Dear Dr. Zaman,

Thank you for sending our manuscript for review. We have attached a modified version of our manuscript that addresses all comments. Please find a point-by-point response to the comments below.

Regards,

Henrik Bringmann

Dear Dr. Bringmann,

Your manuscript JoVE52742R1 "Agarose microchambers for long-term calcium imaging of Caenorhabditis elegans" has been peer-reviewed and the following comments need to be addressed.

Please keep JoVE's formatting requirements and the editorial comments from previous revisions in mind as you revise the manuscript to address peer review comments. Please maintain these overall manuscript changes, *e.g.*, if formatting or other changes were made, commercial language was removed, *etc.*

Please track the changes in your word processor (*e.g.*, Microsoft Word) or change the text color to identify all of the manuscript edits. When you have revised your submission, please also upload a separate document listing all of changes that address each of the editorial and peer review comments individually with the revised manuscript. Please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.

**Your revision is due by Jan 19, 2015. Please note that due to the high volume of JoVE submissions, failure to meet this deadline will result in publication delays.**

To submit a revision, go to the [*JoVE* Submission Site](http://www.editorialmanager.com/jove) and log in as an author. You will find your submission under the heading 'Submission Needing Revision'.

Sincerely,

Sephorah Zaman, Ph.D.

Science Editor

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**Editorial comments:**

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. Please use this updated version for any future revisions.

*Authors:*

*We have used this file as a template*

2) Please upload Table 1 as an Excel file to the JoVE submission site, instead of a Word document.

*Authors:*

*We have changed this file to Excel format.*

3) In step 1.1, are manufacturer's instructions used to set up the LED-EMCCD camera system? If so, please include the words, "using manufacturer's instructions" in this step.

*Authors:*

*We have added these words.*

4) In step 1.2.1, please include the depth of your device, as an example.

*Authors:*

*We have added this information.*

5) In Line 218: Should be Fig 2C instead of 2B.

*Authors:*

*We have changed this.*

5) Throughout the protocol section, introductory phrases (such as "Microscope setup:" in step 1.1) were removed to comply with the JoVE format. If these phrases are added to the protocol, then they must form a numbered protocol step (increasing the total length of the protocol to over 3 pages). For example:

1. Instruments, Culture Media and Dishes

1.1) Microscope setup

1.1.1) Use a microscope..

*Authors:*

*We have noticed this change.*

6) Please be consistent with journal title formatting in your references.

*Authors:*

*We reformatted some of the journal titles.*

7) JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

*Authors:*

*We included the DOIs as far as they were available.*

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

In this work, the authors present a short introduction to a technique that they had developed and previously described, namely the use of agarose microchambers (AMI) for studies of the C. elegans nervous system; they also give a practical description of how to perform imaging in AMIs and a discussion of future possibilities and limitations. Previously, AMIs had been used only in studies of larval development or behavior (especially of "lethargus" sleep-like behavior), while the authors now show that it can also be used to record neural activity or behavior in adults.

Imaging of C. elegans using AMIs allows to combine high-resolution and (to some extent) high-throughput imaging of the nervous system with recording behavioral responses, which makes it a powerful addition to the arsenal of tools available in worms. The assembly of the imaging chambers appears to require care and will clearly benefit from a visual demonstration, as in my opinion it seems to be difficult to reproduce from a written description alone.

*Major Concerns:*

None

*Minor Concerns:*

- In the protocol text, a section describing the strains used is missing. There should also be a description (or reference) of the frame subtraction method for determining worm mobility in the protocol section, as well as for the presentation of the calcium imaging results; how was the baseline of ΔF/F defined in figs. 2B and D?

*Authors:*

*We added a section describing the fluorescent strain, frame subtraction with references, and for presentation of calcium imaging results.*

- It would be helpful to know which objectives the authors had been using, in addition to (or instead of) the total magnification. How was the total magnification achieved - was there a magnifying video coupler in the light path? For example, how was a 140x magnification achieved (line 314)?

Also, the magnification stated does not correspond to the images shown. For example, in figure 3B, the scale bar is ca. 18.5 mm long - i.e. it is 50x magnified from the stated 370 μm, while the caption says it is 100x magnified.

*Authors:*

*We refer to the magnification by the microscope not the print out, which is standard in the field. We now state the magnification of the objective in the text which should prevent such misunderstandings.*

- When performing Ca2+ imaging, especially when using the GCaMP sensor, is there a problem in these chambers with the neurons going out of focus?

*Authors:*

*We have not found this to be a problem. We state now in the text that in case this is a problem for some other neurons a z scan can be used to always obtain fluorescence data.*

- The authors say that when scanning multiple worms the movement of the motorised stage should be set to a slow speed to prevent disturbing the worms, i.e. to prevent a response to this mechanical stimulus. Does a slow scan speed indeed completely suppress a mechanosensitive response?

*Authors:*

*We could not find a mechansensitive response and state this clearly now.*

- The authors write in the discussion section that the chamber cavity is to be filled with a bacterial suspension that should be liquid, not too viscous or solid. Most behaviors in C. elegans are studied while the animals move on a quite solid agar surface, and the animals behave very differently when placed in liquid. Given that the environment in the agarose microchamber is (mostly) liquid, is the behavior of the worms in a microchamber akin to thrashing in a liquid environment or does it resemble crawling on a solid surface?

*Authors:*

*We added a sentence that states that worms are in physical contact with a surface and that their crawling is similar to that on the plate.*

- In discussing the limitations of the technique, it should be mentioned that it will be difficult to expose the C. elegans placed into the microchambers to most sensory stimuli. Also, I expect that the worms and bacteria sealed into these chambers, which are both alive and consuming oxygen, will quickly create a hypoxic environment, which will have considerable effect on the worms' physiology - this should be discussed as well.

*Authors:*

*We added comments to the discussion to address this point.*

- It may be helpful to provide a brief comparison with long-term culture of C. elegans in microfluidic microchambers made of PDMS.

*Authors:*

*We now briefly mention the use of PDMS devices for microfluidics and refer to a recent review that nicely summarizes these technologies.*

*Additional Comments to Authors:*

Line 52: Principal, not principle

*Authors:*

*Corrected.*

Line 68: In my opinion FRET is an acronym for "Förster Resonance Energy Transfer", not "Fluorescence Resonance Energy Transfer".

*Authors:*

*We now use this alternative term in the new version of our manuscript.*

Line 147: What is the purpose of exposing the PDMS surface to air plasma here, if not for bonding to a glass slide (section 1.2.2)?

*Authors:*

*We added a note stating that this treatment makes the PDMS hydrophilic.*

Sections 4. and 5. (Lines 176 to 194): I would talk about calcium imaging first, as it is slightly confusing how the information on frame rates etc. is split up between the two sections.

*Authors:*

*We swapped sections 4 and 5.*

Line 218: This should be Figure 2C, not 2B.

*Authors:*

*We changed this.*

Line 232: Mean intensity - of all pixels of the image?

*Authors:*

*Yes, we added this information.*

Figure 1: I don't understand the meaning of the two red "YA" labels placed in the figure.

*Authors:*

*We added the information that YA means young adult.*

Figure 2: There should be a scale bar to correlate the false colors with ΔF/F.

*Authors:*

*We added the scale bar.*

**Reviewer #2:**

No edits- accept in its current state.

**Reviewer #3:**

*Manuscript Summary:*

This work describes construction and use of an agarose chamber that allows (scalable) imaging of worms (and expression signals) over relatively long times. This sort of development is necessary to move C. elegans beyond a system with only a series of short term measurements. The discussion of practical considerations was very helpful.

*Major Concerns:*

None

*Minor Concerns:*

There are a few almost trivial descriptions that nevertheless might lead to some problems with creating the chambers and executing the experiments. The they should be checked for these. An example is 'sticky tape'. This could easily be listed in the equipment list. Also, could an 'architect's diagram' of the chambers be included?

*Authors:*

*We added the brand name for the sticky tape to the materials list. We would suggest that a cartoon (architect’s diagram) should be added to the movie to help understand the process.*