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Automated behavioural analysis of large C. elegans populations using a wide field of view tracking platform

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Dear Dr Braiman,

Please find enclosed our manuscript entitled " *Automated behavioural analysis of large C. elegans populations using a wide field of view tracking platform"* that we would like to be considered for publication in Journal of Visualized Experiments.

The nematode worm *C. elegans* is a widely used model organism in biomedical research, as with its simplicity and versatility it is an effective system to study a variety of aspects of a wide range of human diseases. A number of automated platforms have been proposed recently for screening worm phenotypes but have largely focused on monitoring the behaviour of single or limited amounts of worms. Because of these limitations, high throughput multi-parameter platforms, giving access to screening for genes or potential drugs, have remained out of reach.

To address these limitations, we recently developed a Wide Field-of-View Nematode Tracking Platform (WF-NTP) (Perni et al. J. Neurosci. Methods 2018, in press) and successfully applied it to drug and antibody discovery. This platform can be used to study the behaviour of more than 5000 animals in parallel and to report metrics of more than a million worms every week. The unprecedented level of throughput provided by this method allows statistical errors to be very significantly reduced, enabling previously undetectable phenotypic differences to be discovered and opening the concrete possibility of carrying out drug discovery in worms.

The platform has already been successfully used into drug discovery for Alzheimer's disease (AD) (Habchi et al, 2016,2017) and Parkinson's disease (PD) (Perni et al., 2017a, 2018 in press). Furthermore the development platform allowed also to set novel protocols to evaluate directly the effects of the administration of antibodies in worms (Aprile et al, 2017, Perni et al., 2017b). Remarkably, one compound identified with this method, squalamine, has being tested in stage 2a clinical trials for PD (Zasloff et al, 2018 in Preparation).

We anticipate that the greatly enhanced ability to detect weak phenotypic differences by using of this platform will have widespread applications in the thousands of laboratories that carry out *C. elegans* research worldwide. The full open source availability of the code and platform, combined with the protocol described in the attached manuscript, will also make it readily accessible to the community.

We suggest the following experts as potential reviewers:

Richard Morimoto, Northwestern University, Chicago, USA.
Franz-Ulrich Hartl, Max Planck Institute of Biochemistry, Munich, Germany.
Judith Frydman, Stanford University, Palo Alto, USA.

Perni M, Casford S and Aprile FAA designed the procedures described in the manuscript, performed the experiments and analyzed the data. Perni M, Casford S and Aprile FAA, Nollen EAA, Knowles TPJ, Vendruscolo M, Dobson CM were involved in the design of the study and wrote the manuscript.

During the preparation and submission of this manuscript, we have been kindly assisted by Dr Jaydev Upponi.

We very much hope that you will find this manuscript to be of interest to the readers of *JovE* and look forward to hearing from you.

Tuomas Knowles, Michele Vendruscolo and Chris Dobson

TITLE:

Automated Behavioral Analysis of Large *C. Elegans* Populations Using a Wide Field-of-view Tracking Platform

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KEYWORDS:

Drug discovery, phenotype-based screening, high-throughput screening, *C. elegans,* amyloid formation, Alzheimer's disease, neurodegeneration, nematode library, large population analysis

SUMMARY:

We describe protocols for using the wide field-of-view nematode tracking platform (WF-NTP), which enables high-throughput phenotypic characterization of large populations of *Caenorhabditis elegans*. These protocols can be used to characterize subtle behavioral changes in mutant strains or in response to pharmacological treatment in a highly scalable fashion.

ABSTRACT:

Caenorhabditis elegans is a well-established animal model in biomedical research, widely employed in functional genomics and ageing studies. To assess the health and fitness of the animals under study, one typically relies on motility readouts, such as the measurement of the number of body bends or the speed of movement. These measurements usually involve manual counting, making it challenging to obtain good statistical significance, as time and labor constraints often limit the number of animals in each experiment to 25 or less. Since high statistical power is necessary to obtain reproducible results and limit false positive and negative results when weak phenotypic effects are investigated, efforts have recently been made to develop automated protocols focused on increasing the sensitivity of motility detection and

multi-parametric behavioral profiling**.** In order to extend the limit of detection to the level needed to capture the small phenotypic changes that are often crucial in genetic studies and drug discovery, we describe here a technological development that enables the study of up to 5000 individual animals simultaneously, increasing the statistical power of the measurements by about 1000-fold compared to manual assays and about 100-fold compared to other available automated methods.

INTRODUCTION:

Approximately half a century ago, Sydney Brenner introduced *Caenorhabditis elegans* (*C. elegans*) as a model system to study development and neurobiology, as this small (1 mm in length), transparent nematode worm is easy to manipulate genetically and to maintain in the laboratory[1]. Today, *C. elegans* is used to study a wide variety of biological processes including apoptosis, cell signaling, the nature of the cell cycle, gene regulation, metabolism, and ageing[2]. Furthermore, the cellular and tissue complexity, protein expression patterns and the conservation of disease pathways between *C. elegans* and higher organisms (80% of worm genes have a human orthologue), linked with the simplicity and cost-effectiveness of cultivation, make it an effective *in vivo* model organism amenable to high-throughput genetic[3-13] and drug[14-16] screenings. For all these reasons, *C. elegans* has been employed for the characterization of normal and disease-related molecular pathways; in the field of neurodegeneration, for example, it has enabled the exploration of the effects of ageing on protein aggregation[3,4,7,15,17,18], and the characterization of promoters and inhibitors of protein aggregation[3-7,14,18].

The overall fitness of the worms, which is an important behavioral parameter to be defined in this type of study, can be measured manually in a variety of ways, such as by counting the number of body bends per minute (BPM)[4,6,19], or by measuring the speed of movement[20-22], as well as by measuring the average lifespan and rates of paralysis. Although manual measurements of body bends and speed of movement have led to many important insights into a variety of molecular pathways and mechanisms[3,4,14,19,20,23], manual assays remain currently low-throughput, highly labor-intensive, and time-consuming whilst being prone to errors and to human biases. These issues present considerable challenges in the collection of data with sufficient statistical power to distinguish subtle behavioral changes. This limitation is particularly relevant for drug screening as treatments with potential drug molecules often lead to small phenotypic changes[24], therefore requiring the study of large numbers of animals in order to acquire reproducible results. To illustrate this point, recent studies have shown that a high power of detection (POD) is necessary to define with confidence any significant changes in behavior and to limit false positive results[25]. This has resulted in a strong motivation in the *C. elegans* community to replace manual counting with reproducible, automated, time- and cost-effective measurements. To meet this demand, several laboratories have recently developed methods for high-sensitivity measurements and accurate worm tracking of larger number of worms[22,26-33].

In order to expand further the process of automation to the large cohorts of animals needed for statistically significant measurements, we have recently developed a wide field-of-view

nematode tracking platform (WF-NTP)[15,34-36], which enables the simultaneous investigation of multiple phenotypic readouts on very large worm populations, a key factor in statistically relevant phenotypical detection[25]. Not only can the WF-NTP currently monitor up to 5000 animals in parallel, but the phenotypic readouts also include multiple parameters, including the rate and amplitude of body bends, the speed of movement, the fraction of the population that is paralyzed, and the size of the animals. It is therefore readily possible to screen thousands of worms in parallel and to combine the different readouts into a behavioral map to create a multidimensional fingerprint[36]. The associated open-source software is written in Python, which is also required to operate it and is completely customizable. A graphical user interface (GUI) is also provided to enable users to adopt this technology.

Here, we present a series of protocols that illustrate some of the potential applications of the WF-NTP. In particular, we discuss the administration of compounds, ranging from small molecules to protein therapeutics, and describe how to screen their effects directly over large populations of worms, thus effectively removing the need to sample small sub-populations. The use of the WF-NTP for such a purpose has already brought significant advantages in procedures aimed to design drug discovery programs of Alzheimer's disease (AD)[15,34,35] and Parkinson's disease (PD)[18] using *in vivo* data for the assessment of therapeutic candidates[35,37].

PROTOCOL:

1. Preparation of Materials for *C. Elegans* Protocols

1.1. To prepare a 10x M9 buffer solution, add 30 g of monobasic potassium phosphate, 60 g of dibasic sodium phosphate, and 50 g of sodium chloride to a 1 L autoclavable bottle.

1.1.1. Add 1 L of purified water and autoclave at *ca.* 121 °C for 15 min.

1.1.2. Dilute 10 times in purified water and autoclave at *ca.* 121 °C for 15 min.

1.1.3. When cooled, add 1 mL of a solution of 1 M magnesium sulphate.

Note: Only 1x M9 (M9) should be used for worm handling in the following steps.

1.2. To prepare the nematode growth medium (Rich-NGM), mix 2.7 g of sodium chloride, 15.75 g of agar, 5.75 g of casein, 900 mL of purified water, and autoclave at 121 °C for 15 min.

1.2.1. Transfer the Rich-NGM medium to a warming oven (*ca.* 70 °C) if not used immediately; the Rich-NGM may be kept molten at *ca.* 70 °C for up to 12 h before becoming unusable.

1.2.2. To the Rich-NGM mix, add 900 µL of a solution of 1 M calcium chloride, 900 µL of a solution of 1 M magnesium sulphate, and 900 µL of a solution of 5 mg/mL cholesterol in absolute ethanol.

Note: Cholesterol does not require autoclaving prior to use.

1.2.3. To prepare the experimental plates that maintain an age synchronous population, add 92 µL of 5-fluoro-2'-deoxyuridine (FUDR) at 812 µM to 900 mL of Rich-NGM.

Note: FUDR is both toxic and carcinogenic, so it is essential to wear nitrile gloves in addition to lab coats and safety glasses. Do not allow waste to enter general waste stream and dispose of by incineration with an afterburner.

1.2.4. Pour 20 mL of the autoclaved Rich-NGM into a 9 cm sterile Petri dish to generate a growth plate (Rich-NGM plate). Pour 15 mL of the autoclaved Rich-NGM into a 9 cm sterile Petri dish to make a motility plate (Mot plate). Pour 20 mL of Rich-NGM with FUDR into a 9 cm sterile Petri dish to generate an FUDR plate.

Note: The low agar volume of the Mot plate facilitates the camera focus and worm tracking (see step 3.2.5).

1.3. Prepare the OP50 *Escherichia coli* strain, by autoclaving 1 L of LB broth at 121 °C for 15 min and inoculate with starter culture when cooled.

1.3.1. Allow the bacterial culture to grow overnight at 37 °C and sterilize the tube to be used for centrifugation.

1.3.2. Transfer the bacteria to sterile tubes and centrifuge at 4600 x g for 15 min.

1.3.3. Decant the supernatant and, using sterile water, suspend in 10% of the original volume to create 10x (10 times concentrated) OP50 stock.

1.3.4. Dilute 15 mL of the 10x OP50 stock to 150 mL to generate 1x OP50, using sterile water, before being used to seed Rich-NGM plates. Use the remaining 10x solution to seed FUDR plates.

Note: The Mot plates will remain unseeded and will be used only in the image acquisition step.

1.4. To seed plates with bacteria, segregate the Rich-NGM and FUDR plates.

1.4.1. Under sterile conditions, add 350 µL of 1x OP50 to Rich-NGM plates and spread evenly.

Note: Do not spread to the edge of the plate as worms will crawl up the sides of the plate and die.

1.4.2. Under sterile conditions, add 350 µL of 10x OP50 to FUDR plates and spread evenly.

1.4.3. Allow the plates to dry and incubate at 20 °C for 2 d before use.

2. Preparation of *C. elegans* for Use with the WF-NTP

2.1. Maintain *C. elegans* on Rich-NGM plates or by transferring a small amount of agar containing worms face down onto a fresh bacterial layer. Maintain the *C. elegans* strains in this manner prior to initiating the experimental protocols.

2.2. Using the transferring technique (see step 2.1), seed 20 plates of each strain using well-fed, mixed stage worms and allow them to grow for 2 d at 20 °C.

2.3. Wash off 5 plates containing worms using 15 mL of M9 and pool in one 15 mL tube. Repeat for all 20 plates of each strain.

2.4. Centrifuge the worms at 2000 x g for 2 min, remove the supernatant, and suspend in 2 mL of M9 solution.

2.5. Prepare 10 mL of bleaching solution by mixing 13% sodium hypochlorite and 4 M sodium hydroxide at a ratio of 3:2 vol/vol.

Note: This solution does not require autoclaving. Both components and the resulting solution are corrosive and should be handled with care using nitrile gloves. Sodium hydroxide is mildly corrosive to glass and should be stored in plastic containers.

2.6. Add 1 mL of bleaching solution to each centrifuge tube and shake vigorously for approximately 3.5 min or until the cuticle has fully dissolved.

2.7. Under sterile conditions, dilute the samples to 15 mL M9 and centrifuge at 2000 x g for 2 min. Remove the supernatant and suspend in 15 mL of M9 solution.

2.8. Repeat step 2.7 6 times, until only worm eggs remain, and the solution no longer has an odor of chlorine.

2.9. Centrifuge the samples at 2000 x g for 2 min, remove the supernatant, and suspend in 2 mL of M9 solution.

2.10. Under sterile conditions, use a sterile glass pipette to transfer 2 mL of M9 containing eggs into each well of a 12-well tissue culture plate and incubate at 20 °C for a minimum of 16 h.

Note: Use separate plates for each strain to prevent cross contamination. The transferring step from the centrifuge tubes to the multi-wells helps to avoid worm suffocation.

2.11. Transfer the worms hatched overnight from the 12-well plates into 15 mL centrifuge tubes. Take 3 drops of 5 µL of worm solution and count the number of worms present at first larval stage (L1).

2.12. Calculate the number of worms per µL of solution.

2.13. Seed L1 worms onto Rich-NGM plates at 3000 worms per plate and allow them to grow for 2 d at 20 °C, until they reach last larval stage (L4) stage.

3. General WF-NTP Protocol

3.1. Add 2.2 mL of each drug compound at the appropriate concentration (such as 25 or 10 µM for drugs like squalamine[18] and bexarotene[15], respectively) to 6 FUDR plates and allow to dry under sterile conditions for each desired worm strain.

Note: This step is repeated for each compound under examination, for each concentration and each solvent used.

3.1.1. Wash the worms off 5 Rich-NGM plates prepared at step 2.13 using 15 mL of M9 buffer and transfer the worms to a centrifuge tube.

3.1.2. Centrifuge at 2000 x g for 2 min, remove supernatant, and suspend in 3 mL of M9. Take 3 drops of 5 µL of the M9 solution containing worms and count the number of worms present at the last larval stage L4.

3.1.3. Calculate the number of worms per µL of M9 buffer.

3.2. Seed 700 L4 larvae onto 6 of the dry FUDR plates containing a given compound, repeat for each concentration of each compound and allow to dry under sterile conditions.

3.2.1. Incubate the worms at 24 °C from L4 for those strains where the worm paralysis is induced only by raising the temperature, such as the AD strain[38]. Count the day following the L4 larval stage as D0 of adulthood.

3.3. Turn on the stage lights for the tracker.

3.3.1. Clean the glass stage with 70% ethanol and ensure that no visible residue remains, then remove lens cap. Clean the imaging lens using an air duster. Ensure that the camera is plugged in correctly and installed and start recording with the image capture software.

3.3.2. Adjust the camera settings to record 20 frames per second, with mono 16 recording parameters setup; record 1200 frames, saving in MJPEG format at a 95% compression rate. Use an empty 9 cm Petri dish to ensure that the stage is set to the correct height and adjust it if necessary.

Note: Ensure that the edges of the plate are visible in the recording as this will allow the 'keep-dead' algorithm to work correctly (**Figure 1a**).

3.3.3. Under sterile conditions, take 2 plates previously prepared at step 3.2 with the same conditions and 1 Mot screening plate prepared in step 1.2.4. Add 3 mL of the M9 solution to the Mot plate. Use other 2 mL of M9 to wash 1/3 of the surface area of 2 plates prepared at step 3.2 onto the Mot plate.

3.3.4. Place the Mot plate containing approximately 5 mL of the M9 solution and approximately 600 worms onto the tracker stage. Focus the camera using an individual worm and begin recording.

3.3.5. When the recording is complete, discard the Mot plate with the worms; mark the FUDR plates as being used once and return it to the incubator.

3.3.6. Repeat steps 3.3.3 to 3.3.5 for each experimental condition.

3.3.7. Repeat steps 3.3.3 to 3.3.6 for each time point in the adult lifespan.

Note: The screening procedures can be carried out at any point of the worm's adult lifespan (*ca.* 3 weeks). These protocols facilitate screening up to 9 time points; the authors recommend screening every 2 d starting from day 1 of adulthood.

4. Optimization for Discrete Dose Studies

4.1. Prepare worms by repeating steps 3.1.1 to 3.1.3.

4.1.1. Seed *ca.* 1000 L4 worms onto 5 FUDR plates. Incubate the plates at 24 °C until D3 of adulthood, for experiments involving AD[38] worms.

4.2. Using a sterile glass pipette, transfer the worms prepared under sterile conditions from the plate to a 15 mL centrifuge tube, centrifuge at 2000 x g for 2 min and remove the supernatant.

4.2.1. Suspend the solution of worms in 1 mL of M9 buffer. Transfer the worms to a microcentrifuge tube, centrifuge at 300 x g for 2 min, and remove the supernatant.

4.3. For protein transduction, add 40 µL of the transfection delivery reagent and 20 µL of native protein at the desired concentration (typically 40 µM), and incubate at room temperature for 30 min.

4.3.1. Under sterile conditions, use a sterile glass pipette to transfer worms from step 4.2.1 into a microcentrifuge tube containing encapsulated protein to give a total volume of 1060 µL. Place tubes horizontally and shake at 60 rpm in a 24 °C shaking incubator for 8 h.

4.4. Under sterile conditions, add 4 mL of M9 solution to a Mot plate and transfer one microcentrifuge tube of worms plus drug onto the Mot plate.

4.4.1. Set up the tracker by repeating steps 3.3 to 3.3.2.

4.4.2. Under sterile conditions, add 4 mL of M9 solution to a Mot plate and transfer one microcentrifuge tube of worms plus drug onto the Mot plate.

4.4.3. Place the Mot plate onto the tracker stage, focus the camera on an individual worm, and begin recording. When complete, label the Mot plate and set to one side.

4.4.4. Repeat steps 4.4.1 and 4.4.3 for all samples.

4.4.5. Using a glass pipette, transfer worms under sterile conditions from the Mot plate to a 15 mL centrifuge tube, and centrifuge at 2000 x g for 2 min. Remove supernatant, transfer the pellet onto an FUDR plate, and allow to dry under sterile conditions.

4.4.6. Repeat step 4.4.5 for all samples.

4.5. Incubate the worms at 24 °C until D6 of adulthood.

Note: Multiple antibody incubations are also possible.

4.6. Add 3 mL of the M9 buffer to the Mot plate and use 2 mL of M9 solution to wash all the worms from an FUDR plate onto the Mot plate.

4.6.1. Set up the tracker by repeating steps 3.3 to 3.3.2.

4.6.2. Place the Mot plate onto the tracker stage, focus the camera as necessary, and begin recording. When the recording is complete, discard the Mot plate or recover it for further screening if desired.

4.6.3. Repeat steps 4.6 to 4.6.2 for all samples.

5. Analyzing the Video Data

5.1. After acquiring the videos, select the appropriate parameters in the software GUI (**Figure 2**) for data analysis.

5.1.1. Load single or multiple videos *via* the **Browsing** function. Select an output destination folder. Ensure that 600 to 1200 frames are used for a 30 s analysis.

Note: Any range of frames can be analyzed.

5.1.2. Insert a pixel to mm conversion factor, which will be 0.029 for imaging a 9 cm Petri dishes at full resolution. Select the tracking algorithm 'keep dead'.

Note: The conversion factor will depend on the field of view used. **Z-transform** algorithm can be also used as an alternative.

5.1.3. Fill in the parameters in the **Locating** section (**Figure 2**): **Z use images** = 9, **Z padding** = 3, **Std pixels** = 54, **Threshold** = 9, **Opening** = 1, **Closing** = 2. Adjust the **Filtering** parameters to **Minimum size** = 20, **Maximum size** = 180, **Worm-like** = 0.94.

5.1.4. Tune the **Forming trajectories** parameters: **Maximum distance move** = 10; **Minimum length** = 150, **Memory** = 10.

5.1.5. Set **Bends and Velocity** parameters. Use 1.8 for **Bend threshold**, 0 for **Minimum bends**, and 150 for **Frames to estimate velocity**.

5.1.6. Tune the **Dead worm statistics**. Use 5.0 for **Maximum beat per minute** and 1.0 for **Maximum velocity**.

5.1.7. Choose the output folder. Set the number of **Output frames** to 50. Set **Font size** to 10.

Note: Optionally, select one or more **Regions of interest** (ROIs).

5.2. Test the parameters by using the **Example** function. Output the example images and check that the worms are visible throughout the 8 thresholding steps (**Figure 1**).

5.3. Use the **Start job** function.

5.4. Analyze the text result files by combining the individual results for the whole dataset. Use the **Export to tsv** function in the GUI.

Note: Optionally, individual metrics of the worms can also be considered for population studies.

5.4.1. Analyze data with a statistical software package.

REPRESENTATIVE RESULTS:

The ease of operating, multiparametric analysis, and high throughput of the illustrated WF-NTP protocol (**Figures 1 and 2**) makes it possible to determine very small changes in worm behavior in a very accurate manner. The imaging platform is based on custom-made opto-mechanics,

and it can be assembled using a 6 MP monochrome camera combined with 16 mm focal length high-resolution lens for 1'' sensor, illuminated with a 8'' by 8'' white backlight (see **Table of Materials** and also Ref[36] for additional details). The associated WF-NTP software is written in Python and was developed to run on Windows platforms. It runs on a custom assembled computer with 3.00 GHz octa-core processor and 64 GB of random-access memory (RAM). The software was also designed to parallelize the work and video analysis based on the RAM and CPU of the computer; *i.e.,* a machine with a lower calculation power will result in less videos run in parallel. The setup we are currently using is optimized to run up to *ca.* 16 videos in parallel and can complete an analysis of *ca.* 100 videos overnight. Moreover, the high level of detail of customization provided in the GUI of the WF-NTP allows great control of the quality of the imaging analysis. The GUI can be used to directly upload large datasets in parallel or individual videos; specific frames can also be selected for detailed sub-analysis, together with a pixel conversion factor, which can be used to estimate behavioral metrics. One of the two different tracking algorithms (keep dead and Z-transform) can be chosen depending on whether or not the user would like to consider paralyzed animals in the analysis. The thresholding parameter can be tuned accordingly with the video and experimental quality. The opening and closing parameters allow the user to remove the noise and further implement the thresholding functionality. The skeletonizing algorithm offers an alternative method of analysis. The object size cut-offs (filtering) provide an additional filter for background noise and the worm-like parameter allows the user to consider worms only objects with an ellipsoidal shape, hence distinguishing from other objects that may have the same pixel size of the worms. After these thresholding operations, all the resulting labelled regions can be identified as individual worms and the positions of those regions are then stored for each frame for subsequent analysis and tracking. The eccentricity of each tracked worm is used to estimate the worm metrics such as the extent of worm bending (BPM) as a function of time. The users are also allowed to select the number of frames used to keep a worm in the memory of the software following collisions, and the number of pixels that a worm can move between frames can also be tuned to distinguish the animals from the noise. The minimum track length option allows the user to discard worms that have been tracked for only a few frames. Other key parameters, such as bends and velocity, allow the user to select the degree of bending necessary to be counted as a body bend and the number of frames considered to be needed to estimate the speed of the animals. Cut-off parameters can be further tuned for the inclusion of paralyzed animals. The output is automatically shown in the result files. These values are considered as upper limits for the evaluation of the fraction of paralyzed animals. The user can also select one or more regions of interest. This feature is particularly useful to analyze subpopulations of the worms and the output is sorted automatically in the results files. The output option allows the user to select the output folder and the number of tracking frames that will be produced for it. Various tool sets can also be used for further data analysis, such as the plot path tool that shows individual worm tracks and the fingerprinting tool that allows the user to create fingerprint maps.

This methodology enables new approaches to be adopted, not only for biological studies of *C. elegans* but also for pharmacological and medical research purposes, such as high-throughput screening of genetic modifications and drug treatments. We have illustrated this potential by

describing the characterization of the phenotypes for large population studies (N > 1000) of various worm models of neurodegenerative disease (frontotemporal dementia (FTD)[39], Parkinson's disease (PD)[7], Alzheimer's disease (AD)[16,40], and amyotrophic lateral sclerosis (ALS)[19] (**Figure 3a**), and characterizing the effects of potential therapeutic molecules using worm models of PD[18] and AD[15,12] (**Figure 3b**). Two small molecules, squalamine[18] and bexarotene[15], were administered at concentrations up to 25 µM to PD (**Figure 3b**) and 10 µM to AD[38] (**Figure 3b**) worms, respectively. Both compounds showed clear dose-dependent effects over the range of concentrations tested. We have shown that this high accuracy of the measurements is achieved by increasing the number of worms that can be analyzed compared to traditional methods (**Figure 3c**). We illustrated the importance of the sample size (**Figure 3c**) in molecular screening as well as in characterization of mutant strains. The increase in temperature from 20 °C leads to approximately half of the AD worms becoming paralyzed after 3 days of adulthood. Worm populations were screened under different conditions, *e.g.,* when worms over-expressing the 42-residue form of the amyloid-β peptide ($A\beta_{1-42}$) (AD worms) were exposed to subtle temperature variations (**Figure 3c**, left panel), when $A\beta_{1-42}$ was expressed in all the neurons (**Figure 3c**, central panel), or when AD worms were exposed to bexarotene (**Figure 3c**, right panel). Worms were also analyzed from small ROIs randomly selected from the full field of view of the videos acquired with the WF-NTP (N < 50, yellow bars) highlighting the comparison of the motility of these worms with the average motility of the whole worm population (N < 1000). In all the panels, the difference measured on the whole worm population appears to be statistically significant with $p \leq 0.0001$ (****).

The WF-NTP protocol described here also allows the simultaneous recording of multiple parameters (**Figure 1b**) to support, in an optimal manner, both internal validation and the development of a comprehensive fingerprint of a wide range of conditions relative to a control sample, allowing for meaningful comparisons across multiple studies. This multi-parametric approach includes the simultaneous analysis of multiple behavioral features, including bending frequency, speed, paralysis rate, size, bend amplitude, and bend displacement[36]. This allows thousands of animals to be monitored in great detail and at a very high sensitivity and statistical significance and provides opportunities for large populations studies. This tracking procedure also has the advantage of allowing paralysis studies to be performed in parallel with other behavioral measurements, a key feature in molecular screening studies.

The results that have been achieved so far in AD[15,34,35] and PD[18] drug discovery demonstrate the importance of wide field-of-view data acquisition to greatly increase the numbers of animals that can be monitored in a single experiment, significantly decreasing the experimental errors and greatly improving the statistical validity of the studies. Based on these results, we anticipate that the WF-NTP protocol, which we made readily available for the community[36], will significantly extend the use of *C. elegans*.

FIGURE LEGENDS:

Figure 1. WF-NTP analysis steps and example of a fingerprint. (**a**) 1. Original video. 2. Background image. 3. Background subtracted image. 4-6. Thresholding steps. 7-9. Single

labelling of worms. (**b**) Multiple phenotypes are reported with a fingerprint for wild-type worms and worm models of PD and AD.

Figure 2. **Graphical user interface (GUI) of the WF-NTP.** Specific videos and selected frames can be selected in the GUI for analysis, and a pixel conversion factor can be inserted, after which the analysis is carried out with one of the two given tracking algorithms. It is possible to select the degree of bending necessary to count as a body bend as well as the number of frames needed to estimate the speed of the animals. Body bends and speed thresholds can determine the paralyzed worm statistics.

Figure 3. Examples of applications enabled by the WF-NTP method. (**a**) Application of WF-NTP in large population studies (N > 1000) of BPM measurements for *C. elegans* models of a range of neurodegenerative diseases including FTD, PD, AD, and ALS. (**b**) Application of WF-NTP in drug discovery. (**c**) Importance of the population studies in temperature sensitivity, drug efficacy, and mutant strain characterization. Phenotypes of subpopulations N < 50 (yellow bars) are compared with those of the whole worm population (N < 1000). The error bars indicate the standard error of the mean (SEM).

DISCUSSION:

Because of the rapid expansion of techniques within the field of optical sciences, it is now possible to address the requirement for automated methods in *C. elegans* studies in substantially new ways. As a result, a number of digital tracking platforms[20,41-46] have been designed and made available over the last few years in order to replace manual counting of parameters such as speed of movement, bending frequency, paralysis rate, as well as more complex forms of behaviors such as omega turns, and lifespan measurements. Most recent automated platforms have greatly improved the reproducibility and sensitivity of *C. elegans* studies[41] and provided high quality data on small cohorts or even individual animals. We decided to extend the automation of the analysis of worm behavior to make it also possible to evaluate the phenotypes of cohorts of thousands of animals in parallel. The main advantage of the approach of studying worm cohorts is that it allows for accounting for the high intrinsic variability of worm behavior[24] and for the fact that drug treatment studies often lead to subtle phenotypic variations, which are difficult to detect with sufficient statistical significance when using a small group of animals. A high power of detection (POD) is indeed necessary to detect with confidence any significant variation in behavior and to limit false positive results[25].

Here, we have described a series of protocols based on a recently reported automated screening method for *C. elegans*, the wide field-of-view nematode tracking platform (WF-NTP)[36]. The protocol described here is divided into 5 parts. Parts 1 and 2 describe the preparation of large worm populations. Critical steps are the sterility of the working conditions and preparation of reagents and plates necessary to run the experiments. We note that, due to the increased throughput provided by this protocol compared to other screening methodologies[36], it also requires increased quantities of reagents; this factor needs to be considered carefully in the experimental design. We also note that the bleaching step is critical and needs to be tested in advance as a large number of eggs and healthy larvae are necessary

to run these experiments. Part 3 of this protocol details how to deliver drugs in solid media and screen worm populations. We note that this part of the protocol is strongly dependent on the number of drugs and drug concentrations to be tested by the user in parallel. The complete automation of the screening procedure and rapid data acquisition shift the limiting step from behavioral observation to reagent preparation and growth and synchronization of large worm populations. The key steps during the behavioral screening are the timings of the recording and any worm handling steps (*e.g.*, the transfer of worms from the NGM plates to the tracking platform). The protocol described here is an example designed to screen the worms for up to 9 days during the adult lifespan; however, this protocol can be easily adapted to screen as many time points as the user desires, *e.g.*, 18 consecutive days[36]. Part 4 then illustrates the application of the protocol to deliver protein molecules (*e.g.*, antibodies and molecular chaperones) into *C. elegans*, and shows how the protocol illustrated in Parts 1-3 can be easily customized, depending on the desired application. We demonstrate how this procedure can be extended not only to the delivery of drug-like molecules but also for the administration of molecular chaperones or antibodies[37]. The first four steps (parts) are carried out under sterile conditions, unless noted otherwise. All liquid components should be autoclaved prior to use and the incubation steps should be performed at 70% relative humidity. In Part 5, we describe how to use the software package provided in combination with the tracking stage. This software has been custom designed for the analysis of WF-NTP data related to the behavior of large worm populations. We suggest that the user follows the guidelines provided in Part 5 for the data analysis; however, these parameters are dependent on the specific features of the recorded videos (*i.e.*, fps, field of view, video resolution, number of acquired frames). The example function provided in the GUI has been designed to facilitate the evaluation of the correct parameters prior to the analysis.

These series of protocols make it possible to analyze the phenotypes of large populations of *C. elegans* (currently up to 5000 individual worms in parallel) effectively, reducing artefacts due to the intrinsic variability of the behavior of the animals, in agreement with preliminary studies on the power of detection necessary to achieve statistical significance for studies of *C. elegans*[25]. The platform uses a system of high-resolution cameras, capable of recording images of large numbers of animals at a high speed, while simultaneously recording multiple large cohorts. The high performance and high throughput of the WF-NTP protocol makes it possible to determine very small changes in worm behavior in a very accurate manner. Therefore, this methodology enables new approaches to be considered not only for the study of the biology of *C. elegans*, but additionally for pharmacological and medical research, such as the high-throughput screening of genetic modifications and drug treatments. This procedure also has the advantage of allowing paralysis studies to be performed in parallel with other behavioral measurements, a key feature in molecular screening studies.

The results that have been achieved so far in the drug discovery programs of AD[15,34,35] and PD[18] demonstrate the importance of wide field-of-view data acquisition in substantially increasing the numbers of animals that can be monitored in a single experiment, thereby significantly decreasing the experimental errors and greatly improving the statistical validity of studies. While the current approach described in this protocol has focused on addressing challenges in

the field of drug discovery, we hope that the methodology will be widely adopted in the community, and that its application will be extended to complex genetic and behavioral studies and expand the number of phenotypes that are currently detectable.

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DISCLOSURES:

The authors declare that there are no conflicts of interest.

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Figure 1

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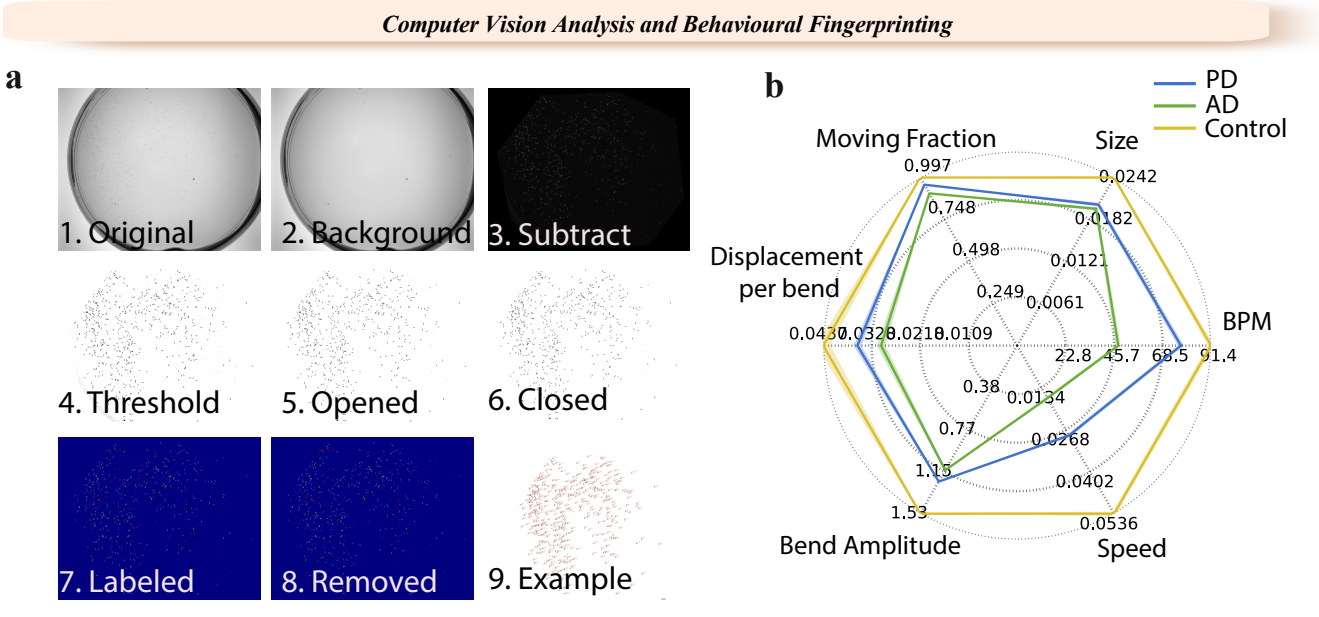

Figure 2

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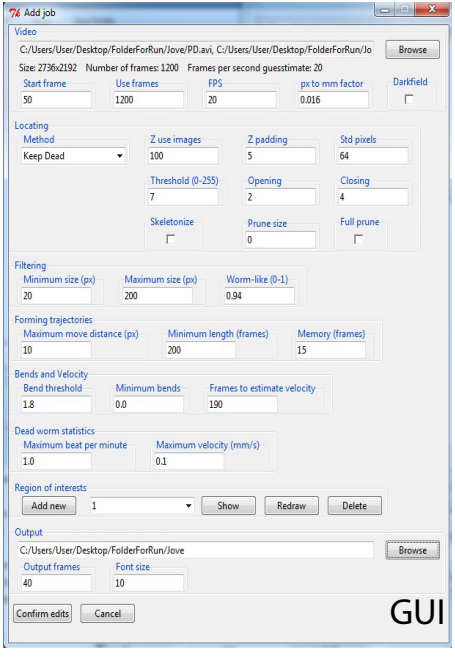
Graphical User Interface (GUI) and Analysis Tools



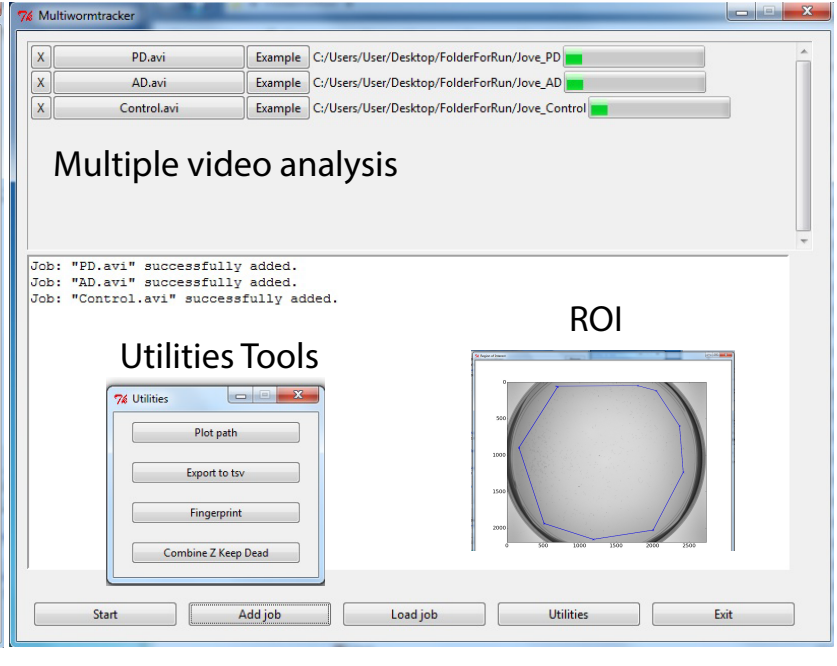


Figure 3

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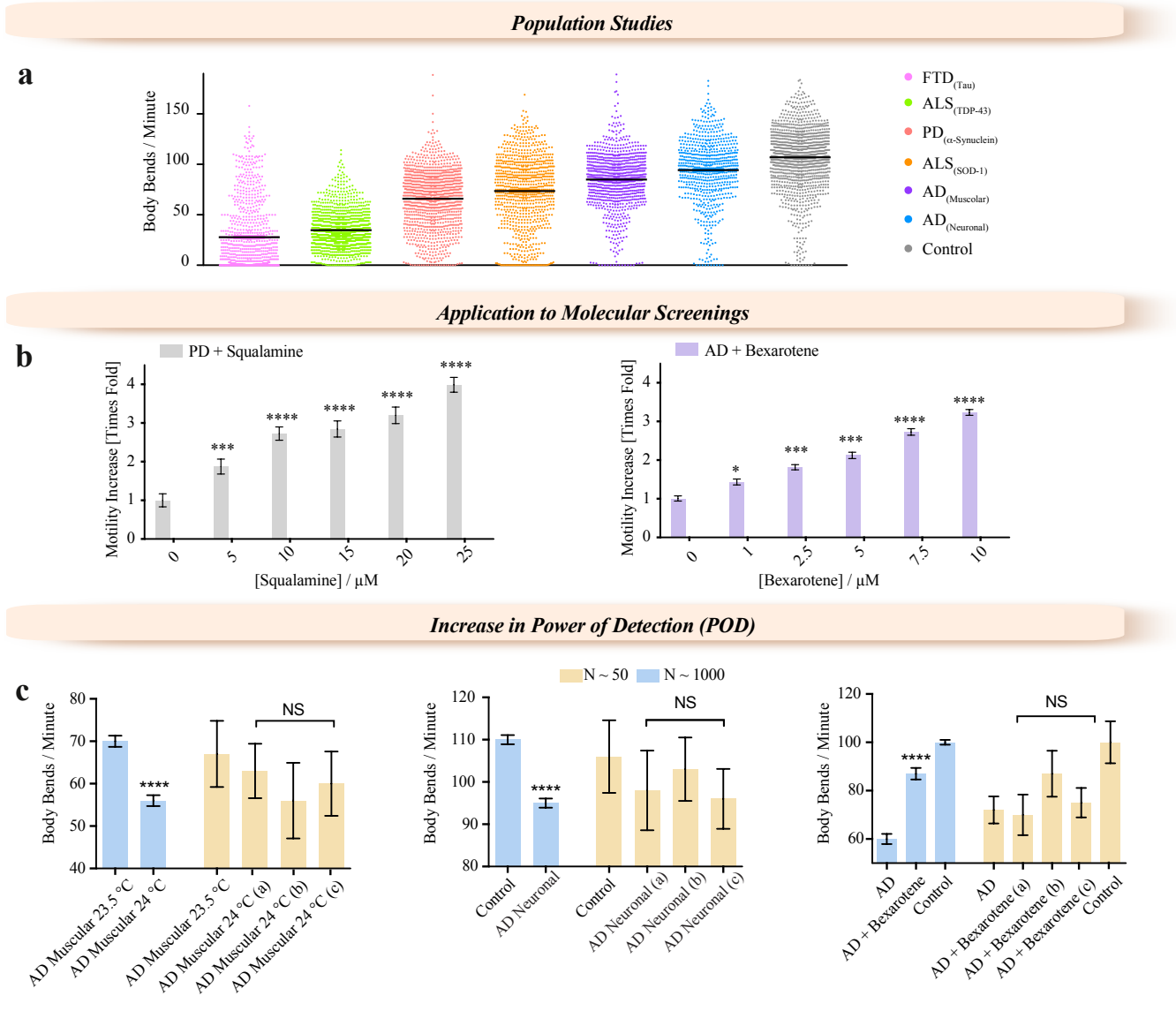

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Consumable reagents			
monobasic potassium phosphate	Sigma Aldrich	P0662	
dibasic sodium phosphate	Sigma Aldrich	S3264	
sodium chloride	Sigma Aldrich	13422	
magnesium sulphate	Sigma Aldrich	M7506	
Agar	Sigma Aldrich	A1296	
Difco casein digest	Scientific Laboratory Supplies	211610	
calcium chloride dihydrate	Sigma Aldrich	C3881	
cholesterol	Acros	110190250	
absolute ethanol	Vwr	20821.33	
5-Fluoro-2'-deoxyuridine 98%	Alfa Aesar	L16497.ME	
LB medium capsules	MP biomedicals	3002-031	
13% sodium hypochlorite	Acros Organics	AC219255000	
Sodium hydroxide	Fisher Chemical	S/4880/53	
Strains			
E coli strain OP50	Supplied by CGC	Op50	E coli strain
C. elegans strain wild type	Supplied by CGC	N2	C. elegans strain
C. elegans strain AD	Supplied by CGC	GMC101	C. elegans strain
C. elegans strain PD	Supplied by CGC	NL5901	C. elegans strain
C. elegans strain ALS	Supplied by CGC	AM725	C. elegans strain
C. elegans strain Tau	Supplied by CGC	BR5485	C. elegans strain
Equipment			
Tactrol 2 Autoclave	Priorclave		
9 cm sterile petri dishes.	Fisher Scientific	11309283	

2 L erlenmeyer flasks	Scientific Laboratory Supplies	FLA4036	
Nalgene 1 L Centrifuge pots	Fisher Scientific	3120-1000	
RC5C plus floor mounted centrifuge	Sorvall	9900884	
15 mL centrifuge tubes	Fisher Scientific	05-539-12	
Heraeus Multifuge X3R	Thermofisher scientific	75004515	
Inoculating Spreaders	Fisher Scientific	11821741	
M4 multipette	Eppendorf	4982000012	
P1000 pipette	Eppendorf Research Plus		
P200 pipette	Eppendorf Research Plus	3123000055	
P10 pipette	Eppendorf Research Plus	3123000020	
1000 µL low retention tips	Sarstedt		
300 µL low retention tips	Sarstedt	70.765.105	
10 µL low retention tips	Sarstedt	70.1130.105	
pipeteboy 2	VWR	612-0927	
50 mL serological pipette	Appleton Woods	CC117	
25 mL serological pipette	Appleton Woods	CC216	
10 mL serological pipette	Appleton Woods	CC214	
glass pipette 270 mm	Fisherbrand	FB50255	Camera for videos recordin
pulsin	Polyplus Transfection	501-04	Transduction reagent
Multitron Standard shaking incubator	Infors HT	INFO28573	
air duster	Office Depot	1511631	

WF-NTP Tracker Components and Image Capture Software

8'' by 8'' Backlight	Edmond Optics	88-508	Tracker component
16 mm FL high resolution lens for 1'' sensor	Edmond Optics	86-571	Tracker component
6 Mpx camera	Edmond Optics	33540	Tracker component
FlyCapture Software	PointGrey	SDK v2.12	Image capture software
WF-NTP Software	Cambridge Enterprise	v1.0	Image analysis software

Office Package	Microsoft	Office 365	Statistical analysis software

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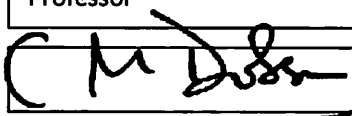
Signature:	
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Dear Editor,

Thank you for your comments on our resubmission.

We are really glad that you found our work of interest for the community and gave us such positive comments.

We have now implemented the manuscript as indicated by the new editorial guidelines and we hope you will find the finalised manuscript fully suitable for in JoVE.

Very best wishes,

Tuomas Knowles, Michele Vendruscolo and Chris Dobson

Editorial comments:

1. Please remove the titles from the uploaded Figures 1 and 3.

- **The titles have been removed**

2. please highlight only 2.75 pages or less 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.

- **Only less than 2.75 pages are now highligthed**

3. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

- **This has been revised and relevant steps are included**

4. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

- **This has been revised accordingly**

5. Table of Materials: Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

- **This has been revised accordingly**