Journal of Visualized Experiments

Planarian motility provides a sensitive and accessible means to gauge the biomodulating properties of natural products

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61070R1
Full Title:	Planarian motility provides a sensitive and accessible means to gauge the biomodulating properties of natural products
Section/Category:	JoVE Biochemistry
Keywords:	planarian, pLmV, motility, behavior, stimulant, withdrawal
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Morrisville, NY

TITLE:

Planarian Motility Assay to Gauge the Biomodulating Properties of Natural Products

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KEYWORDS:

planarian, pLmV, motility, behavior, stimulant, withdrawal

SUMMARY:

Planarian motility is used to gauge the stimulant and withdrawal properties of natural products when compared to the movement of the animals in spring water alone.

ABSTRACT:

A straightforward, controllable means of using the non-parasitic planarian, *Dugesia tigrina*, a free-living aquatic flatworm, to study the stimulant and withdrawal properties of natural products is described. Experimental assays benefitting from unique aspects of planarian physiology have been applied to studies on wound healing, regeneration, and tumorigenesis. In addition, because planarians exhibit sensitivity to a variety of environmental stimuli and are capable of learning and developing conditioned responses, they can be used in behavioral studies examining learning and memory. Planarians possess a basic bilateral symmetry and a central nervous system that uses neurotransmitter systems amenable to studies examining the effects of neuromuscular biomodulators. Consequently, experimental systems monitoring planarian movement and motility have been developed to examine substance addiction and withdrawal. Because planarian motility offers the potential for a sensitive, easily standardized motility assay system to monitor the effect of stimuli, the planarian locomotor velocity (pLmV) test was adapted to monitor both stimulation and withdrawal behaviors by planarians through the determination of the number of grid lines on the path crossed by the animals with time. Here, the technique and its application are demonstrated and explained.

INTRODUCTION:

The protocol described uses planarian motility to provide a means of assessing the biomodulating effects of natural substances. It was specifically adapted to determine if these substances function as stimulants, and if they then were associated with a measurable withdrawal behavior[1]. This assay, known as the planarian locomotor velocity (pLmV) test, was first used to test known pharmacological agents[2,3]. The application of this planarian motility-based assay has since grown in popularity and has been adopted by different laboratories interested in a substances other than natural products[4,5]. A means to assess planarian motility is presented here, where an animal is placed on a plate with a grid, and the number of grid lines crossed within a period of time are counted to determine how much the animal moved. The light/dark test, otherwise referred to as the conditioned place preference test (CPP), is another variation on the theme of monitoring planarian motility, and assesses how quickly the animals respond and migrate to a darkened environment[6,7]. Video tracking of planarian movements can also be analyzed using computer programs and center of mass (COM) tracking[8-11].

Using the planarian as an animal model for such studies offers several advantages over other animals in that the experimenter can easily control the assay environment. Specifically, starving the planarians before experimentation can prevent their exposure to other nutritional or pharmacological agents that might otherwise confound the results, and the specific biomodulator under investigation can be introduced to the planarians simply by adding it directly to the culture water, thereby standardizing exposure. Furthermore, because planarians have a nervous system and neurotransmitters that are reminiscent of 'higher-order' animals, the physiology and experimental responses of these animals to neuromuscular stimuli are considered relevant to other organisms[12-16]. Also, because planarians are relatively inexpensive and straightforward to maintain in the laboratory, they offer an accessible biological model for many investigators.

As an experimental animal, planarians are suitable to a wide range of studies. For example, investigators use planarians to study tumorigenesis[17-19]. Planarians also exhibit a host of response behaviors to chemical, thermal, gravitational, electrical, photo, and magnetic stimuli that have formed the basis of other assay systems. Some of these effects have been used to study learning and memory in these animals[20-27]. The primary use of the planarian model in the literature at present focuses on the activity of planarian pluripotent stem cells, called neoblasts, and their role in regeneration[28-30]. Thus, adopting the model described here allows for further study using other planarian-based assays to provide a broader understanding of how natural products and other biomodulators affect the organism.

PROTOCOL:

1. Planarian husbandry

1.1. Use planarians purchased from a biological supply company or wild-caught if needed. The planarians used in this protocol are *Dugesia tigrina*, as listed in the supply list. This species is also

referred to as *Girardia tigrina*[31]. Other aquatic planarian species are also acceptable[2,3].

NOTE: The protocol described is geared to animals purchased from a biological supply company. This assay system has not been tested with wild-caught planarians. However, if wild-caught planarians are used, it is recommended that they be habituated to the water used in the experiments as well as the laboratory environment for at least 1 week before use.

1.2. Upon arrival, transfer planarians to plastic food storage containers containing clean spring water and keep the lids ajar.

1.3. Maintain planarians in a darkened environment.

1.4. Feed newly delivered planarians after 24–36 h in their new environment.

1.5. Allow planarians to acclimate to the laboratory at least 1 week prior to experimentation.

1.6. Feed planarians on a regular twice-a-week schedule.

1.6.1. Allow planarians to feed ad libitum on chopped organic boiled eggs or blended organic beef liver for 1–2 h.

1.6.2. Place fed planarians into a clean container after feeding.

1.6.2.1. Remove the soiled water off the planarians.

1.6.2.2. Use a small flat watercolor paintbrush (number 3–6) to transfer food debris and slime adhering to the container from around planarians to a paper towel.

1.6.2.3. With fresh spring water and gentle swirling or agitation, dislodge planarians and pour them into a clean container.

1.6.2.4. Any planarians remaining adhered to the container can be transferred using a round watercolor paintbrush (number 3–6) or transfer pipette with a wide bore.

NOTE: When manipulating planarians, use a small, clean, flat, or round watercolor paintbrush (number 3–6). When moving planarians, the brush should be placed under the animal to lift it gently. To ensure planarians are not damaged when using the brush, the bristles of the brush should not be splayed out under the animal. Spreading out of the bristles could harm the planarian if it is caught between the fibers of the brush.

1.6.2.5. Decant the transfer water.

1.6.2.6. Cover the planarians with clean spring water.

1.6.2.7. After 24 h, remove the planarians from any expelled food waste by transferring them to a clean container as described above (steps 1.6.2.1–1.6.2.6).

1.7. To clean containers and utensils used for planarian husbandry, do not use soap or detergent. Clean these items by rinsing them well with clean water (tap water is acceptable) and drying them with a clean cloth or paper towel.

2. Preparation of planarians for experiments

2.1. Allow newly delivered planarians to acclimate to their environment at least 1 week prior to experimentation.

2.2. Starve planarians for 5–10 days prior to experimentation.

2.3. Change the culture water at least 1x during the starvation period.

3. Planarian locomotor velocity (pLmV) test: Stimulant behaviors

3.1. Prior to experimentation, ensure starved planarians are fully formed, with a complete and pigmented head and tail.

3.2. Prepare a glass or plastic 10 cm Petri dish and a habituation container for the pLmV test prior to starting the experiment.

3.2.1. Place a clean 10 cm diameter Petri dish to be used for the pLmV test on prelaminated grid paper with 0.5 cm squares).

3.2.2. Add 20 mL of unadulterated spring water for controls, or spring water containing the appropriate concentration of the natural product being tested, to the 10 cm diameter Petri dish to be used for the pLmV test.

3.2.3. Position a camera (e.g., cell phone or high-resolution camera) above the prepared 10 cm diameter Petri dish to record planarian motility over the grid paper during the experiment. A ring stand is a convenient way to position the camera at a distance that can record the entire view of the 10 cm Petri dish and grid lines.

3.2.4. Prepare a habituation container with 5–10 mL of unadulterated spring water (controls), or spring water containing the appropriate concentration of the natural product being tested. A container similar to a scintillation vial or small 5 cm Petri dish is suitable.

3.3. Use a small, clean, flat, or round watercolor paintbrush to gently transfer a planarian from the stock container with spring water to the habituation container having 5–10 mL of unadulterated spring water or spring water containing the natural product being tested.

NOTE: A wide bore transfer pipette can also be used for transferring the planarian into a clean and dry habituation container.

3.3.1. If using the pipette, remove excess water moved with the planarian from the habituation container using the transfer pipette.

3.3.2. Carefully add the habituation solution (i.e., spring water for controls or spring water containing the concentration of natural product being tested) to the habituation container containing the planarian.

3.4. Habituation periods will depend on the stimulation dynamics assessed for the natural product being tested. A habituation time of 2 min proved acceptable to detect stimulation in this work[1].

3.5. Following the 2 min habituation period use a watercolor paintbrush to gently transfer the planarian to the center of the prepared 10 cm Petri dish for the pLmV stimulation experiment. Gently move the animal from the habituation container to the 10 cm Petri dish.

3.6. Start the camera to record the movement of the planarian. Record 10–11 min of video.

3.7. Prepare the habituation container and the 10 cm Petri dish for the pLmV experiment with fresh solutions for each planarian.

3.8. Having dedicated pipettes, dishes, containers, and paintbrushes for each experimental concentration of the natural product being tested is a convenient way to avoid inadvertently exposing planarians to the wrong solution during experimentation.

3.9. Because planarians exhibit learned behaviors, each planarian (control or test) should only be used once[21,22].

4. Planarian locomotor velocity (pLmV) test: Withdrawal behaviors

4.1. Prior to experimentation, ensure starved planarians are fully formed, with a complete and pigmented head and tail.

4.2. Prepare a 10 cm Petri dish (glass or plastic) for the pLmV experiment, a 5 cm Petri dish (glass or plastic) for rinsing the planarian following habituation, and a habituation container prior to starting the experiment.

4.2.1. Place a clean 10 cm diameter Petri dish to be used for the pLmV experiment on prelaminated grid paper with 0.5 cm squares).

4.2.2. Add 20 mL of unadulterated spring water to the 10 cm diameter Petri dish to be used for the pLmV experiment.

4.2.3. Position a camera above the prepared 10 cm diameter Petri dish as in step 3.2.3 to record planarian motility over the grid paper during the experiment.

4.2.4. Prepare the planarian rinse container by adding 5 mL of spring water alone to the 5 cm Petri dish.

4.2.5. Prepare a habituation container with 5–10 mL of unadulterated spring water (controls) or spring water containing the natural product being tested. A container similar to a scintillation vial or small 5 cm Petri dish (glass or plastic) is suitable.

4.3. Use a small, clean, flat, or round watercolor paintbrush to transfer a planarian from spring water to the prepared habituation container having 5–10 mL of unadulterated spring water (controls) or spring water containing the natural product being tested. Gently move the animal from the stock container to the habituation container. Ensure the planarian is not damaged by the brush.

NOTE: A wide bore transfer pipette can also be used to transfer the planarian into a clean and dry habituation container.

4.3.1. If using the pipette, excess water moved with the planarian should be removed from the habituation container using the transfer pipette.

4.3.2. Carefully add the habituation solution (i.e., unadulterated spring water for controls or spring water containing the natural product being tested) to the habituation container containing the planarian.

4.4. Habituation periods for withdrawal will depend on the stimulation dynamics assessed for the natural product being tested; 2–5 min have proven sufficient.

4.5. Following the habituation period use a watercolor paintbrush to gently transfer the planarian to the prepared 5 cm Petri dish containing spring water to rinse off any natural product from the habituation container. Ensure the planarian is not damaged by the brush.

4.6. Immediately transfer the planarian to the center of the prepared 10 cm Petri dish containing spring water for the pLmV withdrawal experiment. Ensure the planarian is not damaged by the brush.

4.7. Start the camera to record the movement of the planarian. Record 10–11 min of video.

4.8. Prepare the habituation container, the rinse container, and the 10 cm Petri dish for the pLmV experiment with fresh solutions for each planarian.

4.9. Use dedicated pipettes, dishes, containers, and paintbrushes for each experimental

concentration of the natural product being tested to avoid inadvertently exposing planarians to the wrong solution during experimentation.

4.10. Because planarians exhibit learned behaviors, use each planarian (control or test) only once[26,27].

5. Data analysis

5.1. Prepare a data collection table to document the behavior and the motility of planarians as the number of grid lines crossed for each minute during the pLmV run. The table should allow for the accumulated number of lines per minute crossed by the planarian to be documented as well. Include lines for notes and a table of definitions to tally the observation of behaviors during the experimental period, such as 'wander' and 'stop' (see **Discussion**).

5.2. Using the video, count the number of full grid lines crossed by the planarian per minute for 10 min, and record that number on the data table. Typical planarian behavior consists of continuous velocity, forward-directed, horizontal movement, with periodic turns, and without stops.

5.2.1. Begin to time the experiment at the point that the planarian has moved off the paintbrush used to transfer it to the 10 cm Petri dish. Record this start time.

5.2.2. To determine when the animal crosses a full grid square, focus on the head and score one line when the head fully crosses a square.

5.2.3. To score a full grid when the worm moves around the edge of the dish, visualize a distance of 0.5 cm by referring to the lines as they extend out from the margins of the dish. If the planarian crosses the corner of a box, refer to the second line crossed to score one grid line. Again, focus on the head to make these determinations.

5.2.4. Stop the video after each minute to record the data.

5.2.5. When restarting the video to count the next minute, if the head of the worm was between grid lines when the video was stopped, record the first line crossed as a full box.

5.2.6. Score the number of grid lines crossed for 10 min.

5.3. If the animal stops moving during a pLmV test and no longer crosses grid lines during the 10 min recording time, document the behavior of the planarian in the behavioral chart (e.g., 'wander' or 'stop'). Animals that cease their forward track during the pLmV assay should instead be taken note of and the data presented as a frequency of the total number of animals exposed to that reagent concentration. Coiling or convulsive behavior (known as a C behavior) preventing any forward movement during the habituation period indicates that the concentration of the natural product is not appropriate for use in a pLmV assay because the pLmV assay is motility-

based. C-type behaviors can be analyzed using a different type of analysis (see **Discussion**).

5.4. If possible, test multiple experimental concentrations of the natural product using at least 9–12 worms on different days and different times of the day if determining the overall effect of the reagent on planarian physiology. However, if researchers endeavor to reduce circadian rhythm-induced variability, experiments can be conducted with constant lighting at a set time of day using worms that are cultured with timed light/dark cycles and set feeding times. Have at least two experimenters involved in the project to allow for the option of having one individual record data, while the second individual counts the grid lines 'blind' to the conditions used for data collection. Having different individuals involved in data collection, as well as statistical calculations and analysis, also reduces possible bias.

5.5. Calculate grid line counts for each natural product concentration on each day as relative to control counts for each minute so that data from different days, times, and experimenters can be combined. These data can be averaged and then analyzed using Student's T-test. P-values for each test can be assessed per minute compared to the control and between reagent concentrations. ANOVA assessments using data sets derived from different experimental concentrations provide a further method of analysis.

REPRESENTATIVE RESULTS:

The laboratory setup and preparation of the workspace for the pLmV assays should be completed before experimentation begins. This includes the preparation of the habituation container, rinse container if needed (for withdrawal experiments), Petri dish over laminated grid paper, and properly placed camera (**Figure 1**). Once all the videos are taken, it is advisable to use a common datasheet to standardize data collection and presentation between investigators (**Figure 2** and **Figure 2 Supplement**).

The camera setup allowed for a clear view of the planarian and the grid paper to permit an accurate assessment of the progress of the animal for the duration of the experiment (**Figure 3A** and **Figure 3A Supplement**). Data collection included the number of grid lines crossed, as well as the cumulative total number of lines crossed per minute of the experiment (**Figure 3B**). During pLmV analysis, planarians had a continuous velocity, forward-directed, horizontal movement, with periodic turns, and do not stop. When beginning, the first full grid box crossed was scored as one, not the first line crossed as seen in the example video. It was important to record the start time of directional movement after the planarian was free of the paintbrush used to transfer it to the pLmV dish, and then followed each minute thereafter. If a planarian was part way through a box at the minute time, the next grid line crossed after restarting the video was counted as a full box. When the animal moved around the edge of the dish, the lines as they extend out from the dish were referred to in order to determine a distance of 0.5 cm. When worms encountered the corner of a box, the second line crossed was referred to in order to score one grid line. These determinations were always made relative to the position of the head. If the planarian began to cover a tight area, the experimenter followed the head of the worm to monitor the distance of a full grid box. An example of this behavior is included in the supplementary video (**Figure 3A Supplement**).

To standardize the results from each trial, the pLmV runs were calculated and plotted as the number of boxes crossed relative to the progress of the matching control worm (**Figure 3B**). Each user should be trained to perform the assay and counting grid lines prior to beginning tests using reagents of interest. As a benchmark, using the experimental set up, planarians in spring water typically covered approximately 24 boxes in 3 min (**Figure 4A**; data from 4 users, average of 24.8 ± 4.8). A range of test reagent concentrations were examined to determine the type of behavioral analysis to be used for each. For pLmV analysis, researchers should determine if the animals display motility when exposed (**Figure 4B**). Other types of behavioral analyses may be more effective for different types of behaviors (see **Discussion**). Relative to the spring water control, stimulant data showed an increasing number of grid lines crossed as the animal moved through the pLmV container with the desired concentration of the test reagent in spring water after being habituated in the same concentration of the test reagent. In contrast, withdrawal data showed a decreasing number of grid lines crossed relative to the spring water control, when the planarian moved through the pLmV container having spring water alone after being habituated in the desired concentration of the reagent mixed in spring water (**Figure 4C** and **4D**). Notably, plain withdrawal data can lead to fewer grid counts than those of the controls, as has been observed in drug-induced withdrawal data by other groups[2,6].

Spring water control planarians moved over grid lines for the duration of the experiment. At times, however, the test planarians ceased crossing grid lines during the assay. When this occurred, these data were tallied separately. Documenting these data as a percentage of the total number of animals exposed to that particular reagent concentration was an effective way to illustrate the relative frequency of these findings (**Figures 5A–C, Figure 5A Supplement** and **Figure 5B Supplement**). Behaviors not preventing the planarians from crossing grid lines were included in the pLmV analyses, even if these movements impeded the steady progress of the animals.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative setup for the pLmV assay. The laboratory space was prepared prior to beginning the assay. Shown is a typical setup with a 10 cm Petri dish placed over laminated 0.5 cm grid paper. A document camera was positioned such that a clear view of the 10 cm Petri dish could be recorded on a linked computer. However, any camera can be used for recording the progress of the planarians during the experiment including a cell phone camera positioned above the Petri dish using a ring stand. In the background is a 5 cm Petri dish to rinse worms for withdrawal experiments, as well as small white containers used for habituating the planarians. Also, in the background are labeled, dedicated round watercolor paintbrushes, pipettes, and Petri dishes for each product concentration.

Figure 2: pLmV data sheet. A prepared data sheet to record the number of grid lines crossed for the tested substance and the control data, as well as a means to tally any behaviors useful for data collection purposes. See **Figure 2 Supplement** for a downloadable PDF version of this figure.

Figure 3: Representative pLmV experiment and data sheet. (**A**) A camera was placed above the

10 cm Petri dish used for the pLmV assay such that the planarian and the full 0.5 cm grid under the dish was clearly visible. The full pLmV run was recorded (**Figure 3A Supplement**), and the number of grid lines crossed each minute placed into a prepared data table (**B**). The accumulated total grid lines crossed were tallied, and then these were converted to the number of accumulated lines crossed relative to the corresponding spring water control worm (**B**). The data on the table provided (**B**) matches the counts in the supplementary video (**Figure 3A Supplement**). Corresponding control counts are not shown.

Figure 4: Representative graphs of stimulant and withdrawal data. Researchers should be trained to use and score the pLmV assay grid lines using spring water alone prior to experimentation. Typically, planarians travelled about 25 grid lines in 3 min. (**A**) Data from four users; 10 spring water control worms each. To appreciate the effect of a reagent on planarian motility a series of concentrations were surveyed using the pLmV assay and the total number of grid lines crossed were examined relative to the corresponding control counts at 3 min. (**B**) Control data represented by white bar. Test data for different concentrations (mM) of test substance represented by black bars. Test data were plotted relative to control data to best represent all the data collected. (**C**) Stimulant data represented by blue line/diamonds show a rise in grid lines crossed, versus (**D**) withdrawal data represented by blue line/diamonds, showing a decreasing slope from the initial start value relative to the control data, represented by red line/squares in both figures **C** and **D**. Error Bars = ±S.D.

Figure 5: Observation and documentation of behavioral data. Planarians in the pLmV assay that did not maintain directional movement during the experiment, but stopped with characteristic behaviors and no longer moved over the grids were tallied. Typical behaviors included displays 'wander' (**Figure 5A** and **Figure 5A Supplement**), and 'stop' (**Figure 5B** and **Figure 5B Supplement**). These tallies of behavioral data are presented to document the frequency of these behaviors compared to all the animals exposed to that product concentration (**C**). Shown are sample data documenting the percentages of spring water control animals (**Ci**), and animals exposed to a stimulant (**Cii**), having a wander (blue), stop (red), or no (green) behavior.

DISCUSSION:

A straightforward and accessible planarian motility assay is described to determine the stimulant and withdrawal effects of natural products. As a behavioral model it is necessary to have stringent protocols for scoring movement and clear definitions of any behaviors to standardize observations between different experimenters. The ideas presented offer a demonstration of how this can be achieved. Each laboratory using this protocol should adapt the presented information to suit the effects of the particular product being tested. It is recommended to carefully set up the workspace to ensure tests can be done in consistent conditions by each investigator involved in the study (**Figure 1**). Standardized recording sheets can be compiled to assist with precise record keeping and data collection on motility and behaviors (**Figure 2** and **Figure 3B**).

Motility tests should be done at various times of the day, using different batches of planarians if possible, to account for diverging inherent circadian activities. While these analyses have not

been conducted, it is suggested that investigators monitor how the planarian circadian rhythm might affect motility by maintaining planarians using a standard light/dark cycle (e.g., 12/12), and when conducting the pLmV assay, use standard lighting conditions and run the tests at the same time of day[32-34]. Researchers should practice the mechanics of the pLmV assay and learn to count grid lines to standardize both the handling of the planarians and the assessment of planarian motility (**Figure 3**). This should be done using unadulterated spring water controls. Typically, after 10–20 such practice runs, data becomes quite standard for all laboratory members. Counts after a set pLmV time should be chosen to compare progress in learning the assay. Investigators typically observe approximately 24 grid lines crossed by 3 min in the pLmV assay in spring water following a 2 min habituation in spring water (**Figure 4A**; representative data for four investigators with 10 random spring water controls each; 24.8 ± 4.8 grid lines). Having two or three trained investigators conducting each set of tests can further improve the reliability of the results because any user-specific effects on the method and counts can be taken into account in the error analysis. In this way, tests and counts can be swapped between investigators so that counts can be done 'blind' to the experimental conditions. To decrease the chance of bias, different individuals could conduct statistical tests on the count data and perform the subsequent analyses. Outside of the presence of the product being tested, environmental variations in water quality such as temperature and pH should be avoided. Because planarians are light sensitive, the setup of the experiment should ensure that the lighting of the workspace is even. Finally, because planarians can exhibit learned behaviors, each worm, including controls, should only be used once[26,27].

It is useful to have an experimental checklist in the lab if multiple experimenters are working with the assay, particularly in an undergraduate research laboratory, to avoid common pitfalls that might occur when running the pLmV test, which would affect the statistical value of the results. All solutions should be at room temperature because planarian motility is reduced at lower temperatures. Care should be taken to ensure that the specimens are starved 5–10 days prior to their use and that each specimen is fully formed, with a completely pigmented head and tail. It is also suggested that dedicated flat or round watercolor paintbrushes (number 3–6), habituation containers, and Petri dishes be used for each experimental concentration to make the workflow smoother for the experimenter. The technique of transferring the planarians using small flat or round watercolor paintbrushes should be practiced extensively by the investigators to ensure efficient transfer of the worms between containers without causing injury or distress to the animals (see discussion above; **Figure 4A**). The use of these paintbrushes minimizes the transfer of liquids between the containers and reduces potential stress on the planarians. However, planarians can be damaged by the bristles if the fibers are spread or splayed out when they contact the animal

Because the pLmV assay relies on behavioral data, it is essential to use a sufficiently large data set to ensure robust data despite innate worm-to-worm response variability. As such, most laboratories use a minimum of nine to twelve planarians to test each concentration of the product being examined, particularly because natural products may not have as marked an effect as standard pharmaceuticals[1-7,13-16,35,36]. The number of grid lines crossed each minute is calculated relative to the spring water control data for each test day and time. These data are

485 averaged for each test concentration for each minute and compared to the control data, as well
486 as to other time-matched concentrations using both Student's T-tests and ANOVA.
487
488 The pLmV assay is geared to studies of reagents affecting the motility of the planarians for the
489 duration of the assay time. Detailed studies of planarian movements can be conducted using a
490 number of other assessments described in the literature[4,35-38]. Therefore, it is prudent to conduct
491 a series of habituation experiments to take note of how the planarians react to the test reagent
492 using a range of concentrations and comparing any effects with the behavior of the worms in
493 spring water prior to embarking on behavioral tests. In this way, the appropriate behavioral test
494 can be selected to study the individual mannerisms induced by the reagent on the planarian. The
495 planarians can be placed in various concentrations of the reagent for 5–10 min to determine if a
496 concentration permits them to maintain their typical swimming behavior. Behaviors that do not
497 permit motility, such as those resulting in a C-type, or convulsive or seizure-like behavior, have
498 been the focus of studies using separate types of behavioral analyses that can be applied to this
499 method[4,39,40]. pLmV analyses can be conducted using a range of concentrations after short
500 habituation times and the number of total grid lines crossed at 3 min scored relative to spring
501 water controls prior to time course stimulation and withdrawal analyses (**Figure 4B**)[1-5].
502 Investigators are encouraged to sample other times, such as 15, 30, and 60 min, to see if the
503 stimulation dynamics change with time of exposure[1]. As has been reported, in spring water
504 control worms maintain a steady, forward-directed horizontal movement for the 10 min assay
505 time[1,4]. In contrast, product-treated worms may stop and cease their directional motility during
506 the assay and no longer cross grid lines. The investigator can make a judgment whether to limit
507 the length of the experimental pLmV run or derive a means to assess these behaviors as
508 described. It is important, however, to assess the frequency of these behaviors, asf they provide
509 additional data affecting motility. Motility and movement data are discussed separately in the
510 field because combining the information confounds the assessment of such data[4,35-38]. Two
511 behaviors were observed when the worms stopped and no longer crossed grid lines during the
512 assay. These movements are referred to as 'wander' (**Figure 5A** and **Figure 5A Supplement**), and
513 'stop' (**Figure 5B** and **Figure 5B Supplement**). The frequency of these behaviors are documented
514 as percentages of all the animals exposed to the particular product concentration together with
515 the control data (**Figure 5C**). Importantly, random behaviors that do not prevent the planarian
516 from crossing grid lines should be included in the pLmV analysis, even if these movements
517 impede the steady progress of the animals (**Figure 4B**, 3 mM and 10 mM bars). As mentioned,
518 investigators have described a number of behavioral categories that are beyond the scope of this
519 discussion of the pLmV rate of motility protocol and have not been observed using the products
520 described[4,35-38].
521
522 The pLmV assay relies on the water solubility of the product under investigation. Many of these
523 substances, however, are not fully soluble in water and, as such, only the water-soluble portions
524 can be tested by this assay while the rest must be filtered from the solution as has been done in
525 previous work[1]. If pLmV assays are run using reagents solubilized using solvents other than water,
526 these require a volume equivalent control in addition to the spring water control. While this
527 method has not been used with such substances, such vector controls should likely be treated as
528 a test, and the motility scored relative to controls as would any other test substance. Another

possible means to test non-soluble substances would be to feed them to the planarians by mixing them into food gels. This technique is used to introduce siRNA to planarians in gene knock-down/RNAi experiments[41]. Feeding planarians biological products and siRNA, however, presents complications to this assay in that the planarians would not be starved and cannot be transferred to the motility assay container within a standardized time that ensures equal exposure or intake of the product under investigation by the experimental animals prior to testing.

Once stimulant and withdrawal dynamics are established using the pLmV assay, further experimentation can involve comodulators, the introduction of siRNA, as well as biological pathway modifiers or drugs to test for the augmentation or inhibition of the observed motility effects compared to the initial results collected when not using these biomodulators[1,3]. For example, downregulating the expression of a gene or adding pathway inhibitors could decrease the rate of movement, while others may change the dynamics of withdrawal. Through the observation of changed pLmV results, these additional experiments can provide insight into the underlying physiology affected by a natural product or other test reagent.[5,42-44].

The procedure described is amenable to any laboratory interested in determining the stimulant and withdrawal effects of a variety of biomodulators, including many natural products. Advantages of the application of the planarian pLmV assay include how inexpensive and easy to maintain these animals are, and that they can also be subjects for other planarian-based assays to provide a broad understanding of the physiological effects of the product under investigation.

ACKNOWLEDGMENTS:

The authors wish to acknowledge the office of Institutional Advancement, and the Morrisville College Foundation for a publication grant to support this work, as well as the SUNY Morrisville Collegiate Science and Technology Entry Program (CSTEP) for their ongoing assistance and support of undergraduate research at SUNY Morrisville. We also wish to thank Sophia Hutchens for helpful comments on the technique described.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

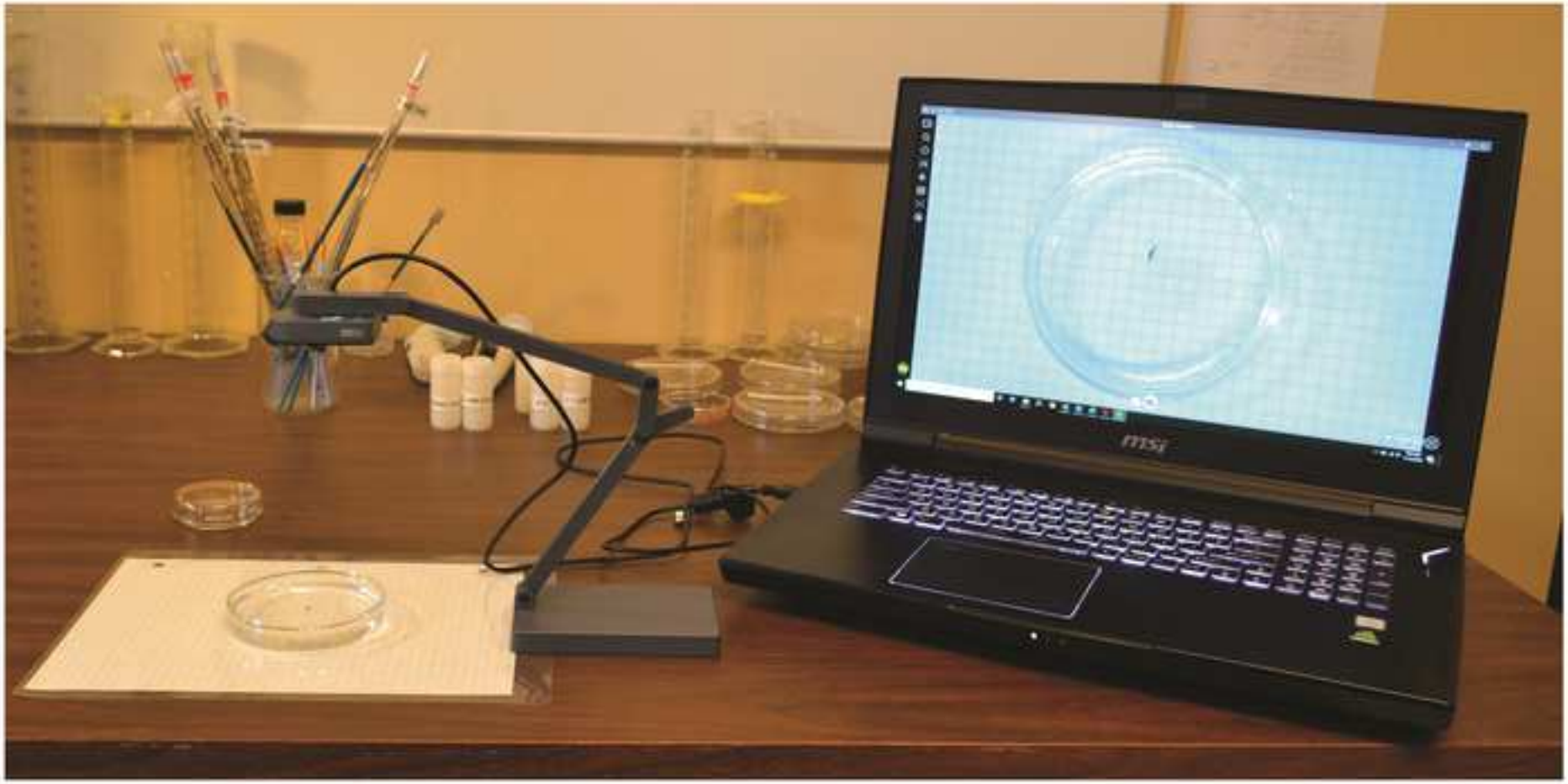


Figure 2 Click here to access/download;Figure;Figure2Final.tif

Name: _____ Date: _____

Habituation (Concentration of Product and Time): _____

pLmV (Concentration of Product): _____

Count Start Time: _____

Time (minutes)

	1	2	3	4	5	6	7	8	9	10
Grid Count										
Accumulated Totals										
Relative to Control										

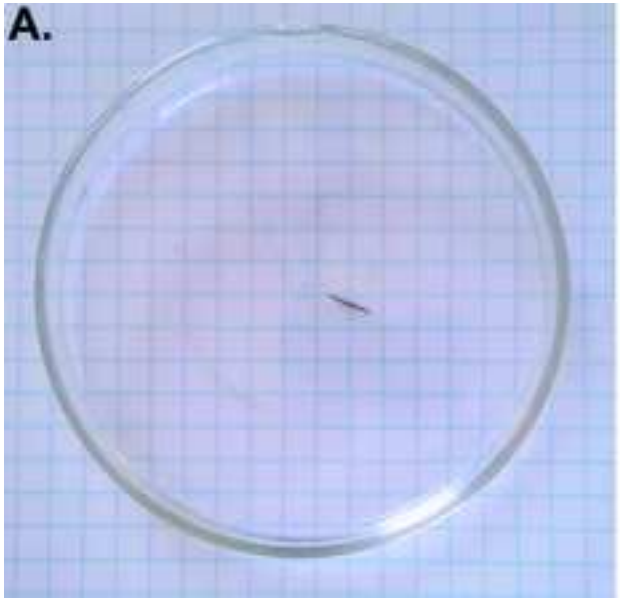
Notes: _____

Behaviors

Wander	Stop

Figure 3

A.



B.

	Time (minutes)									
	1	2	3	4	5	6	7	8	9	10
Grid Count	7	8	9	9	9	8	10	8	10	9
Accumulated Totals	7	15	24	33	42	50	60	68	78	87
Relative to Control	1.40	1.36	1.41	1.44	1.56	1.51	1.60	1.58	1.59	1.58

Figure 4

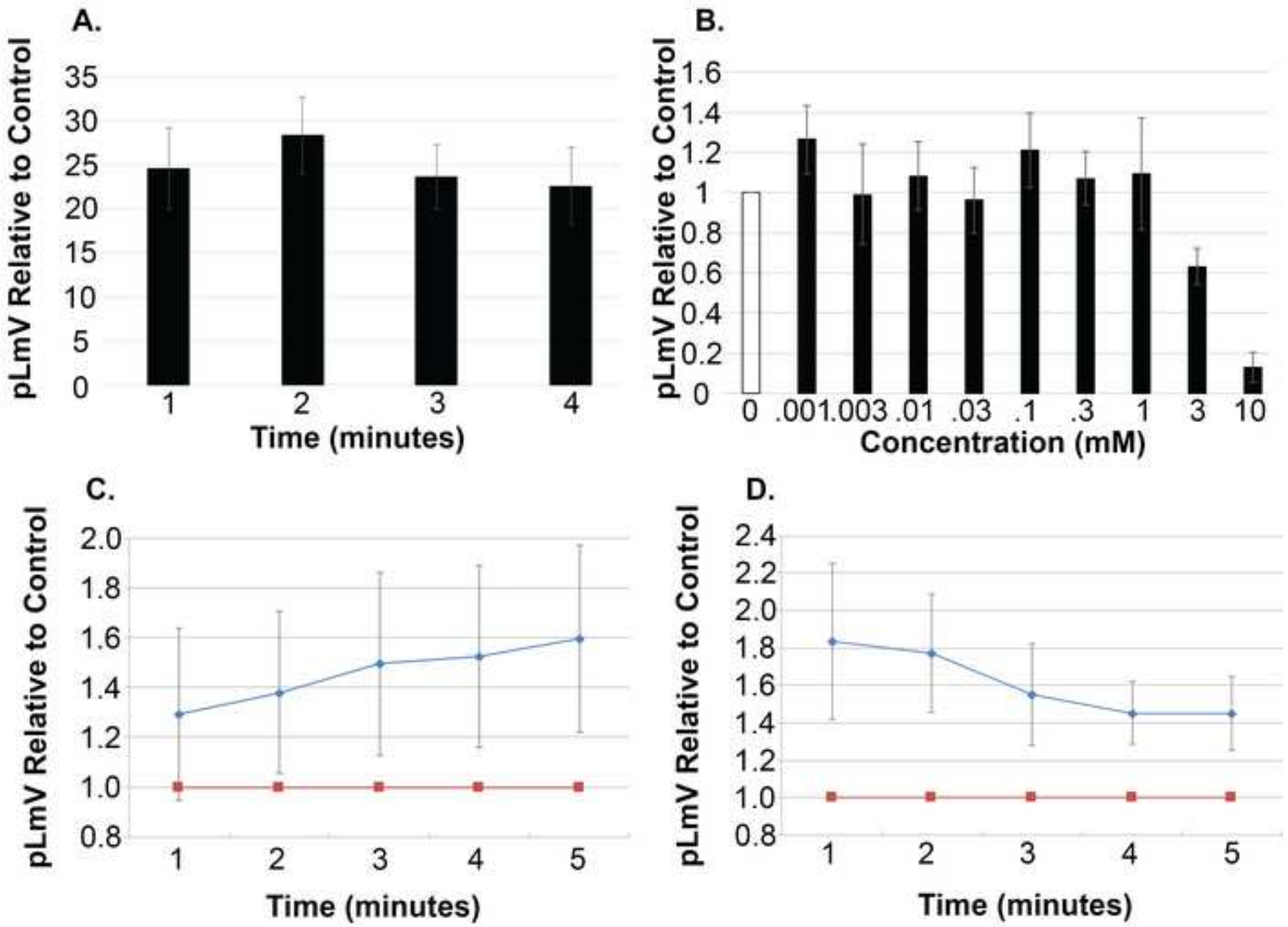


Figure 5

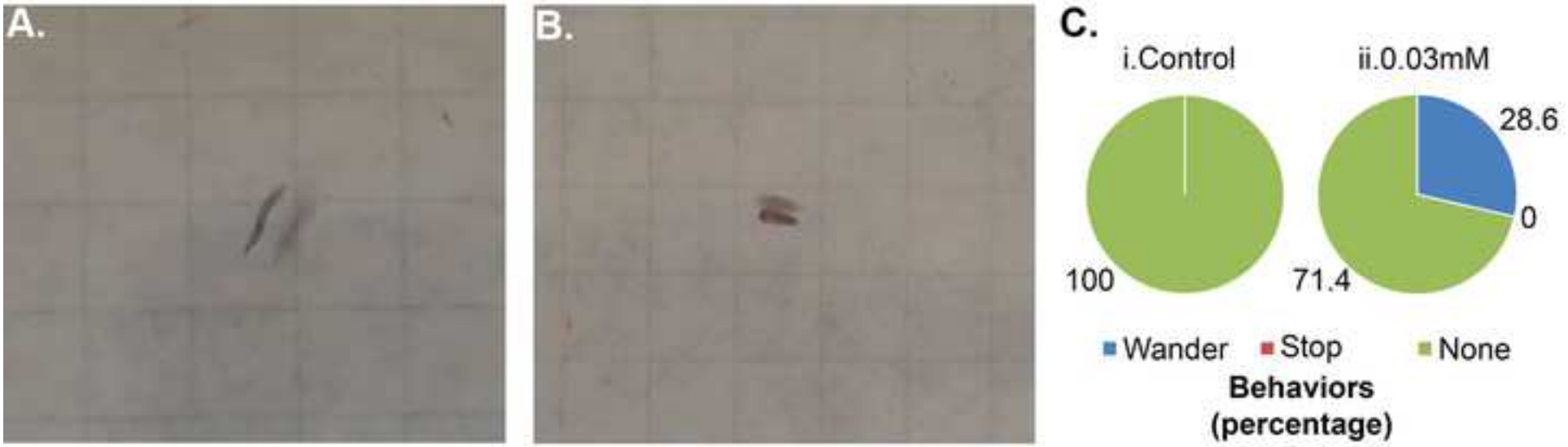


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_VouraEtAlpLmV.xlsx

Name of Material/ Equipment	Company	Catalog Number
Bottled Water - 1 Gal.	Poland Spring	N/A
Brown Planaria (*Dugesia tigrina*)	Carolina Biological Supply Company	132954
Flat Paintbrush	Royal Crafter's Choice	9159
Glass Petri Dish - 10 cm	Kimax	N/A
Glass Petri Dish - 5 cm	Kimax	N/A
Grid Paper	Any	N/A
iPEVO Visualizer (software)	iPEVO	https://www.ipevo.com/software/visualizer
Metalware Set with Support Stand and Retort Ring	Any	N/A
Organic Egg	Any	N/A
Polycarbonate Bottle w/ Screw-on Cap - 10 mL	Beckman	N/A
Round Storage Container - 10 cm	Ziploc	N/A
Round Water Paint Brush	LOEW-Cornell	N/A
Transfer Pipette	Any	N/A
USB Document Camera	iPEVO	CDVU-06IP

Comments/Description
Spring water for planarian culture and to prepare solutions
Brown planaria living (other species are acceptable)
Flat watercolor paintbrushes for cleaning planarian culture containers
10 cm diameter (glass) Petri dishes for pLmV assay
5 cm and Petri dishes for rinsing planarians during withdrawal experiments and for stimulant habituation
Standard 0.5 cm grid paper for pLmV assay
Document camera software for video capture and recording
Standard chemistry lab ring stand to hold a cell phone camera if used
Organic egg or beef liver for feeding planarains
Plastic vials to hold 5 to 10 mL volumes for stimulant habituation
10 cm Round food storage containers for approximately 90 planarians or fewer
Small round watercolor paint brushes (numbers 3 to 6) - soft
Wide bore (5 mL) plastic transfer pipettes to move planarians
Document camera (or other camera or cell phone camera)

EST. 1908
SUNY MORRISVILLE

March 8, 2020

Dear Dr. Steindel,

We wish to thank the reviewers for their input into our protocol. We believe their comments have helped us to increase the clarity of what we have presented, and will also help others adapt this protocol for their use. We have addressed the points made by the reviewers here, as well as in the text (as identified by the line numbers or sections listed).

We wish to stress to the reviewers that the page limit outlined by JoVE was a consideration in the original writing of the paper. Our focus on the mechanics of the protocol itself restricted the topics we elected to include in the Introduction and the Discussion. In other words, it was not the lack of awareness or respect for the importance of the works suggested that drove our decision of which references to include. Since we did not invent the pLmV assay, we assumed that researchers interested in adapting the method for their use would be aware of aspects of its history before arriving at our adapted version of the protocol. As such, we attempted to provide a selection of the many publications that have benefitted from the assay to assist the reader in understanding our perspective on the method.

In addition, we hope to clarify that we do not believe or support the idea that any data be 'excluded' from analysis. When studying behaviors, any changes or deviation of from what is typically observed with untreated planarians is data, and should be considered as such. We regret that we did not sufficiently clarify our approach on this front. We hope the additions to the protocol sufficiently address our analysis. However, we ask that the reviewers understand that we cannot demonstrate or assess behaviors in our experiments that we do not observe using the reagents tested in our laboratory. The inclusion of terms outside of what are presented here (wander and stop) in our original version, were meant only to represent the types of terms used in the field to describe behaviors observed in the literature. There are many more behaviors seen – head bop for example - that we did not even list in the original manuscript. Each of these behaviors will no doubt affect the end user's choice on how to assess behavioral changes in their own lab. It is our purpose to describe the pLmV method for observing planarian behavior, and point out that observations may vary depending upon the natural product or reagent tested.

Editorial Comments:

1. We have adjusted the manuscript for left alignment, and provided spaces between each step and sub-step of the protocol.

2. We took care to ensure that the length of the protocol proper is within the stipulated page count, and also highlighted the filmable content sections as required.

3. We have clarified steps 3.3, 3.5, 4.3, 4.5, and 4.6 (as well as others) in response to the 'how?' suggestion. These are also reflected in the discussion. Other clarifications in addition to changes specifically requested by the reviews are listed below.

4. We have listed the email address of each author on the first page of the manuscript. We were uncertain, however, of what format would be preferable for this list.

5. We removed commercial names – Poland Spring and Carolina Biological Supply – from our manuscript. These now only appear in the Excel spreadsheet listing the materials used. To accommodate this change, steps 1.1 and 1.2 have been reworded. More detail has been added to the spreadsheet as well.

Reviewer 1:

1. The reviewer questioned the reasoning behind the repeated usage of 'natural products' in the text. The reason for this specificity is because the submission is a response to the call for protocols applicable to a JoVE special collection on natural product research.

https://www.jove.com/methods-collections/108/current-methods-in-natural-products-research

We have added a statement to increase the inclusivity of the work at lines 64, 94, 581, and 584. We did, however, describe the use of this assay, as well as other behavioral assays, taking advantage of planarian physiology in the long abstract and the first part of the introduction. We wish to emphasize to the reviewer that our research is focused on natural product work and, as such, we do not have experience using standard pharmaceuticals in our laboratory. To emphasize this point, we added a statement at line 59. The response of planarians to pharmacological agents may be more robust than what we observe.

2. We wish to ask the reviewer to refer to the sixth and seventh paragraph of our discussion section on the potential use of RNAi targeting receptors or even signaling molecules prior to running a pLmV assay. There, we briefly describe potential caveats that should be kept in mind if adapting this protocol for such studies. However, we again wish to emphasize that we have not yet applied the pLmV assay using RNAi treated specimens, so we only intended to introduce the concept in the discussion of this protocol to acknowledge that others may choose to use the pLmV assay in this circumstance. We have added the JoVE specific reference should readers wish to view how to perform RNAi work with planarians – see Reference 44. Due to the fact that we are not performing any sort of genetic analysis in our protocol, we did not provide a further reference to *Planmine*.

3. So far all of our work has been performed using water-soluble reagents, but in the event that an investigator requires the use of a solvent to solubilize their reagent of choice, we added a statement to include a corresponding control on lines 563-567.

4. We thank the reviewer for this comment. We have only recently begun to observe behaviors that cause the planarians to stop their movement during a pLmV run, so we do not have sufficient numbers to calculate meaningful statistics. As such, we have decided to change our representation of these analyses to a pie chart for the purpose of demonstrating the concept behind this analysis.

5. This point is well taken, and we thank the reviewer for pointing out how our word choice over-emphasizes this idea. We adjusted the abstract as suggested. Please see lines 45-47, and 77-80.

6. We kept 'biomodulating properties' in the title because using the word 'action' does not quite fit either. There are, however, references to biological response modifiers in the literature and in medical texts as having biomodulating properties. We felt this terminology captured the full meaning of what we hoped to express in the title. We thank the reviewer for their additional thoughts on this subject.

Reviewer 2:

We are grateful to the reviewer for recognizing the need for a video version of, as well as detailed notes on, how to practically apply the pLmV assay as described by the Raffa lab in 2001. We did visit both Raffa and Rawls to see them demonstrate the assay. Realizing that such an opportunity is not possible for many investigators we were motivated to share how we have worked to implement the system in our lab in the format provided by JoVE. A rotating cohort of undergraduates with various levels of ability and competing schedules staffs our lab, so an ancillary motivation with this publication is to provide a strong resource for the students to refer to as they join the group. As the reviewer noted, we have taken care to include each detail to assist in the use and basic maintenance of planarians in our lab, and we hope that the additions/clarifications noted below will provide the necessary information to describe how we have implemented the pLmV assay for our purpose that will equal that description. It was our goal to provide a detailed outline of our methods, so that they can be used and adapted by other investigators. We have added more details describing how we train our lab members to use the assay and score gridlines, along with notes that we hope will provide a benchmark for researchers that are new to the system.

1. The reviewer requested clarification on how we reduce bias and standardize the interpretation of specific behaviors among our lab members. The various comments reflecting these ideas included: *Original Line 206*: These terms are ones that have appeared in the literature and were not meant to reflect behaviors that we routinely encounter in our laboratory. We apologize for this confusion. Using our choice of test reagents, the only behaviors of note are those that cause the planarians to cease progress in the pLmV assay. We have not encountered these behaviors with spring water control animals. It is also important to note that once the animal displays these behaviors, it does not resume its progress over gridlines. These animals either 'wander' in place, or 'stop' in place as shown. We have now added notes to clarify this point. See lines 305-308, 311-313, 333-341, 373-374, 406-414, 455-461, 541-558.

Original Line 214: It is important to note here that all planarians travel around the edge of the dish during a pLmV run. It is also critical to convey that there is no guessing involved on the part of the user. The counting of the grid lines is quite straightforward with minimal training and practice. We have added more detail on how we keep track of these grid lines and information involving how users are trained to score these boxes. See lines 321-324 and 379-384.
We have also added greater detail in our analysis section to explain how we address the possible introduction of bias to our results. We hope these additions will address the reviewer's concern on this point. See lines 348-351, 355-358, 389-392, 441-447, 490-495, 521-524.
Original Lines 220/221: Here we wish to stress that we do not drop data from our behavioral analyses. We, as well as others as cited in the text, have assays to address the specific behaviors that the planarians present once exposed to a test reagent. As mentioned above, we fully include data on grid counts during the pLmV assay that takes place during the continuous horizontal movement of the animals (See added Figure 4B - 3 and 10 mM data). There are a number of tests in the literature that describe planarian movements in the absence of motility. The C-behavior is one of these. The field typically provides specific analyses of planarian behaviors when planarians are 'in place'. This is separate from 'speed of movement' analyses such as is collected using the pLmV assay. One of many examples can be found in reference number 4. The behaviors we note in our paper – 'wander' and 'stop' – are only assigned and highlighted if the planarians no longer cross grid lines for the duration of the experiment. This specific data describes the relative frequency of the occurrence. Combining motility data with movement data can confound the study of these behaviors. For example, of the behaviors reported in the literature, only the 'C-type' behavior is one that we occasionally observe, and only at high concentrations of the test reagents. With these concentrations, further study is required to determine what sort of physiological response is taking place. It may be, for example, that the osmotic balance of the planarian is compromised at these concentrations, that the substance is an irritant to the animal, or that there is a change in the neurological signaling taking place. Our work is focused on motility, so we work with the concentrations of our test product that cause a change in the pLmV, and use those for further study on stimulation and withdrawal, or other studies examining a possible mechanism as it applies to motility (see added Figure 4B). We hope the added discussion clarifies these ideas. As with the other point above, see lines 305-308, 311-313, 333-341, 373-374, 406-414, 455-461, and 541-558.

2. We thank the reviewer for pointing out the poor quality of the video in Figure 5A Supplement. This was not a representative video showing the experimental quality that is acceptable for analyses, and was used for practice purposes only. We have replaced the video with one that is more reflective of the quality needed for proper analysis.

Reviewer 3:

Response to General Comments.

We thank the reviewer for their thoughtful review. We would like to convey to this reviewer that the purpose of this JoVE submission is to present the pLmV assay as we are using it in our lab for our purposes. Our goal is to provide a visual interpretation of the protocol to address the issues we had in learning how to use the assay despite both reading the published work by Drs. Raffa, Rawls and co-workers, and visiting their labs to observe the way they perform the assay themselves. This method has proven suitable for our work in the lab, and it remains a staple for others as well. We are also familiar with publications using the COM analysis, however, the pLmV assay, and other assays using grid counts, remain in use by many investigators. In the end, it is up to the end user to decide which methodology best suits the needs of their particular laboratory. The pLmV assay remains a necessary and useful system to track planarian rate of movement. Because this protocol is based on the use and implementation of the pLmV assay to track rate of movement in response to natural products, the discussion of other stationary behaviors displayed by the planarian is beyond the scope of what we are addressing in this work.

As far as addressing planarian behaviors outside of pLmV, as the reviewer noted, there is a rich body of literature describing the many types of planarian behaviors observed in different circumstances, of which we are definitely aware. In our work, the only behaviors of note include the 'wander' and 'stop' behaviors shown in our protocol. Our purpose in listing the other reported behaviors was only meant as an example of what sorts of behaviors the end user might observe while using their own reagent of interest. We cannot show data or discuss behaviors we do not see when running our pLmV analysis. Likewise with the discussion above, the many works describing these other behaviors are quite descriptive, and these analyses are still routine, and analyzed without COM software. Again, it is up to the end user to decide if they would like to perform these analyses. The COM analyses are not feasible for use in our laboratory (should we endeavor to study other behaviors outside of rate of movement) for a variety of reasons that are beyond the scope of this discussion of our research protocol. We have, however, added the reference, as well as others (References 8-11) suggested by the reviewer, and we thank them for their choice of reference. We have also included a statement to draw attention to the use of COM and FIMtrack analysis, should any readers not be aware of the possibility and wish to use it to study any behavior of their choosing. See lines 69-70.

The effect of targeting different genes using siRNA, biological pathway modifiers and drugs is highly specific to the genes and reagents selected, as are the effects they might induce. It is not possible for us to anticipate which genes, modifiers or drugs will be tested for any reagent or natural product used by labs adopting the pLmV protocol, let alone which reagent or natural product others will test. As such, we are uncertain how the reviewer envisions we can offer predictions of how such agents will affect the initial results using the reagent or natural product the end user might choose. Furthermore, the steps involved in siRNA work references other protocols, while this paper is focused on the pLmV assay. Introducing the idea of using such agents in addition to the pLmV in our discussion is meant only to offer ideas to the reader of how they may apply the assay after their initial study.

1. We have added considerable discussion to more clearly outline how we begin our analyses, train investigators, perform statistics, assess results and reduce bias. These can be found between lines 392-395, 441-447, and 528-541, as well as in our referenced publication (1). Our statistical analyses are representative of what is done in the field and detailed in lines 348-351, 355-358, and 521-524.

2. We explain the difference between stimulant and withdrawal data, as well as provide actual data to show these responses along with references to other such data in the literature in the Representative Results and Figure Legends. Our other reviewers were satisfied with this presentation. Please see lines 447-451 and 396-494.

3. We have added data documenting the typical number of grid lines crossed using this assay system as 3-minute data in spring water (Figure 4A). These data provides the reader with a benchmark to determine how their work compares to what we typically obtain. See Figure 4, and lines 389-392, 441-444, and 481-490.

4. A detailed analysis of our methods and notes on the nuances of the procedure are discussed at length in the Representative Results and Discussion sections. The Abstract and Introduction provide information on why the pLmV assay is of interest, as well as the context of the methodology in the larger body of work that is found in the literature demonstrating the use of planarians for various types of analyses.

5. The use of clonal planarians is beyond the goal of this protocol, and would require the introduction of cutting/regeneration to the work presented. Additionally, clonal animals are not routinely used for these studies. If users wish to use clones, another excellent JoVE protocol is available for use. Since the use of clones is not typically done with the pLmV assay, it is beyond the reach of this method paper.

6. We have added a qualifying sentence for investigators to source planarians for their work. See lines 100-107.

7. The way food waste and planarians are separated is described in steps 1.6.2.1 – 1.6.2.6. At step 1.6.2.7, the reader is asked to repeat certain steps since the procedure on the second day is the same as on the first. We are uncertain how to better present these repeating these steps, because to list them again, verbatim, would be redundant.

8. The 2-minute habituation period is representative of what is used in the field and reflects the time we use in our work. We have encouraged the user to test other times, because different reagents will no doubt result in different dynamics. It is impossible for us to anticipate what the stimulation and withdrawal dynamics might be for each user. See lines 539-541.

9. The amount of water or solution to use in the 10 cm Petri dish is stated in 3.2.2 and 4.2.2.

10. We have added discussion to clarify how we score grid lines, as well as the associated analysis and behaviors. We do not exclude observed behaviors from the analysis. See lines 305-308, 311-313, 333-341, 373-374, 406-414, 455-461, and 541-558.

11. All video recordings are 10 minutes or more. We do not state that video recordings should stop before 10 minutes.

12. We have included a discussion on circadian rhythms. See lines 345-348, 477-481, and References 32-34.

13. We are uncertain why the reviewer was not able to see Figure 3A Supplement.

14. More details have been added to the figure legends.

15. The number of planarians typically used for pLmV analyses is stated as 9 to 12. This information appears at lines 517-524. The reader is not expected to dig through the literature to determine this number.

16. The references have been reviewed for missing information.

17. We have replaced the video in Figure 5A Supplement.

Again, we thank this reviewer for their interest and thorough review. We hope we have addressed each point and clarified any confusion.

On behalf of the authors of this manuscript, we wish to thank you for the opportunity to provide a revised version and hope that the additions and clarifications provided are sufficient for the publication of our method paper in JoVE.
Thank you,

Evelyn Voura, Ph.D.

Name: _____ Date: _____

Habituation (Concentration of Product and Time): _____

pLmV (Concentration of Product): _____

Count Start Time: _____

Time (minutes)

	1	2	3	4	5	6	7	8	9	10
Grid Count										
Accumulated Totals										
Relative to Control										

Notes: _____

Behaviors

Wander	Stop