*, Indicates that the methodologies can also be used for evaluation of locomotion."**

6. L124: (InVivo Biosystems).

- **This change has been made.**

7. L154: "… well-established mitochondrial complex I disease strain" Perhaps, "model" would be a better word, than strain? i.e. "well-established C. elegans model of mitochondrial complex I disease."

- **This change has been made.**

8. L160: "… gas-1(fc21) locomotor activity…"- as though it is the locomotor activity of an impaired gene. Rather, "locomotor activity of gas-1(fc21) mutants"?

- **This change has been made.**

9. Figure 2B/C: both of these panels are difference images - it is not clear what the difference is between the two panels. Perhaps re-write the caption so the reader has a better idea what each panel is?

Figure 2B is not a difference image, it is an alignment of the image to the region of interest. We have corrected the figure to added 'Aligned to ROI'. The protocol and Figure 2C have been corrected to clarify this shows the difference image .

10. L371: "We highlighted two novel…" Zebralab and WormScan are not novel - these are novel applications and/or adaptations, but the underlying methodology is not novel.

- **This has been corrected to read, "Among these, we highlighted two novel adaptations and applications of semi-automated analyses…."**

11. L377-378: This statement was already refuted in the introduction, as there are automated, high-throughput method that can quantify abnormalities in movement path and speed.

- **We appreciate this point and have amended the sentence to read, "To increase experimental throughput, automated and high-throughput methods should be selected."**

12. L380-381: This passage implies that an automated quantification system is available for tracking worm locomotion in liquid media but not solid media. This is not true! Please see behavioral characterization of over 135 strains in McDiarmed et al 2020 PNAS.

- **This point is well-taken and has now been corrected.**