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.