**Please find all of our responses to reviewer and editorial comments in bold.**

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 be present in the published version.

**We have thoroughly proofread the manuscript for final publication.**

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

**All paragraph indentations have been removed. We have ensured that there are single spaces present between every step, substep, and note in the Protocol. We further confirmed that single-line spacing and Calibri 12-point font are used throughout the manuscript.**

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “This protocol presents …”

**The phrasing has been changed and sits at 45 words.**

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: PDA, Neuroplex, Instant Ocean artificial seawater, Eppendorf, Vaseline, Sylgard, etc.

**We have replaced all commercial language in the manuscript with generic terms.**

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

**We have edited the Protocol section to make sure that every step is indicated using the imperative tense.**

6. The Protocol should contain only action items that direct the reader to do something.

**We have rephrased or omitted any items in the Protocol section that do not involve an action. Please note that this has specifically motivated us to rework Part 8 of the Protocol,**

**which had provided minimal but non-actionable details and appropriates references related to possibilities for post-acquisition data analysis methods, into other parts of the manuscript. Although we provide examples of what sort of analyses these methods may yield in the Representative Results section, a step-by-step protocol outlining the implementation of these methods sits beyond the scope of what we seek to communicate in this manuscript and would furthermore require considerably more space in the Protocol section than what is allowed.**

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

**We have made efforts to convert longer individual steps into shorter constituent steps, and have worked to limit the length and number of notes interspersed between these steps.**

8. Please ensure you answer the "how" question, i.e., how is the step performed? For this please include all the mechanical actions, button clicks in the software, knob turns in the instruments, command lines, etc. If using scripts, please include as supplementary file.

**We have added text to the necessary steps of the Protocol to provide additional implementation details in keeping with this request.**

9. Will this protocol require approval from IACUC? If yes, please include a sentence stating that IACUC approval was obtained.

**As we have described a protocol solely based on the use of invertebrate models, no IACUC approval is required.**

10. 2.2: Please bring out more clarity.

**We have added text to this section to clarify it.**

11. 2.3: How is this done?

**We have added text to section 2.3 to clarify how the superimpose function is carried out.**

12. 3.2.2: How big is the incision? How do you make this incision – scissors, scalpel, etc? How do you identify and remove the CNS?

**We have better specified the size of the incisions, tools required, and the appearance of the CNS for those steps describing the dissection protocol for all three gastropod species.**

13. Please ensure that you use complete sentences and describe all actions associated with the steps.

**We have carefully revised the Protocol to make sure that specific actions are specified in every step.**

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

**We have highlighted steps that can be used for filmable content and checked that their cumulative length doesn't exceed 2.75 pages.**

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

**None of the figures have been published before.**

16. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

**We have modified the figure legends to omit discussion and methodology related to their contents; the figure legends contain the bare minimum information required to understand the contents of the figures.**

17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

**We can confirm that our Discussion section explicitly covers the five topics cited above.**

18. Please do not abbreviate the journal titles in the references section.

**We have corrected this to list the full journal titles in the References section.**

19. Figure resolution is poor, please include high resolution figures.

**We have submitted higher-resolution figures.**

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

**We have reorganized the Table of Materials per these instructions.**

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This methodological article nicely described not only the experimental technique of VSD imaging in non-transgenic invertebrates but also analysis of the imaging data. The authors have described informative protocols with useful movies of physiological recording. This will contribute to not only reproductivity in research but also neuroscience education. As the authors indicated, one of the trends in the experimental neuroscience field is recording/manipulating neuronal population activity in the brain from an unanesthetized, behaving transgenic animal (e.g. experimental system of a tethered fruit fly). The authors have shown a convenient method of application of voltage imaging to the isolated nervous system of multiple non-transgenic invertebrate species, which would be a nice way of introducing a comparative neurobiological study to both students and researchers. I have several concerns regarding the technical details.

Major Concerns:
L.157 3.2.4. Briefly (~10 s) dip the ganglia in a solution of 0.5% glutaraldehyde in saline. This light fix of the connective tissue and its intrinsic muscles will help prevent movement during imaging.

・Can this process kill the nerve roots whose cut end is exposed?
・After washing the preparation, should a new cut end of nerve roots be made for extracellular recording?
・Please describe how to wash the preparation after the 0.5% GA process.
・The time length of the GA dipping would probably depend on the volume of preps. Is there any convenient way to confirm the contractive tissues within the sheath of ganglia are killed? (such as any observable change in the shape of the tissues or its color, or we just need to check if its spontaneous contraction stops?)

**We added text to the Protocol to address some of these issues and have added more detail here for the reviewer.**

**This light fix of the connective tissue and its intrinsic muscles will help prevent movement during imaging. We use a different duration of fix for the three species, depending on the thickness of the connective tissue surrounding the neurons and nerves. Dipping the ganglia in 0.5% GA for 10 to 20 s could very conceivably kill the cut ends of axons in the nerves. However, we typically draw a substantial length of nerve (approx. 1 cm) into the suction electrode for extracellular recording, and we are almost always able to obtain high-quality signals during extracellular nerve recordings. Furthermore, stimulation of the nerves is nearly always effective in eliciting fictive motor programs. After dipping the ganglia in**

**0.5% GA, the ganglia are placed back in the dissection chamber, which is perfused with saline, and after a few minutes of washing, the staining is begun. We have added text to the manuscript describing this.**

**We are not aware of any convenient, quick way of determining (e.g., visible changes to the tissue) if the contractive tissues within the sheath have been killed. We have arrived at the length of time that we dip various ganglia in GA empirically over the years: incubation periods that are too short fail to neutralized stimulatory movement artifacts, while excessive incubation times are known to affect neuronal health and consequently the motor programs they pattern.**

L. 309 Optical recording
In many cases of invertebrate CNS (e.g. arthropods and annelids), electrical signals generated in neurites electrotonically attenuate when propagating to a cell body (in monopolar neurons). Particularly, action potentials are greatly attenuated and look small in intracellular recordings. In this context, CNS of gastropod may possess a great advantage compared with such other invertebrates: many neuronal somata show large action potentials (that might not be simply generalized for many gastropod species, though).
My question is: How large amplitude of action potential is detectable using the PDA system with those absorbance dyes?

**The gastropods we have recorded with intracellular electrodes have overshooting action potentials that are often 80 mV in amplitude. In terms of what the smallest $V_m$ change we can record might be, the optical record in Fig 3G has discernable action potential undershoots (none of our recordings are averaged traces). This suggests that the technique should be able to discern action potentials in other model systems that attenuate to some degree and thus are not overshooting by the time they reach the soma. However this technique may not be ideal for species that are known to have highly-attenuated action potentials when recorded in the soma. We have inserted a paraphrasing of this text into the manuscript.**

Minor Concerns:
L. 132
3. Dissections for three marine gastropod
During dissection (not only in VSD staining and optical recording), was the temperature of saline kept in low? If so, how was it achieved?

**Yes: for *Tritonia* and *Aplysia*, the temperature is maintained at 11 and 15-16 °C, respectively, via saline perfusion through a Peltier cooling device. *Berghia* dissections were conducted at room temperature (22-23 °C), as we maintain *Berghia* in tanks at room temperature. We have added text describing this to the manuscript.**

L. 181
3.4. Dissection of Berghia stephanieae

Isn't the application of 0.5 % GA needed for preventing prep movement in this species? Doesn't this species have contractive tissues in the surrounding sheath of the ganglia?

***Berghia*'s sheath is certainly thinner than that of *Tritonia* and *Aplysia*, such that GA treatment is not needed in *Berghia*. We have not noticed movement of the preparation following a nerve stimulus in *Berghia*. It is also possible that the coverslip flattening in *Berghia* is more effective in immobilizing the CNS, since the coverslip piece can be pressed down more firmly across two chunks of Vaseline than into the silicon used with the larger CNS. Furthermore, movement artifacts are more likely over the course of a longer recording: whereas our VSD recordings in *Berghia* tend to be shorter (e.g., 2 min.), we routinely record in *Aplysia* for 20 minutes. We have added text to the manuscript to clarify the use of Vaseline for *Berghia* and silicon for *Tritonia* and *Aplysia*.**

L. 228 4.3. For Berghia, better results are obtained with the absorbance VSD RH482.
Are there any reasons that RH482 gets better results for Berghia? Is there any relevant physiological difference between Tritonia/Aplysia and Berghia. Or, can the molecular size difference of those VSDs be important to passing through their sheath?

**For *Berghia*, the best results so far have been obtained with the absorbance VSD RH482. RH482 is more lipophilic than RH155. Perhaps it stains this animal's smaller neurons better, or remains in the neuronal membranes more effectively at the higher recording saline temperature we use for this tropical species. We have added these details to the Discussion.**

Grammatical correction
ABSTRACT:
L.39
in the brains "of" non-transgenic gastropod species

**Thank you, this has been corrected.**

Reviewer #2:
Manuscript Summary:
A detailed description of the techniques needed for recording the spikes of hundreds of neurons simultaneously.

Major Concerns:
No.

Minor Concerns:
Lines 46, 76 "1.6KHz" may be changed to "1600 frames/sec" to avoid confusion.

**We have made this change to improve clarity.**

Line 102 : "PDAs are very sensitive to vibration" In fact, it should be "VSD imaging is very sensitive to vibration" . The absorption signal is about 0.1% of the resting light intensity. Floor

vibrations and fluid turbulent all cause large motion artifacts. Also, ~30 Hz vibrations from the air handling system and ~120 Hz light artifact from the room fluorescent lighting tube can contribute to the noise of the recording.

**We have made changes to the manuscript to reflect these suggestions.**

Lines 196, 201 RH 155 is also known as NK3041, RH482 is also known as NK3630 and JPW1132

**We have added this information to the manuscript.**

Lines 280, 532 " control panel gain switch set to 10X" should be "1X"

**Thank you, we have made this change.**

Reference: The authors may consider to cite a previous VSD recording of Aplysia ganglion paper
" Wu, J.Y., Cohen, L.B., and Falk, C.X. (1994). Neuronal activity during different behaviors in Aplysia: a distributed organization? Science 263, 820-823."

**Thank you, we have incorporated this reference into the Discussion section of the manuscript.**

Video: The authors may consider a "slow motion" video expanding the firing sequenced during period of 4-6 seconds of the original video.

**Thank you for the suggestion. Although the period immediately following the nerve stimulus shown in Video 1 is marked by the rapid, synchronous firing of many neurons, likely coinciding with a fictive withdrawal, this pattern quickly subsides and coalesces into the gallop portion of the escape response, which is highlighted in "slower motion" in Video 2. We believe we capture the most revealing period of the immediate post-stimulatory epoch in this video.**

Reviewer #3:
Manuscript Summary:
This manuscript describes a photodiode based imaging technique that can be used to record neural activity in several gastropod species. Two of these species are important model systems that have been studied for decades. This group was, however, the first to develop sorely needed techniques that can be used to record from large numbers of neurons in these species during network activity. The third species is new to neuroscience and its inclusion in this study demonstrates the power of this type of analysis in that it shows that meaningful results can be quickly obtained even without decades of painstaking sharp electrodes recordings to characterize circuitry. The technique is clearly described and the representative data that are included in the figures and videos are of the highest quality. Overall, a first rate job!

Major Concerns:

None

Minor Concerns:
None

Reviewer #4:
Manuscript Summary:
A clear and detailed discussion of using voltage-sensitive dyes to record the activity of 100-200 neurons as three different molluscan nervous systems produce behavioral activity patterns.

Major Concerns:
None.

Minor Concerns:
1. I was curious why the three different animals are dissected in different ways: two of them are anesthetized by cooling and one by injecting magnesium chloride; two are dissected from the ventral body surface and one from the dorsal surface. It would be nice to add a sentence or two to explain the reasons for the differences.

**The reasons are empirical. For *Aplysia*, the PI long ago compared $MgCl_2$, in universal use for that species, with cold and found $MgCl_2$ so convenient he continues to use it. *Tritonia* swim motor programs are better when cold is used. *Berghia* is very new for us, and we have not therefore settled on a standard anesthetic protocol yet.**

2. Referring to Fig. 3D (line 328) and Figs. 4D and 5F (line 347) is a bit jarring because the text has not previously mentioned any of these figures. The reference to Fig. 3D is helpful for understanding the effects of the filters, but the reader does not need to page ahead to Figs. 4D and 5F to see examples of the maps.

**Thank you for the suggestion. We have removed this section of the Protocol altogether, as it did not include actionable items, and integrated its content elsewhere in the manuscript. All premature references to the figures cited have been removed.**

Reviewer #5:
Manuscript Summary:

The contribution by Hill et al would be very useful to the growing number of neuroscientists performing large-scale, single-neuron resolution recordings from neuronal circuits.

Major Concerns:
1. Missing from the files is a video of the investigators performing the experiment procedures. Such videos are typical for JOVE publications and it seems one should be included for the present contribution.

**To the Reviewer: JoVE informed the authors that due to travel restrictions during the pandemic, they will expedite publication of JoVE articles without video, and will then add the standard videos after their team can travel to labs and film.**

2. The resolution of the images are generally rather poor and should be improved.

**We have submitted higher-resolution figures.**

Minor Concerns:
1. While it is mentioned in the article a few times that "Multiple several-minute recordings can be obtained per preparation with little to no signal bleaching or phototoxicity" it would be critical to state explicitly if any supplementary procedures are performed to enhance signal quality for this explicit case of multiple recordings lasting "10-20 minutes" each. For example, do tissues need to be re-stained periodically, or is a single staining period sufficient? Do resting light levels need to be re-adjusted between recordings?

**With these objectives, no supplementary procedures are required to obtain multiple several-minute recordings. The ganglia do not need to be re-stained, and the same light intensity can be used for all the recordings. The most crucial thing, if the experimenter wishes to track neurons from file to file, is that the focal plane not change, and the preparation not move. We have added detail to this effect in the Discussion.**

2. Line 39, "(…) in the brains non-transgenic (…)" should be "(…) in the brains of non-transgenic (…)."

**Thank you, this has been corrected.**

3. Line 153, please specify the type, model and manufacturer of the scissors.

**We have incorporated this information into the Table of Materials.**

4. Lines 238-307. All steps described here are performed after staining (e.g., pressing cover slip, drawing nerve into suction electrode). Are any measures taken to prevent or attenuate photobleaching? Is the experimenter performing these steps in a dark room with minimal illumination of the preparation? Or is the effect of illumination during these steps negligible?

**These steps should be performed with minimal illumination or with green light illumination to minimize photobleaching. We have added this information to the manuscript.**

5. Line 351, "(…) the course the recording (…)" should be "(…) the course of the recording (…)."

**We have removed the section of the Protocol containing this line altogether, as it did not include actionable items, and integrated its content elsewhere in the manuscript.**

6. Line 353, "8.3." should be in regular font, not bold.

**We have removed the section of the Protocol containing this step altogether, as it did not include actionable items, and integrated its content elsewhere in the manuscript.**