Journal of Visualized Experiments

Cultivation of Caenorhabditis elegans in three dimensions in the laboratory

--Manuscript Draft--

Manuscript Number:	JoVE55048R1
Full Title:	Cultivation of Caenorhabditis elegans in three dimensions in the laboratory
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Caenorhabditis elegans; nematode; reproductive fitness; 3D; three dimensions; Behavior; nematode cultivation; Development
Manuscript Classifications:	2.1.50.500.500.294: Nematoda; 2.1.50.500.500.294.700.700.660.250.250: Caenorhabditis elegans (C. elegans); 6.4.96.276: Genetics, Behavioral; 7.7.700.320.124: Aging; 8.1.158.273.200: Developmental Biology; 8.1.158.273.248: Ecology; 8.1.158.273.343.290: Genetics, Behavioral; 95.51.19: genetics (animal and plant); 95.51.9: biological evolution (terrestrial)
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Abstract:	The use of genetic model organisms such as Caenorhabditis elegans has led to seminal discoveries in biology over the last five decades. Most of what we know about C. elegans is limited to laboratory cultivation of the nematodes that may not necessarily reflect the environments they normally inhabit in nature. Cultivation of C. elegans in a 3D habitat that is more similar to the 3D matrix that worms encounter in rotten fruits and vegetative compost in nature could reveal novel phenotypes and behaviors not observed in 2D. In addition, experiments in 3D can address how phenotypes we observe in 2D are relevant for the worm in nature. Here, a new method in which C. elegans grows and reproduces normally in three dimensions is presented. Cultivation of C. elegans in Nematode Growth Tube-3D (NGT-3D) can allow us to measure the reproductive fitness of C. elegans strains or different conditions in a 3D environment. We also present a novel method, termed Nematode Growth Bottle-3D (NGB-3D), to cultivate C. elegans in 3D for microscopic analysis. These methods allow scientists to study C. elegans biology in conditions that are more reflective of the environments they encounter in nature. These can help us to understand the overlying evolutionary relevance of the physiology and behavior of C. elegans we observe in the laboratory.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	10-31-2016

TITLE:
Cultivation of *Caenorhabditis elegans* in three dimensions in the laboratory

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KEYWORDS:
C. elegans, nematode, reproductive fitness, 3D, three dimensions, behavior, nematode cultivation

SHORT ABSTRACT:
We present a simple method to construct 3D nematode cultivation systems called NGT-3D and NGB-3D. These can be used to study nematode fitness and behaviors in habitats that are more similar to natural *Caenorhabiditis elegans* habitats than the standard 2D laboratory *C. elegans* culture plates.

LONG ABSTRACT:
The use of genetic model organisms such as *Caenorhabditis elegans* has led to seminal discoveries in biology over the last five decades. Most of what we know about *C. elegans* is limited to laboratory cultivation of the nematodes that may not necessarily reflect the environments they normally inhabit in nature. Cultivation of *C. elegans* in a 3D habitat that is more similar to the 3D matrix that worms encounter in rotten fruits and vegetative compost in nature could reveal novel phenotypes and behaviors not observed in 2D. In addition, experiments in 3D can address how phenotypes we observe in 2D are relevant for the worm in nature. Here, a new method in which *C. elegans* grows and reproduces normally in three dimensions is presented. Cultivation of *C. elegans* in Nematode Growth Tube-3D (NGT-3D) can allow us to measure the

reproductive fitness of *C. elegans* strains or different conditions in a 3D environment. We also present a novel method, termed Nematode Growth Bottle-3D (NGB-3D), to cultivate *C. elegans* in 3D for microscopic analysis. These methods allow scientists to study *C. elegans* biology in conditions that are more reflective of the environments they encounter in nature. These can help us to understand the overlying evolutionary relevance of the physiology and behavior of *C. elegans* we observe in the laboratory.

INTRODUCTION:

The study of the nematode *Caenorhabditis elegans* in the laboratory has led to seminal discoveries in the field of biology over the last five decades[1]. *C. elegans* was the first multicellular organism to have its genome sequenced in 1998[2], and it has been invaluable in understanding the contributions of individual genes to the development, physiology, and behavior of a whole organism. Scientists now are looking to further understand how these genes may contribute to the survival and reproductive fitness of organisms in their natural environments, asking questions about ecology and evolution at the genetic level[3-5].

C. elegans once again can provide an excellent system to answer these questions. However, little is known about *C. elegans* biology in natural nematode habitats, and there are no current methods to simulate controlled natural conditions of *C. elegans* in the laboratory. In the lab, *C. elegans* is cultivated on the surface of agar plates seeded with *E. coli* bacteria[6]. In nature, however, *C. elegans* and related nematodes can be found sparsely inhabiting soils throughout the globe, but they are specifically found thriving in rotting fruits and vegetative matter[7,8]. These three-dimensional (3D) complex environments are quite different from the simple 2D environments to which worms are exposed to in the laboratory.

To begin to answer questions about the biology of nematodes in a more natural 3D setting, we have designed a 3D habitat for laboratory cultivation of nematodes we called Nematode Growth Tube 3D or NGT-3D for short[9]. The goal was to design a 3D growth system that allows for comparable growth, development, and fertility to the standard 2D Nematode Growth Media (NGM) plates[10]. This system supports the growth of bacteria and nematodes over their entire life cycles in 3D, allows worms to move and behave freely in three dimensions, and is easy and inexpensive to manufacture and employ.

In the current study, we provide a step-by-step method to manufacture NGT-3D and evaluate worm development and fertility. In addition to assessing worm fitness in 3D, we sought to image, video, and assess worm behavior and physiology in 3D cultivation. Thus, in addition to NGT-3D, we present here an alternate method called Nematode Growth Bottle 3D or NGB-3D, for the microscopic imaging of *C. elegans* during 3D cultivation. This will be especially important for the study of known behaviors identified in 2D, and the identification of novel behaviors unique to 3D cultivation.

PROTOCOL:

1. Prepare solutions for NGT-3D and NGB-3D

1.1) Prepare the following sterile solutions: 1 L of 0.1454 M NaCl solution, 1 L of 1 M $CaCl_2$, 1 L of 1 M $MgSO_4$, Lysogeny Broth (LB), 1 L of 1 M KPO_4 buffer (108.3 g of KH_2PO_4 and 35.6 g of K_2HPO_4, and fill H_2O to 1 L). Production of NGM using these solutions can be found in a previous protocol[10].

1.2) Autoclave solutions at 121 °C, 15 min**.**

1.3) Prepare 50 mL of a sterile 5 mg/mL cholesterol solution. In a 50 mL conical tube, mix 0.25 g of cholesterol and 50 mL of 99.99% ethanol and mix well. **Do not autoclave**. Sterilize the cholesterol solution using a 50 mL syringe with a 0.45 µm syringe filter. This will also remove any undissolved cholesterol.

1.4) (Optional) Prepare 10 mL of a 150 mM of 2'-deoxy-5-fluorouridine (FUdR) stock. In a 10 mL conical tube, mix 0.3693 g of FUdR and 10 mL of distilled water. Shake well.

2. Prepare bacteria culture for NGT-3D and NGB-3D

2.1) Inoculate 10 mL LB broth with bacteria. The standard bacteria used for *C. elegans* feeding is *E. coli* strain OP50.

2.2) Culture the bacterial inoculation at 37 °C in a shaking incubator overnight.

2.3) In preparation for a serial dilution, aliquot 9 mL of the NaCl solution into sterile 15 mL conical tubes. For example, aliquot 9 mL into a total of 7 tubes to make a 10^{-7} dilution of OP50 strain *E. coli*.

2.4) Using a sterile 1000 µL pipette, pipet 1 mL of the bacterial culture or diluted bacterial culture into the sterile new tube with 9 ml of NaCl solution and vortex the 10 mL mixture well. Repeat until the desired bacterial dilution is reached.

Note: The desired bacterial dilution depends on the type and condition of bacteria as well as the exact experimental conditions. For example, a 10^{-6}-10^{-8} dilution of OP50 strain *E. coli* was sufficient to produce from 1-200 bacterial colonies per 6.5 mL NGT-3D tube. Worms reliably developed and reproduced normally when the number of colonies was over 60, which occurred at a dilution of 10^{-6}-10^{-7}. For NGB-3D, use a dilution of 10^{-8}.

3. **Making NGT-3D and NGB-3D (200 mL)**

3.1) After preparing the bacterial culture, mix 0.6 g NaCl, 1 g granulated agar, and 0.5 g peptone in a 500 mL flask. Insert a magnetic stir bar into the flask.

3.2) Add 195 mL distilled water and cover mouth of the flask with aluminum foil.

3.3) Autoclave 121 °C for 15 min.

3.4) Place the hot autoclaved flask onto a stir plate, and stir at a moderate speed for at least 2 h. Cool the flask to 40 °C. Be sure to cool sufficiently as continuing from here

at high temperatures may lead to clouding of the finished agar. However, lower temperatures may result in premature hardening of the agar.

3.4.1) (Optional) To speed up the cooling process, place the flask in a 40 °C water bath 15 min before placing on a stir plate.

3.5) When the temperature of the agar media reaches 40 °C, add 200 µL 1 M $CaCl_2$, 200 µL of 5 mg/mL cholesterol solution, 200 µL 1 M $MgSO_4$ and 5 mL 1 M KPO_4 buffer as the solution continues to stir to final concentrations of 1 mM $CaCl_2$, 5 µg/mL cholesterol, 1 mM $MgSO_4$, 1 mM KPO_4.

3.5.1) (Optional) For NGT-3D lifespan assay add 80 µL of 150 mM FUdR to a final concentration of 120 µM[12].

3.6) Add 6 mL of the 10 mL diluted bacterial culture from step 2.4 into the flask directly.

3.6.1) (Optional) For NGT-3D, remove 6 mL of agar media before step 3.6 and keep it warm separately. This media will be used for the bacteria-free top layer.

3.7) Using sterile procedures, dispense the media into a sterile culture chamber. Make sure the cover of the chamber closes tightly.

3.7.1) (Optional) To prevent bacterial colonies from forming at the top surface of the agar, make a layer of bacteria-free agar media from step 3.6.1 on the top before the media from step 3.7 completely hardens. This will create a bacteria-free zone at the top of the NGT-3D.

3.7.2) For NGT-3D, pour 6.5 mL media into the 8 mL clear plastic test tubes to make a bacteria agar layer, and carefully dispense 200 µL of the bacteria-free media on top of the semi-hardened 3D media to make a thin bacteria-free layer on top.

3.7.3) For NGB-3D, pour 65 mL of media into the 25 cm^2 clear plastic cell culture bottle. This amount should fill the body of the 25 cm^2 cell culture bottle.

3.8) Leave chambers vertically at room temperature for one week to allow bacterial colonies to grow to a considerable size of at least 1 mm diameter.

3.8.1) (Optional) For lifespan assay in NGT-3D, cover chambers with aluminum foil to prevent light degradation of FUdR.

4. Measure fitness of worm population on NGT-3D (Relative brood size assay)

4.1) Pick an L4 stage worm using a platinum wire pick and transfer on a bacteria-free NGM plate. Allow worm to freely move around for a few minutes to remove bacteria attached to its body.

4.2) Repeat step 4.1 and make sure that the worm is free from bacteria. Generally, two repeats of 4.1 are enough.

4.3) Carefully place the clean worm on the surface of the 3D media with a platinum wire pick. The worm should eventually enter the agar into the 3D agar matrix.

4.4) Close the cover loosely allowing some air to get into the tube, but preventing drying of the agar media.

4.5) Incubate for 96 h at 20 °C.

4.6) After 4 days, close the lids tightly and place the NGT-3D culture chamber into an 88 °C water bath to melt the agar. The heat kills worms but their bodies remain intact.

Note: Using 8 mL tubes for NGT-3D, 20-30 min incubation is usually sufficient.

4.7) Using a glass pipette, transfer the melted media onto a 9 cm plastic petri dish. Plastic pipettes are not advised, as worms can often stick to them.

4.8) Using a transmission stereodissecting microscope, count the number of worms at the L3, L4, and adult stages. Do not count worms that are L2 stage and younger, as the F1 and F2 generations can be confused here. Thus, this assay is a relative brood size assay rather than a total brood size assay.

5. **Image and record worm behavior on NGB-3D**

5.1) Pick an L4 stage worm using a platinum wire pick and remove any bacteria stuck to the surface by transferring to a bacteria-free NGM plate and allowing it to freely move for a few minutes.

5.2) Repeat step 5.1 to make sure the worm is free from bacteria.

5.3) Carefully place the clean worm onto the center of the agar surface of the NGB-3D near the neck of the bottle with a platinum wire pick. The worm should eventually enter the agar into the 3D agar matrix.

5.4) Close the cover loosely allowing some air to get into the tube, while preventing drying of the agar media.

5.5) Image or record the worm under a transmission stereodissecting microscope. Adjust the focus as the worm moves through the 3D matrix.

REPRESENTATIVE RESULTS:

The construction of NGT-3D is a simple and straightforward protocol that results in an agar-filled test tube with small bacterial colonies spaced throughout the agar (Figure 1A). Worms can freely move through the agar matrix, finding and consuming the bacterial colonies. To confirm whether *C. elegans* can reproduce and grow normally in

NGT-3D, we compared fertility and larval development in 3D with standard 2D NGM plates. In the relative brood size assay, adult *C. elegans* hermaphrodites in NGT-3D reproduce just as well as hermaphrodites in the standard 2D NGM plates when bacterial colonies are plentiful with at least 60 colonies (Figure 1B). Furthermore, larval development in NGT-3D also proceeds normally when there are plenty of bacterial colonies with the majority of worms in the adult state after 96 hours (Figure 1C). However, if bacterial colonies are sparse in the 3D matrix, both relative brood size (Figure 1B, left bar) and larval development (Figure 1C, left bar) are negatively impacted.

To test whether habitation in 3D has any effect on the long-term physiology of the worm, we conducted a lifespan assay comparing NGT-3D and NGM plates. The average lifespan for worms on NGT-3D was 15.6 ± 3.6 days compared to 14.8 ± 3.1 days for standard NGM plates. The lifespan curves for worms living on NGT-3D and NGM were nearly identical. Thus, it seems that worms survive equally well in 3D and 2D conditions.

Manufacturing the NGB-3D is also not difficult and should result in a clear, agar-filled culture bottle like that seen in Figure 2A. To easily view the bacterial colonies, we have expressed deoxyviolacein, a dark purple bacterial metabolite[11], in the OP50 strain *E. coli* (Figures 2A, 2B; strain available on request). Live worms can be imaged under a transmission stereo-dissecting microscope (Figure 2B, Supplemental Movie 1).

Storage of NGT-3D and NGB-3D at room temperature is sufficient to maintain both of these culture chambers for up to 1 month. Desiccation of the agar is not a concern for either NGT-3D or NGB-3D if a plug-type or screw-type cap that fits the tube or bottle is applied.

Figure 1: Development, fertility and lifespan of *C. elegans* cultivated in NGT-3D is comparable with that of the standard 2D NGM
(A) Images of NGT-3D at several dilutions of bacteria. Left and right contains a 5 X 10^{-7} dilution, and the middle is a 1 X 10^{-7} dilution. (B) Relative brood size of wild-type worms in NGT-3D to fewer than 60 OP50 colonies (n = 24), greater or equal to 60 colonies (n = 14), or on 2D NGM plates (n = 29). Error bars indicate standard error. (C) Percent of F1 generation worms in the L3, L4 or adult developmental stage in NGT-3D with fewer than 60 OP50 colonies, greater than or equal to 60 colonies, or on 2D NGM plates. (D) Survival curve of wild-type *C. elegans* in NGT-3D or NGM plates. N.S. indicates not significantly different calculated by log-rank test. This figure has been modified from Lee[9].

Figure 2: Imaging *C. elegans* in 3D using NGB-3D
(A,B) Images of NGB-3D (C) Images of adult *C. elegans* near a colony of OP50 strain *E. coli* expressing the dark purple pigment deoxyviolacein. Scale bar is 500 μm.

Supplementary Movie 1:
Adult *C. elegans* approach an *E. coli* OP50-violacein colony in NGB-3D.

DISCUSSION:

The laboratory cultivation of *C. elegans* using the classical nematode growth media plates was crucial to the hundreds of important discoveries that research in *C. elegans* has provided. Here, we present new methods to cultivate *C. elegans* in an environment that more accurately reflects their natural three-dimensional habitats. Although other methods have been used to observe *C. elegans* in 3D[13], this is the first protocol that allows cultivation of worms in a solid 3D matrix. The two methods shown here, NGT-3D and NGB-3D, allow scientists to ask questions of fitness, development and growth in 3D cultivation, and also image and record the worms in 3D. Although NGT-3D and NGB-3D have some differences to the standard 2D NGM plates, they are similar to this original method and only adjusted to add worm cultivation in the z-axis. Thus, we find that development, fertility and lifespan proceed normally in our conditions. The goal was to create a protocol that any lab can employ for their research. These methods are very simple, easily reproducible at a very low cost, and allow for adjustment of protocols to fit the users' specific experiments.

Although all of the steps in the protocol are important, the critical step in making NGT-3D and NGB-3D is the cooling temperature of the agar after autoclaving in Steps 3.4 to 3.6 of the protocol. If the temperature cools too low (several degrees below 40 °C), the agar will begin to harden and the added solutions will not mix properly into the agar. However, if the cooling is insufficient (several degrees above 40 °C), the added bacteria may die and the added cholesterol solution may cloud the agar rendering the agar useless for experiments. In addition, an area of caution is in step 4.6. When melting the NGT-3D agar in the water bath, make sure to close the lids tightly to aid in the melting. However, the heat can cause the caps to dangerously "pop" off and become a projectile. For safety purposes it is best to cover the water bath. Many parts of the protocol are quite adaptable to the user. For instance, we have used a clear plastic test tube for NGT-3D and a 25 cm^2 clear cell culture bottle (holds a total of 65 mL) for the NGB-3D. Other types of clear tubes or bottles available to the user should also work. Also, bacterial growth is adjustable. *E. coli* strain OP50 generally takes about a week to grow to at least 1 mm, which was preferred for our experiments. In our experience, slightly shorter and even much longer growth times for bacteria do not seem to affect worm growth or fertility. Adjustment of agar concentration, however, will affect worm movement. Lower concentrations of agar result in worms showing a swimming-like movement, and higher concentrations can inhibit overall movement.

One area of concern in NGB-3D was the issue of condensation and water accumulation. As the warm liquid agar hardens, inevitably some condensation develops over time, particularly at the interface of the bottle and the agar. This can pose problems for the worm, which shows swimming behaviors in liquids[14]and the growth of bacteria, which can spread throughout the bottle by the liquid. Prevention of condensation is difficult. Two possible remedies exist for this. First, we make sure that the worm can easily enter the agar by placing it in the center of the agar surface at the neck of the bottle. Secondly, we intentionally keep the concentration of bacteria low in NGB-3D, thereby diminishing the chance that a colony grows at the interface of liquid and agar.

Another concern is the imaging of worms in NGB-3D. To increase the clarity and quality of images of worms in 3D, colonies should be close to the surface but remain completely embedded in the agar. We attempted to grow only embedded colonies near the surface by placing a thin layer of bacteria-containing agar at the surface, and the remaining media was bacteria-free. However, the issue was not fully solved by this method. Instead, we decided to produce many NGB-3D bottles at one time, and use only the ones that contain embedded colonies close to the surface, thereby increasing the chance that a worm will move to a colony near the surface. One option we have not explored is changing the solid media to a matrix other than the granulated agar we employ here. Imaging worms in the z-axis direction can also be challenging. We do this by manually raising and lowering the coarse focus, but if an automated tracking program exists for this, it may be quite helpful.

The hope is that the techniques presented here for the cultivation of *C. elegans* in 3D will be widely used by nematode biologists. Many questions remain about worm biology, including aging and behaviors in 3D, and whether what scientists observe in 2D is relevant for the worm in 3D. Indeed, a previous study using NGT-3D showed the impact on reproductive fitness that 3D cultivation had on a sensory mutant that reproduces normally in 2D conditions[9]. In addition, one study showed altered movement behaviors in 3D compared to 2D[15]. Finally, this system can be used to begin to answer questions of whether certain conditions, mutants or manipulations confer fitness advantages to the animal in its native 3D environment.

ACKNOWLEDGMENTS:
This work was supported by a New Investigator Grant [2014R1A1A1005553] from the National Research Foundation of Korea (NRF) to J.I.L; and a Yonsei University Future Leader Challenge Grant [2015-22-0133] to J.I.L.

DISCLOSURES:
The authors have nothing to disclose.

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Fig. 1

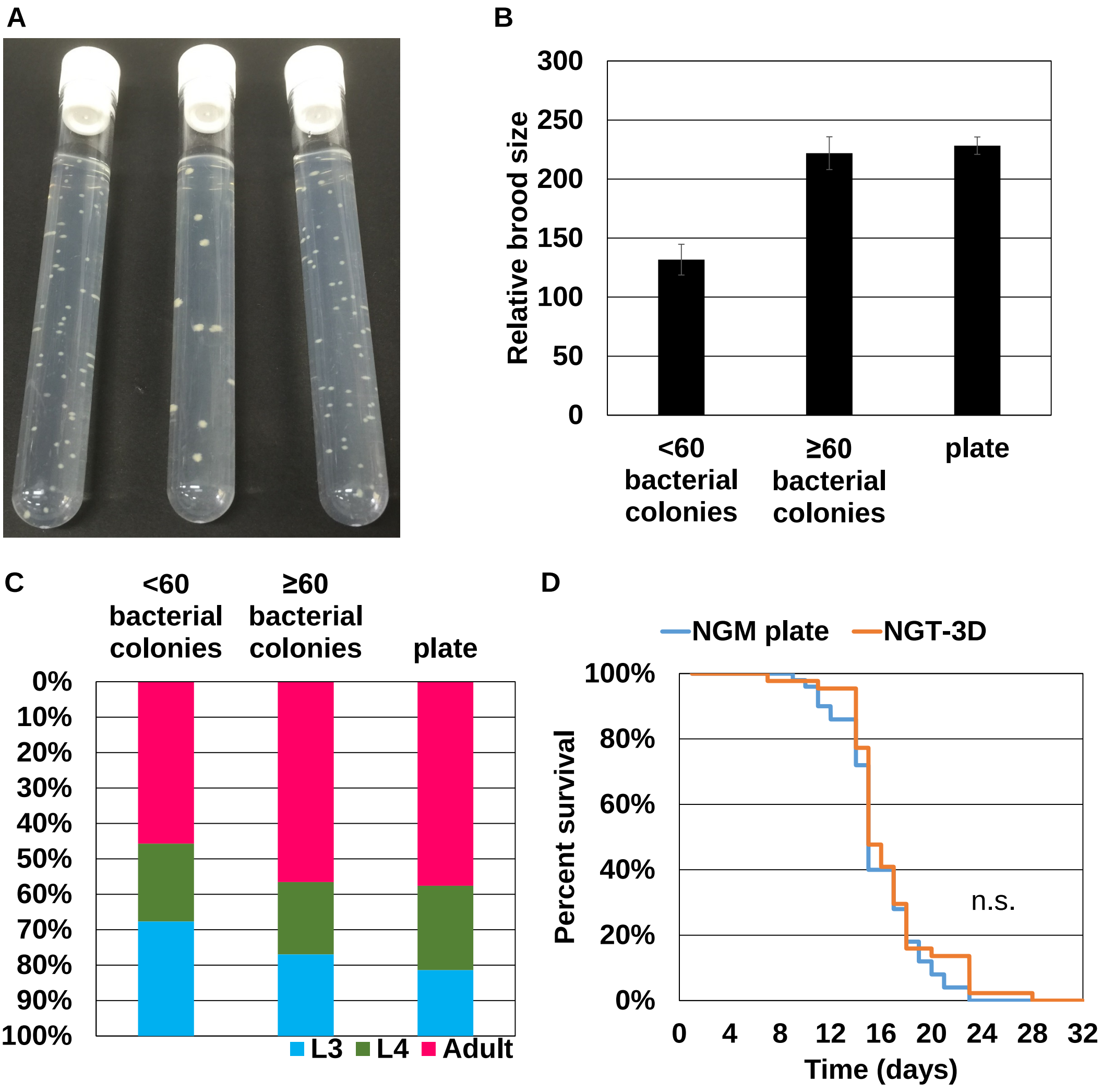

Figure 2

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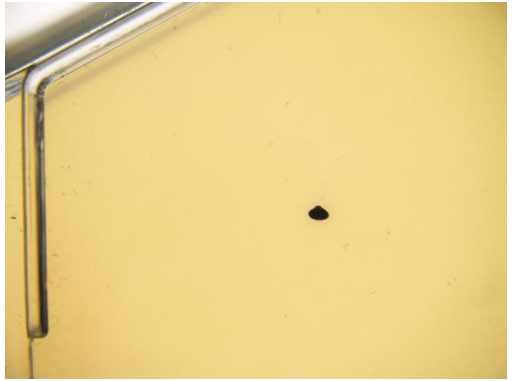
Fig. 2

A

B



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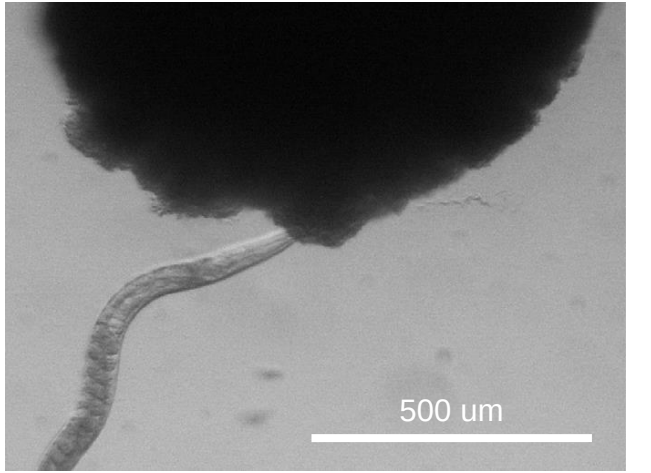

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number
LB broth, Miller (Luria-Bertani)	Difco	224620
Sodium chloride	DAEJUNG	7548-4400
Agar, Granulated	Difco	214530
Peptone	Bacto	211677
Calcium chloride, dihydrate	Bio Basic	CD0050
Cholesterol	Bio Basic	CD0122
Ethyl alcohol	B&J	RP090-1
Magnesium sulfate, anhydrous	Bio Basic	MN1988
Potassium phosphate, monobasic, anhydrous	Bio Basic	PB0445
2'-Deoxy-5-fluorouridine	Tokyo Chemical Industry	D2235
Potassium phosphate, dibasic, anhydrous	Bio Basic	PB0447
Multi-Purpose Test Tubes	Stockwell Scientific	ST.8570
Test Tube Closures	Stockwell Scientific	ST.8575
Cell Culture Flask	SPL Lifescience	70125
Research Stereo Microscope	Nikon	SMZ18
High-Definition Color Camera Head	Nikon	DS-Fi2
PC-Based Control Unit	Nikon	DS-U3
NIS-Elements Basic Research, Microscope Imaging Software	Nikon	MQS32000

Comments/Description
58.44 MW
2*H2O; 147.02 MW
386.67 MW
99.99%; 46.07 MW
120.37 MW
136.09 MW
246.19 MW
174.18 MW
8 mL
25 cm^2

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Article Title:	Cultivation of C. elegans in three dimension in the laboratory
Signature:	
Date:	5/27/2016

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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 10^{9} 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!

This piece of the submission is being sent via mail.

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