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METHODS & TECHNIQUES

Quantitative characterization of planarian wild-type behavior as a platform for screening locomotion phenotypes

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SUMMARY

Changes in animal behavior resulting from genetic or chemical intervention are frequently used for phenotype characterizations. The majority of these studies are qualitative in nature, especially in systems that go beyond the classical model organisms. Here, we introduce a quantitative method to characterize behavior in the freshwater planarian *Schmidtea mediterranea*. Wild-type locomotion in confinement was quantified using a wide set of parameters, and the influences of intrinsic intra-worm *versus* inter-worm variability on our measurements was studied. We also examined the effect of substrate, confinement geometry and the interactions with the boundary on planarian behavior. The method is based on a simple experimental setup, using automated center-of-mass tracking and image analysis, making it an easily implemented alternative to current methods for screening planarian locomotion phenotypes. As a proof of principle, two drug-induced behavioral phenotypes were generated to show the capacity of this method.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/7/1063/DC1

Key words: locomotion, planarian tracking, *Schmidtea mediterranea*, behavioral analysis.

INTRODUCTION

The modern tools of molecular biology promise the ability to probe the molecular basis of complex behaviors in a wide range of systems. To realize this promise we must be able to characterize, ideally with quantitative rigor, the behavior itself, and manipulate individual molecular components, either through genetics or by chemical intervention. Beautiful work has been done in this respect for the nematode *Caenorhabditis elegans* by various groups (Pierce-Shimomura et al., 1999; Zhao et al., 2003; Tsibidis and Tavernarakis, 2007; Ramot et al., 2008; Stephens et al., 2008), but for less-prominent organisms the analysis of behavior remains largely qualitative. To address this, we present a method to rigorously characterize locomotion in the asexual freshwater planarian *Schmidtea mediterranea*.

Several genes involved in planarian locomotion have recently been identified by RNA interference (Inoue et al., 2004; Nishimura et al., 2007; Glazer et al., 2010). In addition, planarians are a popular model to understand learning, behavioral changes due to drug exposure or withdrawal, and general locomotion mechanisms (Jennings, 1906; Moore, 1919; Cole, 1926; Thompson and McConnell, 1955; Pavlova, 2000; Raffa et al., 2001; Hicks et al., 2006; Nishimura et al., 2007; Pagan et al., 2008). Despite this interest in planarian behavior, a quantitative method to assay planarian locomotion in detail is still lacking. To fill this gap, we designed a simple setup that can easily be implemented by any research lab, thus providing a viable alternative to currently used methods such as the planarian locomotor velocity (pLMV) method (Raffa et al., 2001). The pLMV method, although applied in a number of studies (Raffa et al., 2001; Raffa and Valdez, 2001; Pagan et al., 2006; Rawlsa et al., 2007), only quantifies speed based on the number of gridlines crossed in a given time interval, thereby rendering locomotion phenotypes with reduced mobility indistinguishable. We have executed the pLMV method in this study for wild-type (WT) planarians to allow for a direct comparison with our method.

The locomotion of freshwater planarians is characterized by a straight gliding motion, using primarily the beating of ventral cilia in a layer of mucus as a means of force generation (Pearl, 1903; Jennings, 1906; Trueman, 1975; Nishimura et al., 2007). Occasionally, planarians will lift their head to search for food or in response to obstacles, but these events are rare in a neutral environment. The straight gliding motion allows a minimal center-of-mass (COM) tracking approach because there is little additional information in the body shape. This is in contrast to nematode locomotion, which is muscular driven and displays characteristic body shapes (Stephens et al., 2008). Additional differences between nematodes and planarians motivated us to develop our own tracking system. Planarians are an order of magnitude larger than nematodes and they move significantly faster – Walter reports 1.1–1.5 mm s^{-1} for *Planaria maculata* and *P. gonocephala* (Walter, 1907) compared with ~0.2 mm s^{-1} for *C. elegans* (Pierce-Shimomura et al., 1999; Zhao et al., 2003) – while simultaneously being difficult to confine. Further, planarians are negatively phototactic (Walter, 1907; Inoue et al., 2004) and avoidant of open spaces.

We have overcome all of these challenges with an easy-to-implement tabletop experimental setup for planarian tracking. An interesting side effect of this setup was the frequent interactions with the container boundaries, which revealed additional behaviors and raised the question of the limitations of behavioral assays without environmental challenges.

As a proof of principle, we generated two locomotion phenotypes by drug exposure (sulpiride and chloretone) and show that a distinct characterization of these phenotypes is possible. Because our

method can easily be run in parallel using multiple setups, it could become a valuable tool for the creation of a database for locomotion phenotypes.

MATERIALS AND METHODS

Planarian maintenance

We used the asexual strain of *Schmidtea mediterranea* (Benazzi et al., 1975), a gift from A. Sánchez Alvarado (University of Utah, Salt Lake City, UT, USA). Planarians were stored in the dark at 20°C except during feeding, cleaning and recording.

Drug treatment

Sulpiride (Sigma-Aldrich, St Louis, MO, USA; #S8010) is water insoluble at the concentrations used. While soluble in dimethylsulfoxide (DMSO), DMSO itself can cause a locomotion phenotype (Pagan et al., 2006). Therefore, sulpiride solutions were made by adding 0.341 g sulpiride powder to 10 ml of 10% HCl (Schroeder and Packard, 2000), the pH was adjusted with 1 mmol l^{-1} NaOH to pH 7, and planaria water (Cebria and Newmark, 2005) was added to make a 10 mmol l^{-1} solution. We were unable to induce behavioral changes at the concentrations reported previously (Raffa et al., 2001), possibly because of the difference in strains, but *S. mediterranea* show a fully reversible phenotype at 10 mmol l^{-1}. Planarians were incubated in the sulpiride solution for at least 2 h before imaging (ST worms).

Chlotetone (#112054, Sigma-Aldrich, St Louis, MO, USA) was dissolved in planaria water to a concentration of 0.008%. Planarians were incubated in chloretone for 1–2 h prior to imaging (CT worms).

Data acquisition

Planarians were imaged at 5 frames s^{-1} with LabVIEW software (National Instruments, Austin, TX, USA) using a Basler A601f CCD camera (Basler AG, Ahrensburg, Germany) and a double Gauss lens (focus length 25 mm; Edmund Optics, Barrington, NJ, USA) mounted on a ring stand. Homogenous lighting was provided by an incandescent light source. For tracking, single worms were placed in square plastic containers (14×14 cm; up & up #11152081, Target, Minneapolis, MN, USA) filled with 45 ml of planaria water. Minimum intensity projections (MIPs) were constructed using ImageJ (US National Institutes of Health, Bethesda, MD, USA) by projecting the minimum pixel value for the entire image sequence at each pixel position onto a single frame. MIPs were used to review image sequences. Examination of the MIP planarian track allowed us to identify periods of stopping or swimming. Image sequences containing either of these events were excluded, because we were exclusively interested in the behavior of *S. mediterranea* when gliding on a substrate.

To test the effect of substrate composition, we used square containers of glass (13.5×13.5 cm; #3.87870.MB2.3.21.990, Bormioli Rocco, Brescia, Italy), plastic and a plastic container filled with 50 ml of 0.5% agarose (Ultrapure Agarose #$15510-027, Invitrogen, Carlsbad, CA, USA). The square glass container and a 14 cm diameter round glass container (#CZ91688, Anchor, East Dundee, IL, USA) were used to perform the assay on container shape.

pLMV method

Using the image calculator in ImageJ, an image of a grid with 1 cm spacing was averaged with the image sequence to overlay a grid on all images. Planarians were tracked manually across frames (3000 frames, 10 min sequence), and each time the worm crossed a gridline, the count was increased by one. The same process was repeated blindly without access to the previous counts to get an estimate of the count accuracy.

Planarian tracking and data analysis

Image processing and COM tracking was done using custom software written in MATLAB (version 7.6, MathWorks, Natick, MA, USA). The stand-alone executable file (Ptracker GUI) is available with example data and step-by-step documentation by e-mail request. Velocity autocorrelation (vac) was calculated for overlapping time lags (τ_j) as follows:

$$\mathrm{vac}_j = \frac{1}{N-j}\sum_{t=1+j}^{N}\left[\vec{\mathbf{v}}(t)-\bar{\mathbf{v}}\right]\cdot\left[\vec{\mathbf{v}}(t-j)-\bar{\mathbf{v}}\right], \quad (1)$$

where $\vec{\mathbf{v}}(t)$ is the velocity, $\bar{\mathbf{v}}$ (mm s^{-1}) is the mean velocity, j=1,2,...,N–2 is the time lag (τ_j) and N is the number of positions. The velocity autocorrelation data were fit with a simple exponential, $f(t)=ae^{-t/tp}$ (200 frames), to obtain the persistence time (t_p). Orientation correlations (oc) were calculated as:

$$\mathrm{oc}_j = \frac{1}{N-j}\sum_{t=1+j}^{N}\cos(\vartheta_i-\vartheta_{i-j}), \quad (2)$$

where $\vartheta=\tan^{-1}(\Delta y/\Delta x)$; $\Delta y=y_i-y_{i-j}$; $\Delta x=x_i-x_{i-j}$. The curvature [κ (mm^{-1})] of worm trajectories was calculated as:

$$\kappa = \frac{\left|\frac{dx}{ds}\frac{d^2y}{ds}-\frac{dy}{ds}\frac{d^2x}{ds}\right|}{\left(\frac{dx}{ds}^2+\frac{dy}{ds}^2\right)^{3/2}}, \quad (3)$$

where the first and second derivatives of x and y were determined by using a third-order Savitsky–Golay filter with a frame size of 20 positions (s) (Shaevitz and Fletcher, 2008). Curvature and speed *versus* time were then interpolated *versus* distance traveled using a third-order spline fit. Using a bandwidth filter based on identified head movements ('wiggles') and sharp turns in the raw data, areas of high curvature ($\Delta\kappa=\delta\kappa/\delta s>0.05$) were isolated and confirmed by checking the raw data in ImageJ. High-curvature areas with low speed (<0.1 mm s^{-1}) were found to correspond to head wiggles whereas the other high curvature areas with speeds >0.1 mm s^{-1} corresponded to sharp turns. Kruskal–Wallis tests were performed in MATLAB. Unless noted otherwise, data are presented as means ± s.d.

RESULTS AND DISCUSSION

The basis for a quantitative description of locomotion phenotypes is a rigorous characterization of WT behavior. To achieve this goal, we designed a simple experimental setup, composed of an incandescent light source, a Basler A601f CCD camera, an adjustable Gauss lens, a boom stand and an arena with shallow water (Fig. 1A). The trajectories of individual planarians were captured (N=15, 3000 frames, 5 frames s^{-1}; Fig. 1B) using a custom image-acquisition program in LabVIEW software. Next, the COM coordinates were determined (see Fig. 1C and supplementary material Movie 1) using a custom image-analysis routine with a graphical user interface in MATLAB (see Fig. 1D).

For a direct comparison with the pLMV method, we analyzed the number of gridlines crossed by the same (N=15) WT planarians over the course of 10 min (Fig. 2). On average, WT worms crossed a mean of 121±15 gridlines in 10 min. This results in a mean speed of ~2 mm s^{-1}, similar to values reported for other strains (Raffa et al., 2000).

It has been emphasized previously (Walter, 1907) that planarians are complex enough to exhibit a certain degree of individuality.

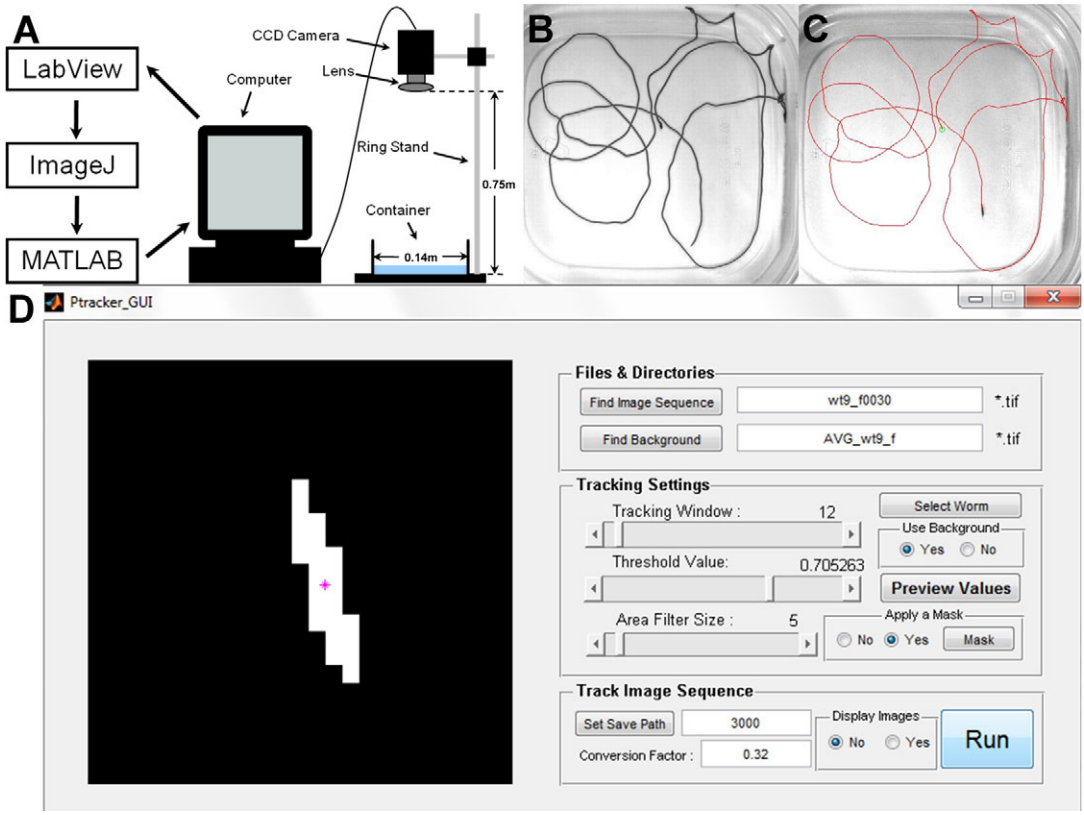

Fig. 1. (A) Schematic of the experimental setup. (B) The MIP displays the planarian track and (C) shows the same data after center-of-mass (COM) tracking in MATLAB, plotted on top of the original image. (D) Screen shot of the P-tracker GUI showing a processed image in the image viewer with the COM indicated by a magenta cross.

Thus, we studied the intrinsic intra-worm *versus* inter-worm variability by comparing three consecutive runs without break with three runs containing 12 h rest periods between runs. Consistent with the results of Trueman (Trueman, 1975), we did not find any significant intra-worm behavioral differences (Fig. 3A). However, the inter-worm variability in the measured speeds was noticeably larger (s.d.=0.23 mm s^{-1}; Fig. 3B).

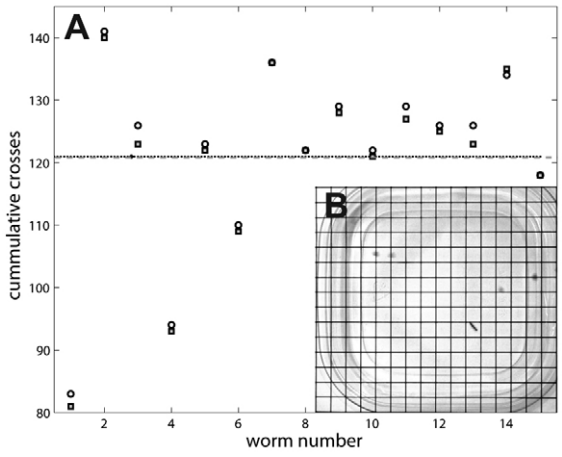

Fig. 2. (A) Cumulative number of gridline crosses in a 10 min time period for *N*=15 wild-type (WT) worms, counted twice manually (circles and squares). The horizontal dashed line represents the mean cumulative number of crosses. (B) Image of a planarian in a dish with overlaying grid (1 cm spacing).

Fig. 3C shows the speed histograms of all WT worms (*N*=15) analyzed. A time lag of τ=2 s was chosen to reduce the influence of tracking error. The mean instantaneous speed was 1.62±0.42 mm s^{-1} whereas the mode was 1.92 mm s^{-1}. This difference is due to the long tail at lower speeds caused by decelerations/accelerations associated with turns and boundary effects (see below). Although these results agree with the speed obtained by the pLMV method (~2 mm s^{-1}), they reveal more details (shape of the distribution).

Because mean speeds stayed constant over the 10 min imaging interval, except for small fluctuations in time due to stopping and turning (Fig. 3D), overlapping time lags (Li et al., 2008) can be used for calculating the velocity autocorrelation (Fig. 3E), which is a measure of the randomness of motion. The persistence time for WT motion is t_p=61±3 s, resulting in a persistence length approximately the length of the dish (14 cm diameter). Thus, a significantly larger container would be necessary to determine the 'true' persistence time; although this is an interesting avenue for future research, we focus here on the effects of confinement on planarian behavior instead.

Effect of confinement and spatial exploration

The angular (orientation) component of the trajectory allows us to characterize the amount of dish exploration over time (Fig. 3F,G). As expected from the MIPs, WT worms show a high orientation correlation. Because they frequently move in circles, their orientation correlation changes between correlated and anti-correlated periods, with a specific time of ~110 s corresponding to a half circle around the dish. Not all worms, however, showed this characteristic time scale (see Fig. 3G).

To determine the influence of container geometry, we compared the behavior of the same worm in different-shaped containers (i.e.

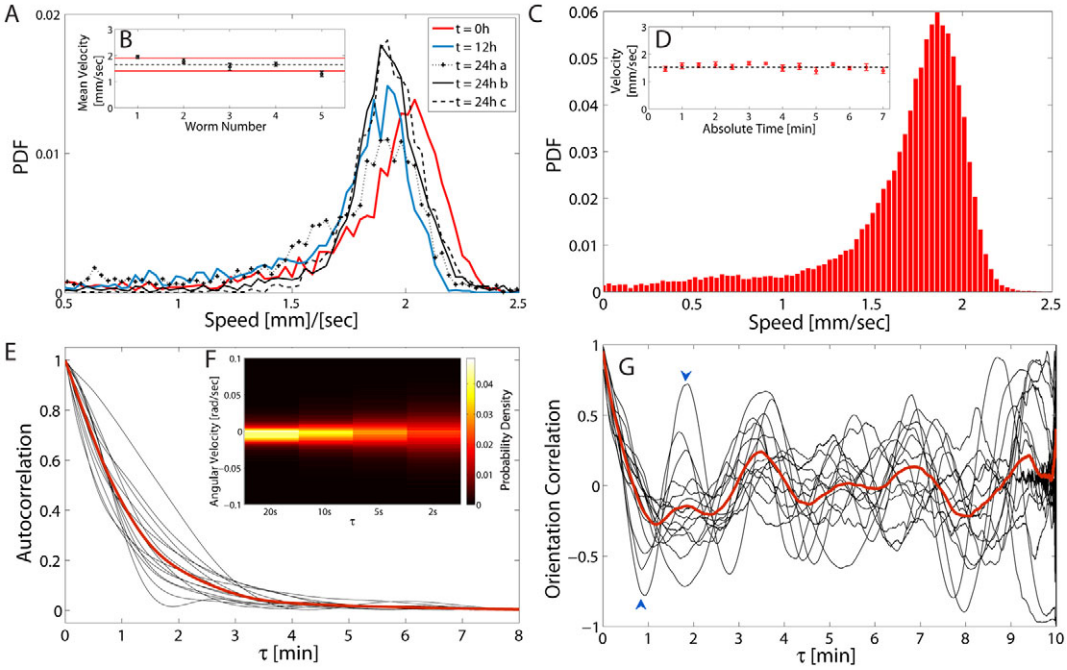

Fig. 3. (A) Speed histogram of an individual WT worm for consecutive and 12 h-spaced-out runs [time lag (τ) 2 s, *n*=5; a, b and c represent consecutive runs without break 24 h after the first run]. PDF, probability density function. (B) Distribution of mean speeds for worm repeats. Error bars (s.d.) show intra-worm variability, whereas the red lines indicate inter-worm variability (s.d.) about the population mean (black dotted line). *N*=5. (C) Speed histogram of WT worms (*N*=15; bin size 0.03 mm s^{-1}). (D) Mean speed over the course of a run of the worms shown in C. Means ± s.e.m. are indicated only every 30 s for clarity. (E) Velocity autocorrelation of individual WT worms (black lines); red line indicates the mean. (F) Angular velocity distribution of WT worms (*N*=15) for time lags of 2, 5, 10 and 20 s. (G) Orientation correlation of individual WT worms (black lines); red line indicates the mean. Blue arrows indicate semi- and full circles around the boundary of the container.

square *versus* circle). Additionally, the influence of substrate on behavior was tested by comparing different materials (plastic, glass and 0.5% agarose). None of the parameters showed any significant differences between container geometries or substrates (supplementary material Figs S1 and S2).

Comparison of drug-induced locomotion phenotypes *versus* WT behavior

We will now show that our method provides a detailed description of phenotype-specific behaviors by chemically inducing two different locomotion phenotypes: (1) the dopamine D2-receptor

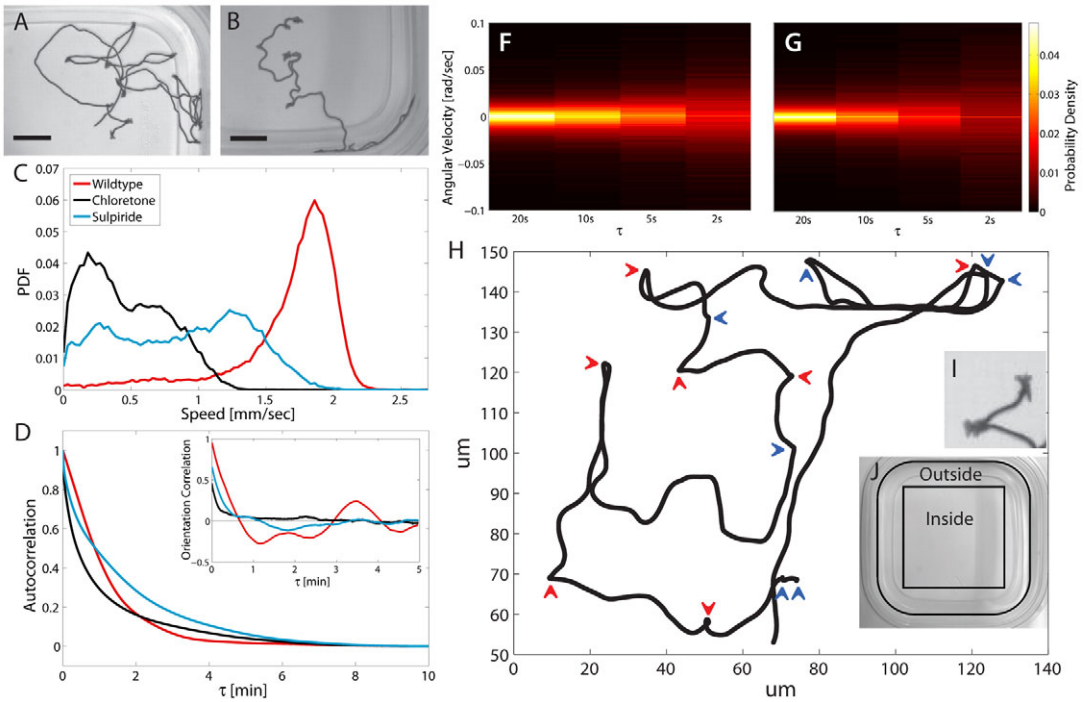

Fig. 4. Minimum intensity projections (MIPs) of an example track of an ST (A) and a CT (B) planarian. (C) Speed histograms of WT, ST and CT planarians. ST and CT planarians display a much broader speed profile due to penetrance variability, and a lower mean speed. (D) Velocity autocorrelation and (E) orientation correlation of WT, ST and CT planarians. (F,G) Angular velocity distribution (rad s^{-1}) for time lags 2, 5, 10 and 20 s for CT (F) and ST (G) planarians, with probability density represented by the color bar. (H) Exemplary COM track of an ST worm indicating sharp turns (red arrows) and head wiggles (blue arrows). (I) MIP example of two head wiggles. (J) Spatial designation of the 'inside' and 'outside' areas of the container.

Table 1. Summary of parameters

Parameter	WT	ST	CT
Speed (v; mm s^{-1})[a]	1.62±0.42	0.89±0.49	0.48±0.31
Persistence time (t_p; s)[b]	61±3	96±2	120±5
No. sharp turns[c]	5±1	6±1	9.3±1
No. head wiggles[c]	1.6±1	12.6±3	14±2
No. high curvature (κ) areas outside[c]	6.1±1	15.1±3	16.6±2
No. high κ areas inside[c]	1±1	3.4±1	6.7±1

CT, chloretone exposed; ST, sulpiride exposed; WT, wild type.
[a]Values are means ± s.d.
[b]Values are means ± 95% confidence intervals.
[c]Values are means ± s.e.m.

antagonist sulpiride [5-(aminosulfonyl)-N-[(1-ethyl-2-pyrrolidinyl)-methyl]-2-methoxybenzamide], a compound known to modulate locomotor behavior (Baik et al., 2002) and shown to alter behavior in planarians (Raffa et al., 2001); and (2) chloretone [1,1,1-trichloro-2-methyl-2-propanol], a known anesthetic (Buchanan, 1922), which is frequently used in planarian research.

Both ST and CT worms (Fig. 4A,B) moved less persistently and displayed more head wiggles (Fig. 4I, supplementary material Movies 2, 3) than WT worms. Quantification and comparison of the ST and CT locomotion phenotypes with WT behavior using the previously mentioned parameters revealed distinct differences (see Fig. 4, Table 1 and supplementary material Fig. S3). In contrast to the straight gliding motion of WT worms, ST and CT worms frequently crossed their own tracks and curved their bodies or wiggled their heads. To fully capture this difference, we extracted the instantaneous speed and curvature *versus* distance traveled. This allowed us to quantify the number of sharp turns ($\Delta\kappa>0.05$, see Materials and methods) and head wiggles ($\Delta\kappa>0.05$, $v<0.1$ mm s^{-1}; see Fig. 4I,H) performed by a worm in a given run over 10 min.

Although the mean number of sharp turns was similar between WT and ST worms ($P>0.4$), ST and CT worms showed a much higher tendency to wiggle their head ($P<0.01$; Table 1). Additionally, spatial distribution analysis of sharp turns and head wiggles revealed a significantly higher occurrence at the container edge for all worm types; this was less pronounced for the drug-induced phenotypes, as these generally show more high-curvature events: WT, ST and CT worms each had 88, 82 and 75% occurrences, respectively. These results indicate the importance of obstacles or challenges for the planarians to display characteristic behaviors, which would be absent in open spaces. Ideally, we would be able to study the behavior in environments as close to natural environments as possible, while still being able to control them and quantify behaviors.

CONCLUSIONS

We have developed a method to track planarians as they move across a substrate in shallow water and showed that a surprisingly rich, quantitative characterization of WT behavior is possible using a relatively simple experimental setup. We tested our method using concrete examples of chemically induced locomotion phenotypes, which current methods, such as pLMV (which studies worm speed in terms of number of gridlines crossed), cannot characterize specifically. Future work may combine this COM tracking with shape analysis because, in contrast to WT locomotion, mutants often display muscular-driven motion, which leads to specific changes in body shape.

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