Dear Nam Nguyen, Ph.D, Science Editor,

Thank you for consideration for publication of our protocol in JoVE. We are grateful for the insightful comments and suggestions that you and all five reviewers made to our manuscript. We have addressed the major concerns, added a paragraph discussing storage conditions and dessication issues in the results, and a paragraph in the discussion addressing the critical steps in the protocol. We have made all the changes to the text that the reviewers suggested, and our story is much stronger than before. I believe that with the new data and revised manuscript researchers will be more convinced that NGT-3D is an excellent way to culture and observe worms in a more natural environment. Nearly all the changes we made are tracked in “Tracked Changes” in Microsoft Word. Below are our responses and edits in accordance with the reviewers’ comments.

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 thoroughly proofread the manuscript the best we can.

2. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi: DOI (YEAR).] For more than 6 authors, list only the first author then et al. We have made these changes in the manuscript. As an extra note, we originally used the Endnote JoVE reference style provided on the JoVE instruction for authors website. However, this style must not be updated or incorrect. Instead, we changed these by hand.

3. Please abbreviate all journal titles. We have made these changes in manuscript.

4. Please include volume, issue numbers, and DOIs for all references. We have made these changes in the manuscript.

5. Please define all abbreviations before use: FUDr, etc. We have made these changes in the manuscript.

6. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). We have made these changes in the manuscript.

7. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). We have made many changes to answer this issue. However, we find that it is nearly impossible to keep the entire manuscript text in a passive voice, and we want to avoid switching between active and passive voices. In terms of this, we have not used any personal pronouns in the Protocols section. However, we found that the writing the discussion without an active voice was awkward and decided to maintain some of the personal pronouns here.

8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Specific details were also requested by the reviewers. We have made those changes in the manuscript. We have added extra references to the protocol.

9. 1.1: 1 L of each solution? We have clarified this in the manuscript.

10. Please provide a figure legend for the supplemental movie. We have added a figure legend in the text.

11. 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].” We have uploaded a file that contains the links to the editorial policy and cited this appropriately in the figure legend.

12. Formatting:

-Please format references according to JoVE style and include DOI where available. Please also cite references using superscript numbers. We have made these changes in the manuscript.

-Please define abbreviations at first occurrence (ie LB). We have made these changes in the manuscript.

-Please include spaces between numbers and units. We have made these changes in the manuscript.

-Please use a dash rather than ~ to indicate a range (see 2.4 note as an example). We have made these changes in the manuscript.

13. Grammar:

-4.6.1 – Please use imperative tense or convert to a note. We have converted this to a note.

-5.3 – “NGT-3B” We have made this change.

-Line 284 – Should be “bacterial growth” We have made this change.

14. Results:

-Figure 3c needs a scale bar. We have added a scale bar to the figure.

-Please provide a legend for the supplemental movie. We have made this change

15. Discussion: Please discuss the critical steps of the method. We have added a paragraph in the discussion mentioning the critical steps.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors present a new method to culture Caenorhabditis elegans in a 3D culture to determine how a 3d environment affects worm physiology and behavior. The paper is generally well written and easy to understand and certainly interesting for the worm community. There are a few details that need to be taken care off to ensure that other labs are able to reproduce their results.

Major Concerns::

none

Minor Concerns:

\*95: 0.85% NaCl solution: Any specific reason on why to indicate the NaCL solution in % and not in molarity like for all the other salts? We have changed the NaCl solution to molarity.

\*101: what solvent is used for cholesterol? Purified EtOh is not a commonly used term to describe ethanol quality. Why is the cholesterol sterlized by a 0.45uM syringe. Et-OH is a desinfectant that is already sterile. Unless Et-OH is less than 40%? Please clarify. The cholesterol was dissolved in 99.99% ethanol, and we have changed the text to reflect that. Filter sterilization of a solution containing alcohol is a standard procedure to make sure a solution is completely sterile. It also can remove any undissolved solid cholesterol, and we have changed the text to reflect that.

\*113: how long have the bacteria to be cultured, 20 min, 12 h , one day, until saturation? Please specify The bacteria is cultured overnight, and we have changed the text to reflect that.

\*146-148: General comment: Whenever a stock is added during the preparation of a solution, please indicate the final concentrations of each of the components, CaCl2, cholesterol, MgSO4 etc. This will avoid errors. We have changed the text and added the final concentrations.

\*150: Same as 146: what will the final FUDR concentration be? We have changed the text and added the final concentration of FuDR (120 µM).

\*General comment: Many Salts used in this protocol are available in multiple forms, for example containing some water (3\*H2O). To avoid confusion it would help if the authors also indicate the molecular weight of each salt to make sure which form is used. We have added the molecular weights in the materials template table.

\*157: Please specify the culture chambers sizes (X ml...) in case other researches have no access to the plates/flasks indicated in the table below. We have added the sizes to the material template table.

\*250: please indicate for how many animals the brood was analyzed for each case (Fig 2B). We have added the sample sizes in the figure legend.

\*Are the dioxyvaleicin expressing OP50 available from CGC? It the plasmid available from addgene? If not please state that. The strain is not yet available in the CGC, nor is the plasmid available from Addgene. We are in process of publishing a separate story using this strain, and will deposit it upon completion. For now, it is available upon request, and we added this into the “Results” text.

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

This manuscript describes a simple method of growing C. elegans in a 3D space, which will better mimic the natural environment.

Major Concerns:

None

Minor Concerns:

1. Is contamination or drying out not worrisome to use 1 week old NGT-3D media after grwoing bacterial for 1 week? This is an excellent question. We routinely use NGT-3D and NGB-3D that are from 1 to 1 month old, and have not had problems with drying out, so long as they are closed with a secure cap. We have added a paragraph pertaining to this and storage in the “Results” section.

2. Is it better to recommend using agarose instead of agar to enhance the imaging quality in the NGB-3D media? We have not tried using agarose, and in our experience the granulated agar was clear enough to image the worms, particularly if they were closer to the surface. But this is an excellent suggestion and we added a sentence in the discussion pertaining to this.

3. Is there any easy way to transfer live worms from an old media to another new one? At this point the answer is “No”. This is why we cannot assess the actual brood size over the reproductive lifetime of the animal, and must use a “relative brood size assay”.

Additional Comments to Authors:

N/A

Reviewer #3:

Manuscript Summary:

This manuscript describes three-dimensional cultivation system to study C. elegans biology. Two methods, called NGT-3D and NGB-3D, provide three-dimensional habitat of C. elegans for study of fitness or behavior. Authors showed consistency in survival rate in 3D and 2D conditions. They also showed an image of a worm in the 3D condition.

Major Concerns:

Major concern of this manuscript is that it lacks description for an advantage of 3D cultivation. Since the representative data shows consistency with 2D cultivation without obvious difference, merits of NGT-3D are missing. Natural condition itself is not convincing if the results are same as the conventional methods. We appreciate this excellent comment here, and fully agree that cultivation in 3D should have merits for the worm for scientific study. The data, however, that is presented here and in our previous paper (Lee et al., Biol Open, 2016) was performed with the specific purpose to identify control conditions in 3D cultivation from which novel studies (eg. developmental, physiologal, behavioral) can use as a basic platform. We show more specifically in our previous paper that maximal reproductive and developmental conditions can be reached with increased bacterial availability, both in 2D NGM and 3D NGT-3D, and these are the conditions we suggest for worm cultivation in NGT-3D and NGB-3D here.

The other concern is that lack of the description for the behavioral assay with NGB-3D. Description for microscopic condition, types of behavior, and behavioral difference between proposing method and conventional methods are missing. Another excellent point that we appreciate. Our personal interest in 3D cultivation is from a behavioral standpoint, and behavioral studies using NGT-3D and NGB-3D are currently occurring in our lab. Our reason for publishing this protocols paper is to provide a platform for other research groups to investigate questions of development, physiology and behavior. We do show in our previous paper that a sensory mutant that can reproduce normally in 2D conditions cannot reproduce in NGT-3D likely due to an inability to find the bacteria in a complex environment. A previous study also showed that there are locomotory behavioral changes in 3D. We have addressed this question by adding these references to the “Discussion” section.

Finally, relationship between this manuscript and the previous paper is not clear. Most of the contents of this paper are same as the authors' previous paper Lee et al. 2016. Although authors cited the paper, descriptions of the cited results are not mentioned properly. I understand that JoVE's unique style allow publication of the previously published methods. However, in that case, authors should clarify the description for what was reported before, and advantages of the describing method. Instead of the claim for the novelty, authors should properly describe previous works and provide what was missed in the previous paper. We appreciate the comment here. We have added correct references to the previous paper, and changed the text in several places to highlight the data from the previous paper. In addition, this paper describes a new method, NGB-3D, to image behaviors in real time, and describes in video and text how other scientists can easily cultivate C. elegans in 3D.

Minor Concerns:

not consistent in line 39 and 57

"seminal discoveries in biology over the last two decades"

"seminal discoveries in the field of biology over the last five decades" We have clarified this and edited the text properly

Additional Comments to Authors:

N/A

Reviewer #4:

Manuscript Summary:

The manuscript submitted by Tong Young Lee and co-workers describes a new three-dimensional cultivation technique for C. elegans, a model system widely used in different disciplines of Biology. In general, the manuscript is well-written and of interest for a broader scientific field.

Major Concerns:

none

Minor Concerns:

l. 38: Why is Drosophila mentioned? We have edited the text and removed the Drosophila reference.

l. 124: Is the dilution correct? 1 mL of a E. coli culture with OD 1 should be about 10 exp.9 bacteria. A dilution of 109 bacteria to 10-6 to 10-7 should result in bacterial colonies in the 100s, which is our goal for cultivating worms in 3D.

References: C. elegans should be written in italics We have made this change.

Additional Comments to Authors:

N/A

Reviewer #5:

Manuscript Summary:

The authors present a novel method of Caenorhabditis elegans growth in a three-dimensional space as opposed to the two-dimensional plate setup that is traditionally used in nematode laboratories. When bacterial colonies were adequate, nematode growth, development, and fertility in 3D environments were comparable to standard 2D growth plates. This technique offers researchers the ability to observe phenotypic properties of nematodes in an environment that more accurately reflects natural nematode habitats. The protocol was detailed and could be followed by other researchers.

I believe this protocol is useful to the C. elegans field because this new cultivation strategy might broaden our understanding of natural C. elegans behavior. However, a few clarification points are necessary.

Major Concerns:

1. The authors just tried the laboratory-adapted strain N2, which is abnormal for nematode physiology and behavior. Specifically, N2 animals interact with food and bacteria in an abnormal way with respect to oxygen concentration. It would be useful to know if this method works with natural C. elegans strains who normally interact with food at low oxygen concentrations. Do they respond better or worse to 3D culturing conditions? This is a very astute comment. For this study, we have only focused on N2, so other researchers can replicate our conditions for their experiments in 3D. Yes, wild strains may be better adapted to reside in 3D conditions where oxygen concentrations are lower. To somewhat satisfy your curiosity, our experiments using Hawaiian strain C. elegans and *npr-1* mutants are ongoing, but on the level of brood size and growth we do not observe many differences.

2. How many independent biological replicates, technical replicates, and number of worms were assayed for Figure 1B and Figure 1C? The error is awfully small, likely because it is standard error. I would recommend showing the data as a box plot with points plotted underneath. Bar plots hide data from the researcher. The statistics can be improved to tell us whether there is a robust difference. For example, show the data as box plots, report t-tests with p-values (or your favorite non-parametric test), and fit a model to deal with assay-to-assay variability. We appreciate your comments here. Your careful analysis of the data presented here is correct, and more specific data will be helpful. We have added sample size numbers to the figure legends here. In addition, our previous paper (Lee et al, Biol Open, 2016) shows several scatterplot analyses of the full set of data points that the reviewer should find more satisfying. We curtailed the data in this paper to only the necessary figures.

3. The clarity and quality of microscope imaging with this strategy seems to be a point of concern. It was stated that the researchers attempted to solve this issue by placing bacteria on the surface of the media, but this seems identical to experiments using the 2D plate strategy and does not offer the benefits of a 3D cultivation strategy. The imaging issue may need further troubleshooting, and the image in Fig. 2C is blurry. We agree that imaging is a major concern. In the text we offered advice in increasing the clarity by choosing NGB-3D where the bacterial colonies had grown close to the surface rather than deeper in the agar. However the colonies were still embedded in the agar and not exposed to the surface. We apologize for the confusion and have clarified this in the text. A new and clearer image for 2C was provided from the same video. However, providing a 3D image is not possible from our part.

4. In step 3.6 of the protocol, "Add the 6 mL diluted bacterial culture from step 1…" is unclear. A 6 mL culture was not mentioned in step 1. Thank you, this was an error on our part. We have corrected this to refer the 6 ml culture back to the correct step.

5. The dilutions used in the tubes pictured in Fig. 1A should be explicitly stated. We have added the dilutions into the figure.

6. Line 287 discusses how shorter and longer growth times do not affect worm growth or fertility, but data to back up this point are not presented. They need to be. To this end, we have added a section in the results pertaining to storage conditions discussing this issue. However, we have not collected any specific data on this.

Minor Concerns:

1. Liquid culture has also been used as a 3D matrix to cultivate worms. Therefore line 271, "this is the first protocol that allows cultivation of worms in 3D" should be changed to "… cultivation of worms in a solid 3D matrix. We have modified the text to reflect this.

2. Line 274, "Although NGT-3D and [NGB-3D] have some differences to the standard 2D NGM pates, they are nearly identical to this original method…" needs clarification. How can they be different and nearly identical? We clarified this in the text.

3. Change the word choice in line 230, "adult C. elegans hermaphrodites in NGT-3D breed just as well…" to "reproduce" instead of "breed". If this sentence is discussing the fertility, hermaphrodites are not being bred. However, if the mating behavior of males and hermaphrodites is the focus, clarify how the mating behavior was assayed. We have modified the text to “reproduce” to reflect this.

4. "NGB-3D" was called "NGT-3B" in many places. Please fix the acronym. Lines 214, 261, 272, 27. We have modified the text to reflect this.

5. Line 318, "confer reproductive fitness to the animal…" should be "confer fitness advantages". We have modified the text to reflect this.

6. Introduce the full term for NGT-3D in line 47. We have modified the text to reflect this.

7. Introduce the full term for NGB-3D in line 49. We have modified the text to reflect this.

8. Line 39 "culture" should be "cultivate". We have modified the text to reflect this.

9. Line 60 "single" should be "whole". We have modified the text to reflect this.

10. Line 61 "looking further to" should be "looking to further". We have modified the text to reflect this.

11. Line 66 "in their natural habitats" should be "in natural nematode habitats". We have modified the text to reflect this.

12. Line 72, add abbreviation "three-dimensional (3D)". We have modified the text to reflect this.

13. Line 73, change to "environments to which worms are exposed in the laboratory." We have modified the text to reflect this.

14. Line 78, add comma after "development". We have modified the text to reflect this.

15. Line 79, change "with" to "to". We have modified the text to reflect this.

16. Line 88, add comma after "NGB-3D". We have modified the text to reflect this.

17. Line 120, add sentence "Pipet 1 mL of diluted bacterial culture into new tube with 9 mL of NaCl" to clarify the serial dilution protocol. We have modified the text to reflect this.

18. Line 204, word choice of "relative brood size" is incorrect. This is not a "relative" estimate, this is a proxy for brood size. I believe that calling it brood size and then being explicit about what is measured is fine. This is an excellent point. However, since we termed this “relative” brood size in our previous paper, we wanted to keep this consistent to prevent any confusion. I wish you had reviewed our previous paper.

19. Line 215, specify if one should place the worm on the agar or the plastic of the bottle. We have specified this in the text for the worm to be placed on the agar.

20. Line 230, "relative" brood size word choice. Addressed above in 18.

21. Line 247, "easily imaged" is incorrect given the difficulty of the imaging discussed later. We have modified the text to reflect this.

22. Line 251, change "with" to "to". We have modified the text to reflect this.

23. Line 252, clarify "several dilutions" with specific concentrations. We have modified the text to reflect this.

24. Line 252, "relative" brood size word choice. Addressed above.

25. Line 270, change "more reflects" to "more accurately reflects". We have modified the text to reflect this.

26. Line 278, delete ", big or small,". We have modified the text to reflect this.

27. Line 279, change "allows" to "allow". We have modified the text to reflect this.

28. Line 297, delete "Instead," We have modified the text to reflect this.

29. Line 316, delete "less or more". We have modified the text to reflect this.

30. Figure 1B axis, "relative" brood size word choice. Addressed above in 18.

31. Line 232, what is considered a "plentiful" amount of bacterial colonies? We have specified the number of colonies as more than 60 as plentiful.

32. Line 38, melanogaster should be italicized. This was deleted

33. Line 39, Only two decades of discovery? Several Nobel Prizes have been awarded for research after 1996. Please edit this sentence. We have modified the text to reflect this.

34. Line 50, it should be clear that the authors are not studying ecology at all. Please remove that wording. We have modified the text to reflect this.

35. Line 63, Choi et al. is not alone for considering ecology and evolution at the genetic level in C. elegans. The authors should reference the long history of QTL mapping (Gaertner et al.) and population genetics (The work of Cutter, Andersen, and Rockman labs). Thank you for helping us here. We have added these appropriate references.

36. Line 71, Braendle and Felix is a better more up-to-date reference. We have added this reference.

37. Throughout the text, the authors should avoid "worms" as a term. It is fine within a small community, but a broad readership might think about annelids. I would argue that “worms” is an appropriate term even for the broader readership, much like “flies” and “monkeys” are general terms but in the context is appropriate.

38. Line 102, remove the comma from "cholesterol, and" We have modified the text to reflect this.

39. Line 235, remove the comma from ", and larval" We have modified the text to reflect this.

40. Line 120, needs to say NaCl solution We have modified the text to reflect this.

41. Line 238, needs a comma to make the preposition easier to understand. We have modified the text to reflect this.

42. Throughout the text, et al. should be italics with al. containing a period. The references have been changed to a number.

43. Spacing in the table is not correct. We edited the table.

44. The manuscript contains a random page at the end entitled, "Comments/Description". Please remove. This must be from the journal editors as we do not see this page in our manuscript.

45. The Oxford comma helps to better understand lists. I suggest the authors use it throughout the manuscript. I would agree the serial comma is better (as I learned it this way originally). However, I’ve had comments and edits in writings and manuscripts that have removed my serial commas. After years of this, I have adopted the non-serial comma as the majority of scientific writers seemed to have gone in this direction. I think I’ll leave it this way, because I’ll likely have another reviewer to say to change it. I apologize to you, reviewer 5. But thank you for the comments!