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TITLE:

Planarian Scrunching as a Quantitative Behavioral Readout for Noxious Stimuli Sensing

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planarians, behavior, noxious, scrunching, peristalsis, TRPA1, AITC, cinnamaldehyde, UV, RNAi

SUMMARY:

Freshwater planarians exhibit three gaits (gliding, peristalsis, and scrunching) that are distinguishable by quantitative behavioral analysis. We describe a method to induce scrunching using various noxious stimuli, quantification thereof, and distinction from peristalsis and gliding. Using gene knockdown, we demonstrate the specificity of scrunching as a quantitative phenotypic readout.

ABSTRACT:

Freshwater planarians normally glide smoothly through ciliary propulsion on their ventral side. Certain environmental conditions, however, can induce musculature-driven forms of locomotion: peristalsis or scrunching. While peristalsis results from a ciliary defect, scrunching is independent of cilia function and is a specific response to certain stimuli, including amputation, noxious temperature, extreme pH, and ethanol. Thus, these two musculature-driven gaits are mechanistically distinct. However, they can be difficult to distinguish qualitatively. Here, we provide a protocol for inducing scrunching using various physical and chemical stimuli. We detail the quantitative characterization of scrunching, which can be used to distinguish it from peristalsis and gliding, using freely available software. Since scrunching is a universal planarian gait, albeit with characteristic species-specific differences, this protocol can be broadly applied to all species of planarians, when using appropriate considerations. To demonstrate this, we compare the response of the two most popular planarian species used in behavioral research, *Dugesia japonica* and *Schmidtea mediterranea*, to the same set of physical and chemical stimuli. Furthermore, the specificity of scrunching allows this protocol to be used in conjunction with RNA interference and/or pharmacological exposure to dissect the molecular targets and

neuronal circuits involved, potentially providing mechanistic insight into important aspects of nociception and neuromuscular communication.

INTRODUCTION:

In addition to their popularity for stem cell and regeneration research[1–3], freshwater planarians have long been used in behavioral studies[4,5], taking advantage of their comparatively large size (a few millimeters in length), ease and low cost of laboratory maintenance, and broad spectrum of observable behaviors. The introduction of computer vision and automated tracking to planarian behavior studies[6–11] have enabled quantitative differentiation of behavioral phenotypes. Animal behavior is a direct readout of neuronal function. Because the planarian nervous system is of medium size and complexity, but shares conserved key elements with the vertebrate brain[12–14], studying planarian behavior can provide insight into conserved mechanisms of neuronal action which may be hard to directly probe in more complex organisms. Thus, planarians are a valuable model for comparative neurobiology studies[8,12,15–21]. In addition, the aquatic environment allows for rapid and facile exposure to chemicals to study their effect on brain function in regenerating and adult planarians, making them a popular system for neurotoxicology[22–26] .

Planarians possess three distinct gaits, referred to as gliding, peristalsis, and scrunching. Each gait is exhibited under specific circumstances: gliding is the default gait, peristalsis occurs when ciliary function is compromised[7,27], and scrunching is an escape gait – independent of cilia function – in response to certain noxious stimuli[7]. We have shown that scrunching is a specific response, elicited by the sensation of certain chemical or physical cues, including extreme temperatures or pH, mechanical injury, or specific chemical inducers, and thus is not a general stress response[7,28,29].

Because of its specificity and stereotypical parameters, which can easily be quantified using this protocol, scrunching is a powerful behavioral phenotype that enables researchers to perform mechanistic studies dissecting sensory pathways and neuronal control of behavior[25,28]. Additionally, scrunching has been shown to be a sensitive endpoint to assay adverse chemical effects on nervous system development and function in neurotoxicology studies[22,24,25,30]. As several different sensory pathways seem to converge to induce scrunching through various mechanisms[28], scrunching differs from other planarian behaviors because various, but specific, stimuli can be used to dissect distinct neuronal circuits and study how different signals are integrated to produce the scrunching phenotype.

Importantly, species differences exist, wherein one chemical may elicit scrunching in one planarian species, but a different behavioral response in another. For example, we have found that anandamide induces scrunching in the planarian species *Dugesia japonica* but induces peristalsis in *Schmidtea mediterranea*[28]. This example highlights the importance of being able to reliably distinguish between the different gaits, because they are the phenotypic manifestations of distinct molecular mechanisms. However, distinction of scrunching from peristalsis is difficult using qualitative observational data, because both gaits are musculature-driven and share qualitative similarities[7,28]. Thus, to distinguish the gaits it is necessary to perform cilia imaging or

a quantitative behavioral study, which allows distinction based on characteristic parameters[7,28]. Because cilia imaging is experimentally challenging and requires specialized equipment such as a high-magnification compound microscope and a high-speed camera[7,28], it is not as broadly accessible to researchers as quantitative behavioral analysis.

Here, we present a protocol for (1) the induction of scrunching using various physical (noxious temperature, amputation, near-UV light) and chemical (allyl isothiocyanate (AITC), cinnamaldehyde) stimuli and (2) the quantitative analysis of planarian behavior using freely available software. By quantifying four parameters (frequency of body length oscillations, relative speed, maximum amplitude, and asymmetry of body elongation and contraction)[7], scrunching can be differentiated from gliding, peristalsis, and other behavioral states reported in the literature, such as snake-like locomotion[15] or epilepsies[15]. Furthermore, while scrunching is conserved among different planarian species[7], each species has its own characteristic frequency and speed; therefore, once the gliding and scrunching speeds of a species have been determined, speed alone can be used as a means to distinguish scrunching from gliding and peristalsis[29]. The protocol assumes no prior training in computational image analysis or behavioral studies and thus can also be applied for planarian behavioral experiments in a teaching laboratory context at the undergraduate level. Example data to facilitate protocol adaptation is provided in the Supplemental Material.

PROTOCOL:

1. Quantitative planarian behavior assays

1.1. Experimental setup

1.1.1. Place a dimmable LED panel upon a flat surface. The LED panel serves two purposes: (1) to provide a uniform white background and (2) to be used as an adjustable light source to obtain appropriate contrast. Place a 100 mm Petri dish arena upon the LED panel.

NOTE: To increase throughput, a multi-well plate may be used as an arena[23,24], but larger arenas facilitate automated image analysis.

1.1.2. Mount a camera on a ring stand above the arena (**Figure 1A**). Adjust the camera position, height, and focus as necessary so that the entire arena is centered within the field of view and is in focus (**Figure 1B**).

NOTE: The camera resolution needs to be high enough to clearly distinguish a planarian from the homogenous background provided by the LED panel.

1.1.3. Fill the arena with the appropriate exposure media (planarian water or chemical solution) to half-maximum volume (this will be referred to as a bath). This corresponds to approximately 25 mL for a 100 mm Petri dish. Turn on the LED panel and turn off any other light sources that

may negatively affect recording quality (i.e., nearby light sources that produce a glare onto the arena).

CAUTION: Manage hazardous chemical solutions appropriately by wearing full personal protective equipment (PPE) and moving the experimental setup to a fume hood if necessary. Follow federal and state regulations on waste disposal.

1.1.4. Drop a planarian toward the center of the arena using a transfer pipette. Begin recording. Record data as image sequences in a native Fiji[31] format (TIFF, GIF, JPEG, PNG, DICOM, BMP, PGM, or FITS; see image analysis section 1.2).

NOTE: Because behaviors and sensitivity to external stimuli vary among individual planarians, it is important to collect data on a sufficiently large number of biological replicates, in addition to performing technical replicates. We have worked with up to 10 medium-sized (4-7 mm) planarians in a 100 mm Petri dish at once. While time efficient, multiple planarians in the Petri dish at once make data analysis more difficult since planarians may cross paths.

1.1.4.1. For gliding experiments, record using at least 1 frame per second (FPS). For scrunching/peristalsis experiments, record using an FPS that is at least twice the scrunching/peristalsis frequency of the planarian species. If the planarian species has an unknown scrunching/peristalsis frequency, use 10 FPS as a starting point and increase/decrease as appropriate.

1.1.4.2. When using a chemical solution, transfer the planarian using as few drops of planarian water as possible so that the concentration of the chemical solution is not significantly changed.

1.1.5. For gliding experiments, record 1-2 minutes of gliding behavior. For scrunching/peristalsis experiments, record long enough to capture at least 3 consecutive oscillations occurring in a straight line. Once the experiment is completed, terminate the recording.

NOTE: For scrunching/peristalsis experiments, if a planarian does not satisfy the termination criterion within a fixed time period that needs to be consistent across replicates and is empirically determined based on the stimulus, terminate the recording and test another planarian.

1.1.5.1. If the planarian reaches the boundary of the arena without satisfying the termination criterion, pipette the planarian back to the center of the arena.

NOTE: Avoid repeated pipetting of an individual for recording, as this may change its behavior.

1.1.6. Remove the planarian(s) from the arena and dispose of the planarian water or chemical solution in appropriate waste containers. Planarians that were in planarian water can be returned to their home container.

NOTE: Avoid cross contamination by using different arenas for different media (i.e., gliding in planarian water experiments should not be run in an arena previously used for scrunching/peristalsis experiments with chemical exposure).

1.1.6.1. Serially rinse planarians exposed to a chemical solution in 3 clean 100 mm Petri dishes filled with 25 mL of planarian water to thoroughly dilute out any chemicals. If scrunching or peristalsis was induced, place these planarians in a separate container. Planarians can be returned to their home container after one month since most cells would have turned over by that time[1].

NOTE: If multiple different experiments are needed for the same population of planarians, e.g., for an RNAi population, allow planarians to recover for 24 hours before running the next experiment. Order the experiments such that the least invasive experiment is first and the most invasive experiment (e.g., amputation) is run last.

1.1.6.2. If running multiple experiments in the same arena, properly dispose of the bath solution and remove any mucus trails by wiping down the arena with a paper towel between runs.

NOTE: The protocol can be paused here.

1.2. Quantitative analysis of planarian behavior

1.2.1. Perform planarian behavior assays as described in Section 1.1.

1.2.2. Open the raw image sequence for an experiment in Fiji (**File > Import > Image Sequence**). Convert the image sequence to 8-bit (**Image > Type > 8-bit**) and use the arrow tool or slider at the bottom of the image stack to watch or pan through the image sequence.

NOTE: For gliding experiments, all data can be used as long as the planarian can be clearly seen throughout the recording. However, it is usually sufficient to analyze the free motion in the center of the arena by extracting the relevant part(s) as described below.

1.2.3. To extract a time period and region of interest, draw a region of interest encompassing the full path of a planarian using the rectangle tool (**Figure 2A, 2B**). Right click on the image stack and select **Duplicate**…, check the box for **Duplicate stack**, enter the first and last frames of the sequence of interest, and click **OK**. If multiple planarians were imaged simultaneously, repeat this region selection and duplication step for each planarian in the arena so that there are as many open image stacks as there are planarians in the arena. The following steps (Steps 1.2.4-1.2.10) should be performed on each image stack, one at a time.

1.2.3.1. For gliding experiments, extract a period of gliding where the planarian moves at least twice its body length.

NOTE: The more gliding data extracted per planarian, the more reliable the data will be. The planarian does not need to be moving in a straight line for the gliding analysis.

1.2.3.2. For scrunching/peristalsis experiments, extract an instance when the planarian undergoes a minimum of three consecutive (ideally more) body oscillations in a straight line, making sure each oscillation is a complete elongation-contraction cycle, as full oscillations are necessary to accurately determine the frequency.

NOTE: The more oscillations that can be extracted, the more reliable the data will be. Do not use sequences where the planarian is turning as these will result in inaccurate length measurements.

1.2.4. Apply a threshold to the duplicated image stack (**Image > Adjust > Threshold**) to binarize the image and extract the planarian from the background. Adjust the sliding bars as necessary such that the entire planarian is highlighted in red. The exact values are dependent on imaging quality. Leave the boxes for **Dark background**, **Stack histogram**, and **Don't reset range** unchecked. Scroll through the image stack to ensure a good threshold range (i.e., the planarian is well separated from the background throughout the stack), and then click **Apply**.

1.2.5. In the **Convert Stack to Binary** window, set the Method to **Default** and the Background to **Light**. Uncheck all boxes in this window and then click **OK**. A binarized image showing a black planarian on a white background will appear (**Figure 2C**). Make sure that the entire planarian is visible in all frames of the image sequence.

NOTE: Unwanted objects in the binarized image sequence that are smaller or larger than the planarian can be filtered out in the subsequent analysis using a size filter (**Figure 2Ciii**).

1.2.6. Set measurements by clicking **Analyze > Set Measurements**. Check the boxes for **Area**, **Center of mass**, **Stack position**, and **Fit ellipse** and click **OK**.

NOTE: These parameters only need to be set once per Fiji session.

1.2.7. Select the open image stack and select **Analyze > Analyze Particles**.

1.2.8. In the **Analyze Particles** window, select **Show > Masks** to open a new stack showing all the objects that were detected with the chosen parameters. This can be used to visually check that only measurements of the planarian are being taken. A size filter may be set at this step to remove unwanted noise by entering the approximate area of the planarian (in pixel^2 units) in the space provided. Check the boxes for **Display results** and **Clear results** and click **OK**.

NOTE: In the **Results** window, if the index (first column) does not equal the slice number for all rows, this means that either too many or too few objects were tracked. One possibility for this discrepancy is the presence of other objects besides the planarian or that the planarian was not tracked in specific frames.

1.2.9. Pan through the mask image stack using the slider at the bottom of the panel. If there is any noise or there are frames that lack a planarian, close the **Results** window and the mask image stack. Repeat steps 1.2.7-1.2.8 by adjusting the area filter to only remove objects other than the planarian.

NOTE: If the planarian is missing from the frame in the mask, this suggests that the lower bound of the area filter was set too high.

1.2.10. On the **Results** window, save the data using **File>Save As**. Add the .csv extension to the filename to save data as comma-separated values. Once data for the image stack is saved, close the respective image stack, and **Results** and **Mask** windows.

1.2.11. Import data and further analyze using any spreadsheet software or freeware. To calculate gliding speed, refer to section 1.3. To calculate the scrunching/peristalsis full parameter set, refer to section 1.4.

NOTE: The protocol can be paused here.

1.2.12. To determine the pixel to actual length conversion, open an image in Fiji with a reference length (e.g., the diameter of the arena). Select the line tool and draw a line over the known length.

1.2.13. Convert pixel units to a standard unit of length by clicking **Analyze > Set Scale**. Enter the length corresponding to the line drawn on the image in the **Known distance** box and change **Unit of length** from **pixel** to the chosen standard unit of length. The conversion factor is written next to **Scale**.

NOTE: A pixel conversion value is not required for gliding or scrunching/peristalsis analyses in sections 1.3 and 1.4.

1.3. Calculation of gliding speed

1.3.1. Using the data file saved in Section 1.2, load the center of mass (COM) x and y coordinates and the major axis data. If the data is saved as a comma-separated values file, these lists correspond to the “XM”, “YM”, and “Major” columns, respectively.

1.3.2. Calculate the displacement *(d)* of the planarian center of mass in pixels for each frame with respect to the next frame using the “XM” and “YM” data columns. Displacement *(d)* is given by:

$$d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$$

where x_1 and y_1 refer to the COM coordinates (XM, YM) of one frame and x_2 and y_2 refer to the COM coordinates (XM, YM) of the subsequent frame.

1.3.3. Set the planarian body length as the 95th percentile of the "Major" column. Since planarians exhibit a wall preference behavior[32], this ensures that the calculated planarian body length is representative of when the planarian is elongated[24].

1.3.4. Normalize displacement by planarian body length by dividing the pixel displacements per frame by the planarian body length *(l)*. Normalized displacement *(d_n)* is given by:

$$d_n = \frac{d}{l}$$

1.3.5. Generate a list of normalized speeds by dividing the normalized displacements by the time elapsed per frame (inverse of the recorded FPS). Normalized gliding speed *(s_n)* is given by:

$$s_n = \frac{d_n}{(FPS)^{-1}}$$

1.3.6. Calculate the normalized gliding speed of the planarian by taking the average of the normalized speeds list *(s_n)*. The standard deviation may be used as an uncertainty measurement for the planarian.

1.3.7. Repeat steps 1.3.1-1.3.6 for each planarian to be analyzed. Average and take the standard deviation of the gliding speeds for all planarians to get the gliding speed and associated uncertainty, respectively, for a planarian population.

1.4. Distinction of scrunching and peristalsis gaits using the full parameter set

1.4.1. Load the major axis data list from the data file saved from Section 1.2. If the data is saved as a comma-separated values file, this corresponds to the **Major** column.

1.4.2. Create a list that numbers each data point in the **Major** column, starting with **0**. Convert this list to time elapsed per frame by dividing by the recorded FPS.

1.4.3. 1.4.3 Plot the **Major** column data with respect to time elapsed to generate a scrunching/peristalsis oscillation plot (**Figure 3A**). Using the oscillation plot, trim the data to at least three consecutive, straight-line oscillations (**Figure 3Bi**). Trim the data to start and end at local peaks (maximum elongation of oscillation) or troughs (minimum elongation of oscillation).

NOTE: If local extrema are not approximately equal (peaks/troughs differ dramatically in heights), this suggests that the oscillations are not straight-line (**Figure 3Bii**). Extract another sequence of at least three consecutive, straight-line oscillations. Refer to Section 1.2.

1.4.4. Confirm that the oscillation sequence of interest has been extracted and trimmed properly by replotting the trimmed **Major** data with respect to time. Use this trimmed data list for all subsequent calculations.

1.4.5. To calculate oscillation frequency *(ν_m)*, divide the number of oscillations *(O_n)* by the total number of data points in the trimmed major axis data list *(N)*. Multiply FPS by this value to get frequency in oscillations per second.

$$\nu_m = FPS * \frac{O_n}{N}$$

1.4.6. To calculate maximum elongation *($|\Delta\varepsilon|_{max}$)*, subtract the absolute minimum body length *(l_{min})* from the absolute maximum body length *(l_{max})*. Normalize to elongated body length by dividing by the absolute maximum body length.

$$|\Delta\varepsilon|_{max} = \frac{l_{max} - l_{min}}{l_{max}}$$

1.4.7. To calculate speed per body length (ν^*_m), multiply the calculated maximum elongation by the oscillation frequency.

$$\nu^*_m = \nu_m * |\Delta\varepsilon|_{max}$$

NOTE: Speed alone can be used to distinguish between scrunching and peristalsis gaits[7].

1.4.8. To calculate the fraction of time spent elongating *(f_{elong})*, take the derivative of the trimmed major axis data list with respect to time. Divide the number of positive data points (i.e., when the derivative is >0 *(n_p)*, by the total number of data points in the major axis data list (n_t)).

$$f_{elong} = \frac{n_p}{n_t}$$

NOTE: Scrunching planarians exhibit an asymmetric fraction of time spent elongating whereas planarians performing peristalsis spend equal amounts of time elongating and contracting[7].

1.4.9. Repeat steps 1.4.1-1.4.8 for each planarian to be analyzed. Calculate a planarian population parameter set by taking the average and standard deviation of each parameter.

NOTE: The parameter set can be used to determine if the oscillation behavior is scrunching, peristalsis or some other form of locomotion with periodic body shape changes. Both scrunching and peristalsis have fixed parameters for a given species[7], with scrunching parameters generally being greater than peristalsis parameters[7]. While it is possible that one of the parameters may fall outside of the species-specific range, as we have previously observed with chemical induction[28], the observed behavior must agree with at least 3 of 4 published parameters to be categorized as either peristalsis or scrunching.

2. Scrunching induction

2.1. Physical stimuli (noxious temperature, UV light, amputation)

2.1.1. For all physical stimuli experiments, refer to Section 1.1 for the experimental setup.

NOTE: It is best to use a large arena, such as a 100 mm Petri dish, for physical stimuli experiments to allow for more open space for maneuvering a pipette and/or razor blade.

2.1.2. To induce scrunching via noxious temperature, heat planarian water in a glass beaker (at least 100 µL per planarian to be tested) to 65 °C on a hot plate.

2.1.2.1. Place a planarian in the center of the arena. Wait until the planarian orients itself upright and begins gliding. Begin recording.

2.1.2.2. Using a P-200 pipette, slowly pipette 100 µL of the 65 °C planarian water post-pharyngeally onto the tail end of the planarian to induce scrunching.

NOTE: Make sure the heated planarian water stays at 65°C. If necessary, reheat the water to 65°C prior to starting another experiment. Since pressure can also induce scrunching, slow pipetting is necessary. Pipetting room temperature water in the same way as in the experiment can serve as a control and practice option.

2.1.2.3. Stop the recording once scrunching has ceased. Place the planarian in a recovery container and exchange the media in the petri dish with fresh, room temperature planarian water if running more experiments.

2.1.3. To induce scrunching via amputation, transfer a planarian to the center of the arena and wait until the planarian orients itself upright and begins gliding. Begin recording.

2.1.3.1. Amputate the planarian using a clean razor blade. Amputations may be done anywhere along the planarian as long as the cut location is consistent across experiments.

NOTE: Scrunching parameters are extracted from the anterior piece. Thus, avoid obstructing the camera's view of this part of the planarian when applying the cut by approaching from the posterior end. Plastic cover slips also work well for cutting and are a safer option, especially in a teaching setting.

2.1.3.2. Stop the recording once the anterior piece has ceased scrunching. Remove both pieces, place them in a separate container and allow them to regenerate for 7 days. Amputated planarians can be reincorporated into the home container once regenerated.

2.1.4. To induce scrunching using near-UV light, attach appropriate filters (e.g., Roscolux filters) to the camera lens to reduce the amount of reflected near-UV light that is collected by the camera and may interfere with imaging the planarian's response. Instead of using the LED panel to illuminate the arena from below, use ambient red lighting to which planarians are insensitive[33].

2.1.4.1. Fill a 100 mm Petri dish arena with planarian water and place a single planarian (5-9 mm) in the center of the arena. Begin recording at 10 FPS.

2.1.4.2. Hold a Class II UV laser pointer (405 ± 10 nm, output power <5 mW) approximately 30 cm from the arena. Position the laser pointer at a 45° angle from the gliding planarian and then shine the laser pointer for 5-10 seconds halfway between the posterior end of the pharynx and the tail tip to induce scrunching.

NOTE: The power of the laser pointer can be measured using a near-UV-sensitive power meter.

2.1.4.3. Wait for the planarian to start gliding again before attempting two more stimulations on the same individual to test for reproducibility of the reaction. If the planarian keeps showing the same behavior, stop recording and put the planarian back in its container. If the behavior changes between stimulations, additional tests will show which response is the most prominent.

NOTE: Planarians can become desensitized to near-UV light and will stop reacting. Consecutive stimulations require a rest period of 8-10 seconds.

2.2. Chemical stimulus (AITC)

2.2.1. To induce scrunching using a chemical, e.g., the TRPA1 agonist AITC[28], planarians are ideally immersed in a bath of the chemical. If necessary, pipetting can be applied as described in section 2.1.2.3.

CAUTION: AITC is flammable, acutely toxic, can cause skin and eye irritation, respiratory and skin sensitization, and is hazardous to aquatic life. AITC oil should be handled in a fume hood. Prior to making stock solutions of AITC, put on appropriate PPE (nitrile gloves and a lab coat) and set up appropriate solid and liquid hazardous waste disposal containers.

2.2.2. In a fume hood, make a 10 mM stock solution of AITC in planarian water in a 50 mL centrifuge tube. This stock solution is useable for up to one month when stored at 4°C.

2.2.2.1. From this stock, prepare a 25 mL working solution of 100 µM AITC in planarian water in a 50 mL centrifuge tube. This 100 µM AITC solution will be used to induce scrunching in planarians.

NOTE: 100 µM AITC induces consistent scrunching in *D. japonica* and *S. mediterranea* planarians[28]. For other aquatic planarians, 100 µM can serve as a starting concentration and can be adjusted accordingly.

2.2.2.2. Set up the experimental setup (refer to Section 1.1). Fill the arena with the AITC working solution and place it in a secondary container. The secondary container should hold at least twice the volume of the arena.

NOTE: Experiments can be carried out inside a fume hood for extra safety.

2.2.2.3. Transfer up to 10 planarians to the center of the arena and begin recording.

2.2.2.4. Once the planarians become desensitized and cease scrunching, stop recording. Remove the planarians from the AITC solution and rinse (refer to Section 1.1). Dispose of solid and liquid AITC waste in appropriate waste containers.

2.2.2.5. Verify the specificity of the response to AITC using RNAi to TRPA1[28] following standard protocols.

REPRESENTATIVE RESULTS:

Extraocular near-UV perception in *S. mediterranea* planarians is TRPA1-dependent and has been proposed to be linked to H_2O_2 release[17]. Because H_2O_2 exposure induces TRPA1-dependent scrunching in *S. mediterranea* and *D. japonica* planarians[28], the steps in Section 2.1.4 can be used to test whether near-UV light exposure induces scrunching in both species. While *D. japonica* planarians scrunch (10/10) when exposed to near-UV light, *S. mediterranea* planarians either exhibit tail thinning (7/10) as previously described[17] or no response (3/10) (**Figure 4A,4B**). A quantification of the scrunching parameters, as outlined in Section 1.4, for the *D. japonica* planarians that exhibited at least 3 consecutive straight-line scrunches reveals characteristic scrunching parameters for this species[7,28] (ν_m = 0.84 ± 0.14, $|\Delta\varepsilon|_{max}$ = 0.56 ± 0.06, ν_m^* = 0.47 ± 0.07, and f_{elong} = 0.56 ± 0.03, values reported as mean ± standard deviation for N=7).

In contrast, exposure to 250 µM cinnamaldehyde, a known TRPA1 agonist in mice[34], causes scrunching in *S. mediterranea*[7,28] (ν_m = 0.46 ± 0.08, $|\Delta\varepsilon|_{max}$ = 0.36 ± 0.08, ν_m^* = 0.16 ± 0.04, and f_{elong} = 0.58 ± 0.04, values reported as mean ± standard deviation for N=8) (**Figure 5A**)**,** whereas *D. japonica* planarians at the same (and 1.6x the concentration) display a mixture of snake-like and oscillatory motion, interrupted by gliding and/or vigorous head turns (**Figure 5A**)**.** A quantification of the (8/24) samples with at least three consecutive oscillations yields significantly lower values for 3 out of 4 parameters than expected for scrunching in this species (ν_m = 0.43 ± 0.08, $|\Delta\varepsilon|_{max}$ = 0.39 ± 0.03, ν_m^* = 0.17 ± 0.02, and f_{elong} = 0.54 ± 0.06, values reported as mean ± standard deviation for N=8). Thus, while *D. japonica* appear to scrunch upon cinnamaldehyde exposure, a comparison of the calculated parameters with the literature values for this species[7,28] shows that the observed oscillatory motion is not scrunching. This example highlights the importance of quantitative measurements in conjunction with careful inspection of the raw behavioral data to properly interpret observed behaviors.

RNAi confirms the specificity of scrunching in response to cinnamaldehyde exposure in *S. mediterranea.* Within 180 seconds of exposure to 250 µM cinnamaldehyde in planarian water 15/15 *unc22* (*control*) RNAi *S. mediterranea* planarians scrunched, whereas 0/16 *SmTRPA1* RNAi planarians scrunched (**Figure 5B**), demonstrating that *S. mediterranea* scrunching in cinnamaldehyde requires *SmTRPA1.* Knockdown of *SmTRPA1* was confirmed through a 60 second exposure to a 100 µM AITC bath[28].

FIGURE AND TABLE LEGENDS:

Figure 1. Planarian behavior experimental setup. (**A**) Sample experimental setup for studying

planarian behavior. (**B**) 100 mm Petri dish arena centered in the field of view of the camera.

Figure 2. Representative examples of the Fiji image analysis of planarians in arena. (**A**) Selected region of interest, encompassing the full planarian path, indicated by the yellow rectangle. (**B**) Sample frames from the region of interest after duplication. (**C**) Subtracting the planarian from background and noise via thresholding (**i**) 8-bit image of planarian with noise, denoted by the asterisk. (**ii**) Binarized image of planarian after thresholding. (**iii**) Mask of planarian after setting filtering by size to remove noise.

Figure 3. Plotting planarian length with respect to time. (**A**) Raw plot of planarian length versus time for a scrunching *S. mediterranea* planarian. The asterisk denotes a moment when the planarian turned while scrunching. (**B**) Possible ways to trim scrunching data. (**i**) A correctly trimmed plot that removes the turning event data. (**ii**) An incorrectly trimmed plot that does not remove the turning event data.

Figure 4. Species specific responses to near-UV light. (**A**) Sample frames of *D. japonica* scrunching and *S. mediterranea* tail thinning in response to near-UV light. (**B**) Representative oscillation plots of *S. mediterranea* and *D. japonica* in response to near-UV light.

Figure 5. Species specific response to 250 µM cinnamaldehyde, a TRPA1 agonist. (**A**) Representative oscillation plots for *D. japonica* and *S. mediterranea* planarians in a 250 µM cinnamaldehyde bath. (**B**) Representative oscillation plots showing loss of scrunching in 250 µM cinnamaldehyde in *SmTRPA1* RNAi *S. mediterranea* planarians.

DISCUSSION:

Using this protocol, one can quantitatively study the effects of physical and chemical stimuli[7,28,29] or genetic manipulation (RNAi)[28,29] on planarian locomotion. To maximize spatial resolution, it is best to move the camera as close as possible to the arena while ensuring the entire arena is in the field of view. To increase throughput, the behavior of multiple planarians can be screened at once by recording multiple planarians simultaneously. When screening more than one planarian in a single arena, regions of interest can be drawn in Fiji to isolate individual planarians as described here or more advanced multi-object tracking can be employed. One issue with having multiple planarians in the same arena is that they can cross paths. This problem can be solved through the use of multi-well plates to isolate planarians from each other while still enabling simultaneous recording of many individuals to quantify behavior[23,24]. However, planarians will spend relatively more time at the wall in smaller arenas, requiring adjustments to the image analysis and limiting the resolution for scrunching/peristalsis quantification.

When stimuli are administered locally (e.g., pipetting[7], amputation[7,28], laser pointer[17]), it is crucial that the planarians are consistently stimulated in the same region because stimulating other body regions can potentially induce different behaviors. Different methods of delivery (such as pipetting or bath of a chemical) can also affect the consistency of the behavioral phenotype. Additionally, planarians can desensitize quickly[28], which needs to be taken into consideration when planning experiments as the same planarians should not be immediately reused for

multiple experiments, either using the same or different stimuli. Finally, as shown here for near-UV exposure and cinnamaldehyde, it is important to be aware that the same stimulus can induce distinct behaviors in different planarian species. *D. japonica* scrunched when stimulated with near-UV light near the tail tip, while *S. mediterranea* planarians displayed tail thinning. In contrast, cinnamaldehyde exposure induced scrunching in *S. mediterranea* but not in *D. japonica* planarians. Thus, while scrunching is a conserved response of various planarian species to noxious stimuli[7], it has species specific parameters[7,28], sensitivities[28], and inducers[28]. Therefore, for a new species for which scrunching has not yet been parameterized, it is best to start with a well-conserved inducer, such as amputation[7], to determine the species-specific parameters before testing the response to other stimuli.

One limitation of the analysis described here is that it does not account for turns and/or mixed behaviors, such as intermittent scrunching with head wiggling, gliding, or other body shape changes. However, close inspection of the raw data can help mitigate these issues if these instances are manually excluded from the analysis, as demonstrated in **Figure 3**. In addition, it is possible to add body shape analysis to the center of mass and length tracking described here and expand the protocol to quantify these other planarian behaviors. Given that the analysis does not make any assumptions about the studied organism, the protocol could in principle also be applied to other organisms that show similar types of behaviors.

The method of quantifying the different planarian gaits and distinguishing scrunching from peristalsis, as described here, assumes no prior training in computational image analysis or behavioral studies and does not require specialized equipment or software. To facilitate protocol adaptation, example data is provided in the Supplemental Material. The ease of obtaining and culturing planarians, as well as the ability to record behaviors without specialized equipment, makes planarian behavioral studies broadly accessible to research across all levels, from primary school classrooms to academic labs. A modified version of this protocol has been successfully used in a teaching laboratory setting that was primarily composed of freshmen and sophomore students and included both prospective STEM and non-STEM majors.

The combination of molecular (RNAi) and chemical tools with quantitative behavioral analysis, as described in this protocol, allow researchers to gain mechanistic insights into the molecular control of behavior. Such work has uncovered some of the key mediators and neuronal circuits involved in planarian gliding[19,20], phototaxis[17,35,36], thermotaxis[9,37], and scrunching[9,28,29]. Although planarian behaviors may not have direct corollary behaviors in higher organisms, such as humans, these behaviors represent fundamental neuronal functions important to all organisms - the ability to sense and process specific stimuli and react appropriately. Because of the conservation of key neuronal functions across different organisms, mechanistic studies in planarians can teach us more broadly about neuronal control of behavior. Additionally, analyzing planarian behavior in response to chemical exposure can be used to study the chemical's effects on the planarian nervous system[23–25], which may inform on potential risks to the human brain. In particular, scrunching induced by noxious heat was found to be a sensitive and specific endpoint for assaying neurotoxicity, because it becomes disrupted by exposure to certain classes of chemicals[22,24,25,30]. Finally, the planarian's unique regenerative capabilities allow researchers to dissect the dynamics

of how different behaviors are restored during neuroregeneration.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

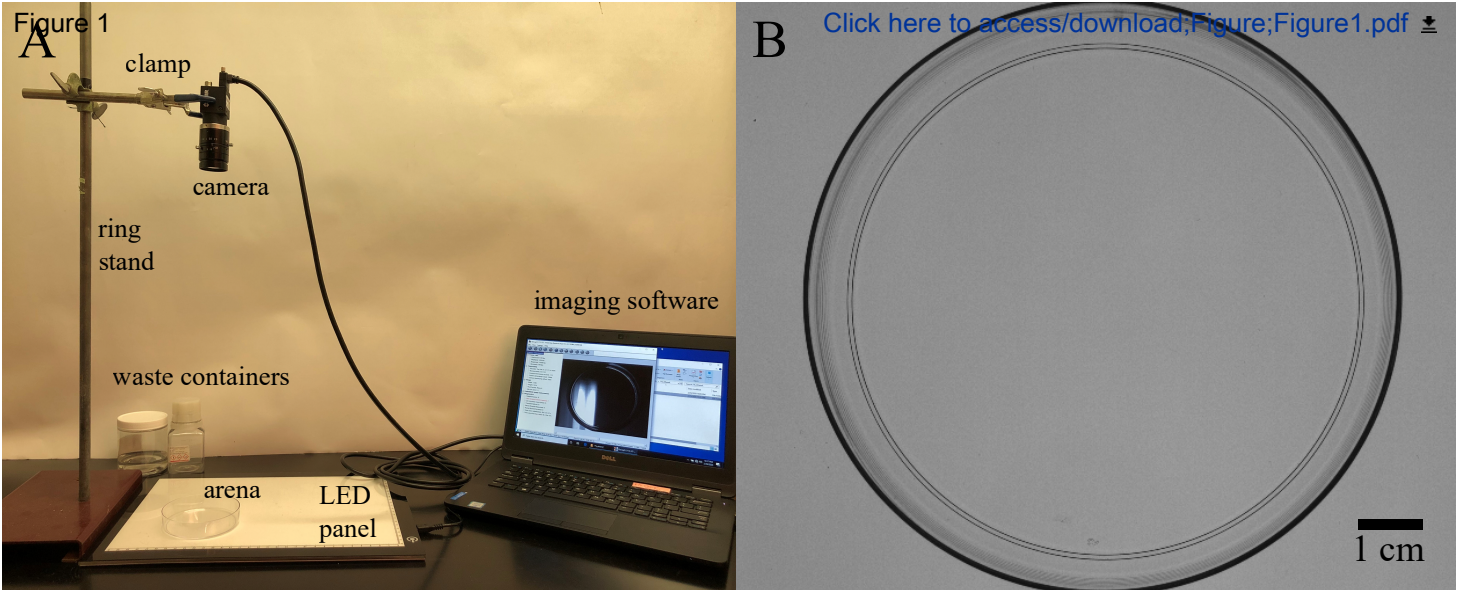
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Figure 2

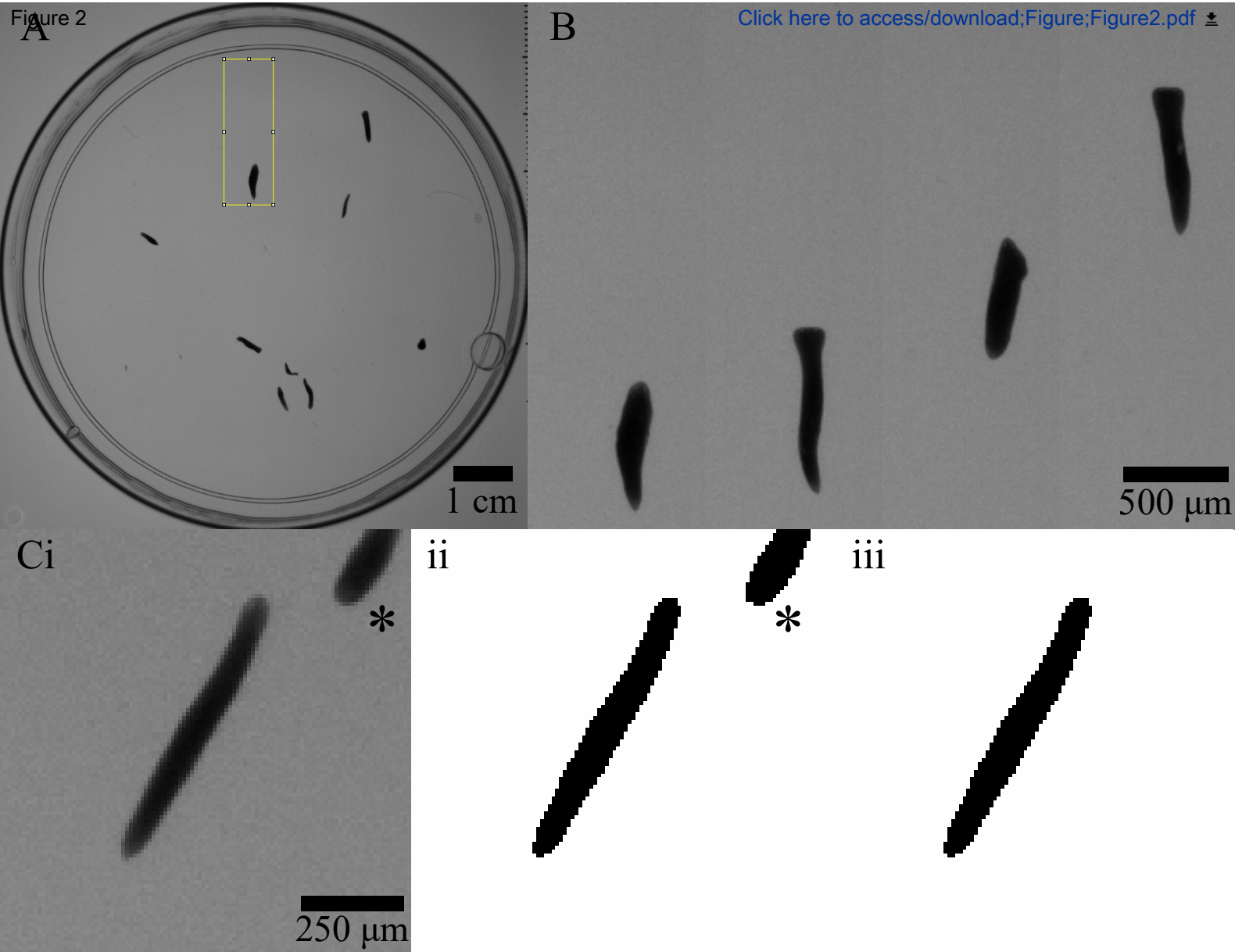
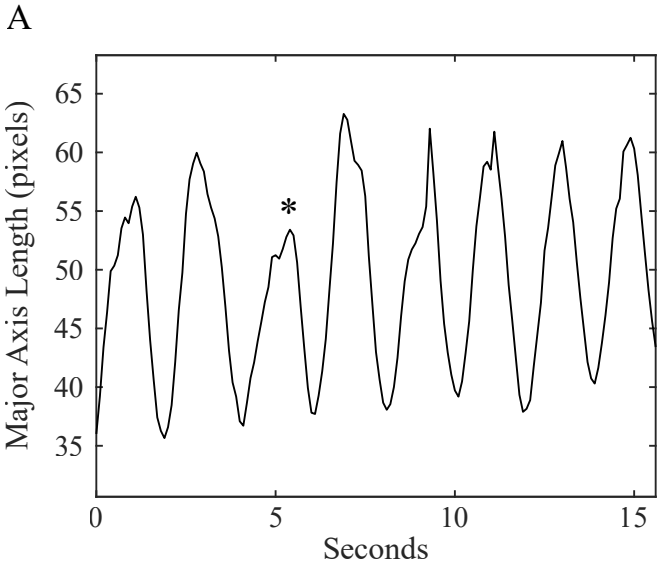
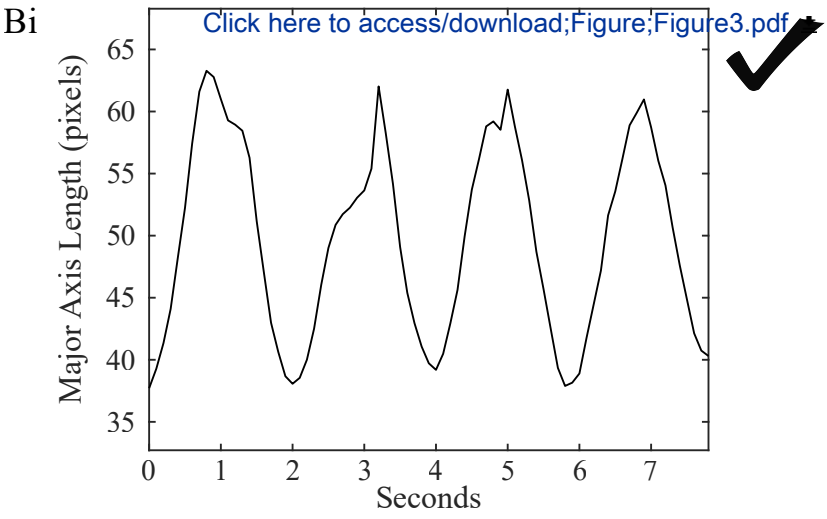
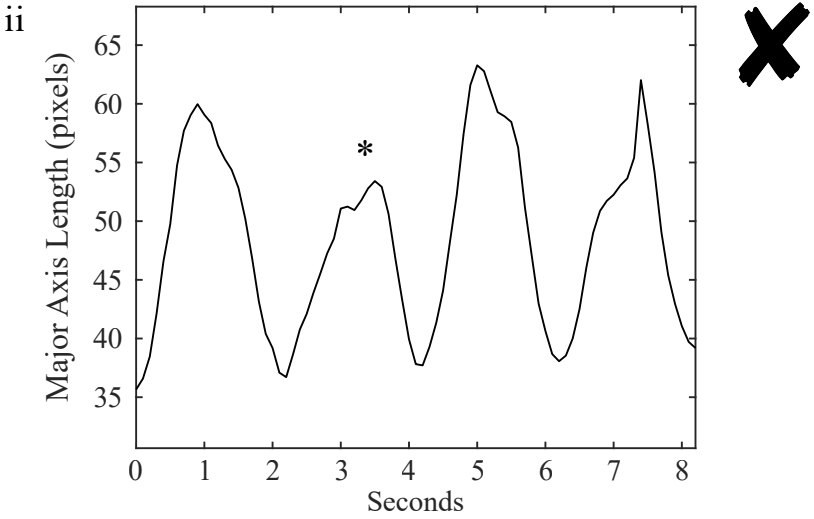
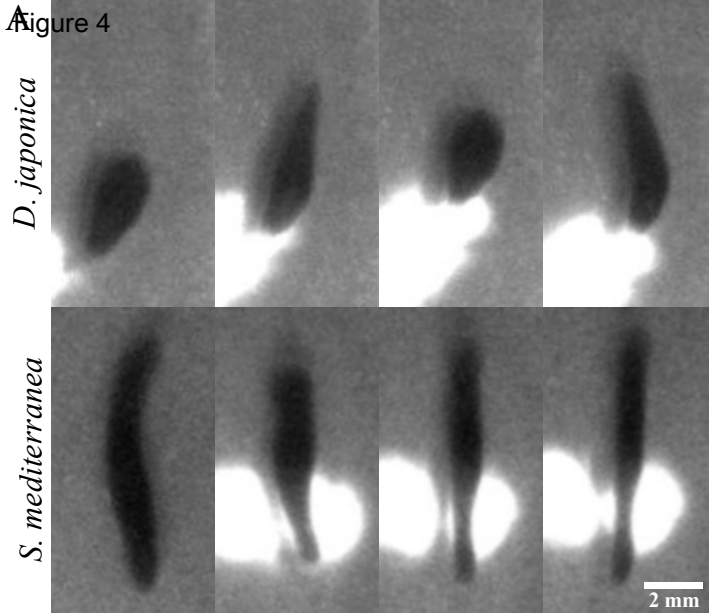


Figure 3








A
D. japonica
S. mediterranea
2 mm

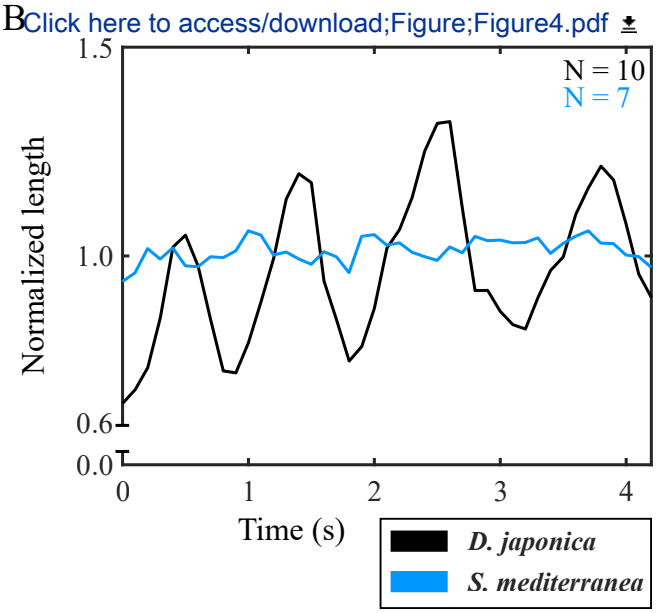
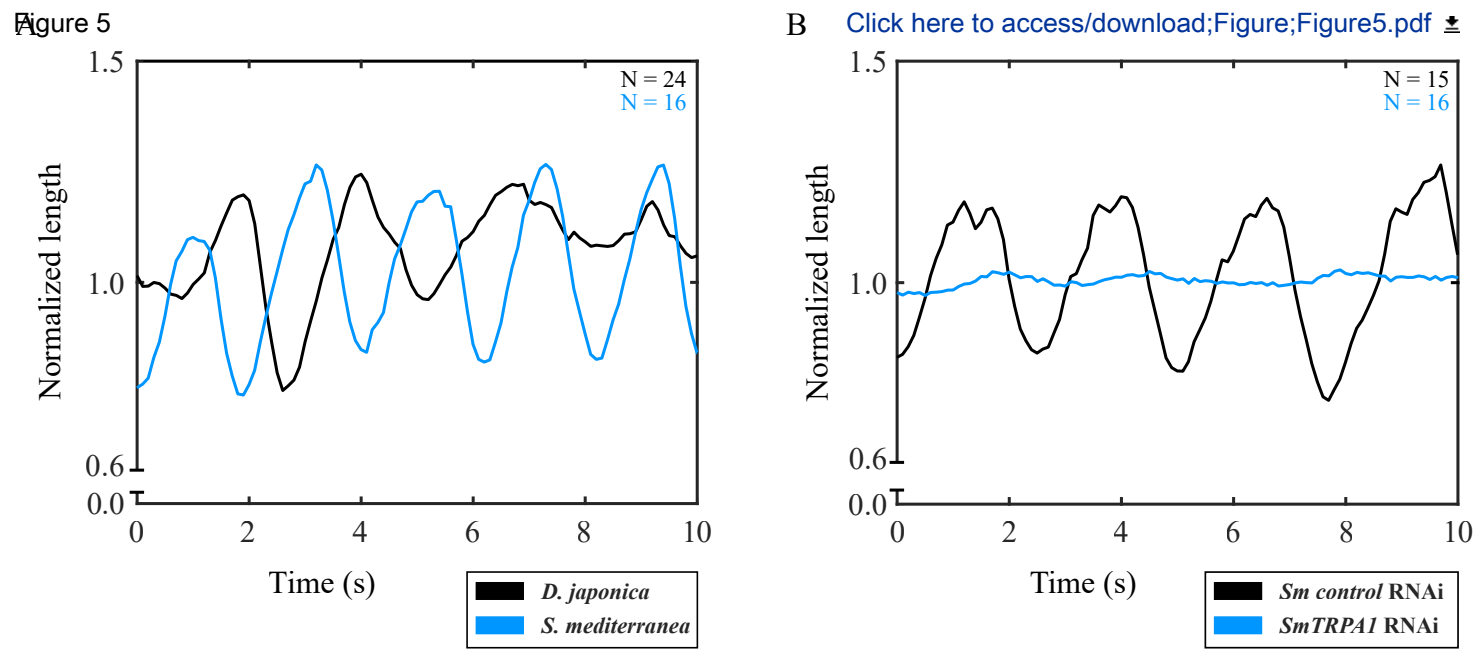

B
Click here to access/download;Figure;Figure4.pdf
1.5
1.0
0.6
0.0
Normalized length
N = 10
N = 7
0
1
2
3
4
Time (s)
D. japonica
S. mediterranea

Figure 5


A
B
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Normalized length
Time (s)
N = 24
N = 16
D. japonica
S. mediterranea
N = 15
N = 16
Sm control RNAi
SmTRPA1 RNAi

Name of Material/ Equipment	Company	Catalog Number
Allyl isothiocyanate, 95% (AITC)	Sigma-Aldrich	377430-5G
Camera lens, 2/3 25mm F/1.4	Tamron	23FM25SP
Cell culture plates, 6 well, tissue culture treated	Genesee Scientific	25-105
Centrifuge tubes, 50 mL polypropylene, sterile	MedSupply Partners	62-1019-2
Cinnamaldehyde, >95%	Sigma-Aldrich	W228613-100G-K
Dimmable A4 LED Tracer Light Box	Amazon	B07HD631RP
Flea3 USB3 camera	FLIR	FL3-U3-13E4M
Heat resistant gloves	Fisher Scientific	11-394-298
Hot plate	Fisher Scientific	HP88854200
Instant Ocean Sea Salt, prepared in deionized water	Instant Ocean	SS15-10
Montjüic salts, prepared in Milli-Q water	Sigma-Aldrich	various
Petri dishes, 100 mm x 20 mm, sterile polystyrene	Simport	D210-7
Pipette, 20-200 µL range	Rainin	17008652
PYREX 150 mL beaker	Sigma-Aldrich	CLS1000150
Razor blade, 0.22 mm	VWR	55411-050
Roscolux color filter: Golden Amber	Rosco	R21
Roscolux color filter: Medium Red	Rosco	R27
Roscolux color filter: Storaro Red	Rosco	R2001
Samco transfer pipette, 62 µL large aperture	Thermo Fisher	691TS
Support stand	Fisher Scientific	12-947-976
Thermometer	VWR	89095-600
UV laser pointer	Amazon	B082DGS86R

Comments/Description
CAUTION: Flammable and acutely toxic; handle in a fume hood with appropriate PPE.
Prepare in deionized water at 0.5 g/L.
Prepare in milli-Q water at 1.6 mM NaCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.1 mM $MgCl_2$, 0.1 mM KCl, 1.2 mM $NaHCO_3$; adjust pH to 7.0 wit
Alternatively purchase the Roscolux Designer Color Selector (Musson Theatrical product #SBLUX0306) which includes all 3 color filters tog
This is a Class II laser (405nm ±10nm) with output power <5 mW.

h HCl.

ether.

Dear Dr. Nguyen,

We would like to thank you and the referees for your constructive comments on our protocol manuscript, "Planarian scrunching as a quantitative behavioral readout for noxious stimuli sensing ". In response to the reviewers' comments, we have edited and expanded the Introduction and Discussion to provide broader context for the need for this protocol. Changes are highlighted in cyan for easy readability. In addition, we have provided example data sets of gliding, peristalsis, and scrunching. We have responded to all of the reviewers' comments, detailed below.

We trust that our revised manuscript is now suitable for publication, and, on behalf of my co-authors, thank you for the expedited review.

Sincerely,

Eva-Maria S. Collins

Response to Reviewers:

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may

Response: We have thoroughly proofread the manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a well-written and clearly presented protocol for conducting a quantitative behavioral readout of noxious stimuli sensing in planaria via quantification of scrunching behavior. The authors utilize a straightforward computational image analysis to differentiate scrunching from peristalsis and enable differentiation of behaviors among species and across experimental conditions.

Major Concerns:

None

Minor Concerns:

The authors mention that this experimental protocol is appropriate for a teaching laboratory course at the undergraduate level. It would be beneficial to students and instructors in those courses, as well as the research community at large, to provide an example dataset that can be processed alongside the video presentation. In my opinion, this would considerably strengthen the adoptability and utility of the methodology described, and bring the protocol presented in line with the author's claim that it requires no prior training in computational image analysis or behavioral studies.

Response: We thank the referee for pointing this out and have now included sample data as supplemental material as suggested.

Reviewer #2:

Manuscript Summary:

The manuscript by Collins and her coworkers provides the tools and methods for developing a system to track planarian "scrunching", locomotory behavior associated with noxious stimuli. The gear and techniques involved to put this system together are readily accessible without the need for great expense or training. Planarians themselves are a very useful model system that can be manipulated via genetic and pharmacological techniques to understand basic neurobehavioral principles. Together, this visual recording technique for Planarians can be used for a variety of research and educational purposes including species-specific changes in locomotion and responses to nociceptive stimuli. In fact, this group has already used this approach in one peer-reviewed study about Planarian responses to noxious stimuli.

Major Concerns:

This is a very well-written submission and I have no major or minor concerns.

Response: We thank the reviewer for their positive comments.

Reviewer #3:

Manuscript Summary:

Sabry et al. present a new and quantitative protocol analyzing the locomotion of the freshwater planarian. This manuscript focuses on three gaits of movement that are distinguishable through video and quantitative analysis. The authors propose a new protocol for the induction of scrunching using various physical and chemical stimuli as well as the quantitative analysis of planarian behavior to these stimuli as an alternative to the difficulties of cilia imaging. Overall, this review is concise and well written. However, to enhance the strength of the paper, some points require clarification and further analysis. The authors may want to consider the aspects described below:

Major Concerns:

1. The paper is clear and easy to follow. However, the authors may expand on the justification of the protocol. In the current version, the need for the protocol is not readily evident. The text in the introduction may be the right location for placing the need in context.

Response: We have expanded the Introduction to more clearly convey the broader context and applicability of this protocol.

2. The discussion could be enhanced by placing the opportunity of measuring scrunching in planarians in the context of the field. Currently, the discussion is more focused on the troubleshooting of the protocol. Thus, incorporating comments that broadly represent the advantage of this protocol and how this could help to illuminate some areas that are difficult in other model organisms or phenotypes may prove beneficial for the field in general.

Response: In the original manuscript, the Discussion was focused on the technical aspects of the protocol, per JoVE guidelines. Following the reviewer's advice, we have expanded the new Discussion to provide broader context of the applicability and benefits of studying planarian scrunching using our protocol, including the unique advantages provided by the planarian system.

3. In 1.1.5.1 the authors mention, "If a planarian reaches the boundary of the arena without satisfying the termination criterion, pipette the planaria back to the center of the arena." However, this may cause inaccurate data as some planaria will be exposed to the scrunching/peristalsis chemical solution for a more extended period than others if they are used for repeated experiments. As stated in 1.1.5, a standard experiment "Consists of completing 1-2 minutes of gliding behavior or recording long enough to capture at least three consecutive oscillations occurring in a straight line." The recommendation for the author is to clarify, is if they are implying on reusing a Planaria or set of Planaria until they get their expected result?

Response: An additional NOTE has been added to clarify this point. While the duration of gliding experiments without chemical stimulus is less important as long as sufficient data is generated, it is important to keep the duration of chemical exposure constant, as pointed out by the referee. The newly added NOTE makes this distinction clear. Planarians should not be reused in the same experiment when exposed to a stimulant; however, they can be and often should be reused for first taking gliding data and then exposing them to a stimulant, to have a record of the baseline behavior.

README

For each planarian gait (gliding, scrunching, and peristalsis), a folder containing a sample dataset is included. Each dataset contains an .avi movie of the respective behavior, which can be opened and converted to 8-bit in Fiji in the same manner as a file sequence, as described in Step 1.2.2 in the protocol. All .avi files were recorded at 10 FPS. As these data have already been pre-processed to extract an appropriate behavioral sequence for one planarian, the reader should start at Protocol Step 1.2.4 to continue with the analysis. The reader can check their analysis by comparing to the provided .csv files containing the tracking data and the .png oscillation plot for scrunching and peristalsis. Parameters for the three sample gaits are provided in the data table below.

The provided data are of *Schmidtea mediterranea* planarians. The various gaits were induced by the following conditions:

Gliding: SmTRPA RNAi planarians in 40 mM H_2O_2

Peristalsis: Wild-type planarians in 100 µM anandamide

Scrunching: Wild-type planarians in 100 µM AITC

Gait	**Frequency (cycles s^{-1})**	**Maximum elongation**	**Speed (body length s^{-1})**	**Fraction of time spent elongating**
Gliding	--	--	0.21	--
Peristalsis	0.25	0.29	0.07	0.50
Scrunching	0.42	0.45	0.19	0.55

Video or Animated Figure

Click here to access/download

Supplemental Coding Files

Smed_100uM_anandamide_peristalsis.mp4

Video or Animated Figure

Click here to access/download

Supplemental Coding Files

Smed_100uM_AITC_scrunching.avi

Click here to access/download

Supplemental Coding Files

Smed_100uM_AITC_scrunching.png

Video or Animated Figure

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Supplemental Coding Files

SmTRPA_40mM_H2O2_gliding.avi

Figure 1

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Supplemental Coding Files

Figure1.svg

Figure 2

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Supplemental Coding Files

Figure2.svg

Figure 3

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Figure3.svg

Figure 4

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Figure4.svg

Figure 5

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Supplemental Coding Files

Figure5.svg