

Video Article

Cultivation of *Caenorhabditis elegans* in Three Dimensions in the Laboratory

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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.

Video Link

The video component of this article can be found at <http://www.jove.com/video/55048/>

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 decades¹. *C. elegans* was the first multicellular organism to have its genome sequenced in 1998², 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 level³⁻⁵.

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* bacteria⁶. 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 short⁹. 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) plates¹⁰. 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. Prepare the following sterile solutions: 1 L of 0.1454 M NaCl solution, 1 L of 1 M CaCl₂, 1 L of 1 M MgSO₄, Lysogeny Broth (LB), 1 L of 1 M KPO₄ buffer (108.3 g of KH₂PO₄ and 35.6 g of K₂HPO₄, and fill H₂O to 1 L). Production of NGM using these solutions can be found in a previous protocol¹⁰.
2. Autoclave solutions at 121 °C, 15 min.
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.
4. (Optional) Prepare 10 ml of a 150 mM of 2'-deoxy-5-fluorouridine (FUdR) stock. In a 15 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

1. Inoculate 10 ml LB broth with bacteria. The standard bacteria used for *C. elegans* feeding is *E. coli* strain OP50.
2. Culture the bacterial inoculation at 37 °C in a shaking incubator overnight.
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*.
4. Using a sterile 1,000 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)

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.
2. Add 195 ml distilled water and cover mouth of the flask with aluminum foil.
3. Autoclave 121 °C for 15 min.
4. Place the hot autoclaved flask onto a stir plate, and stir at a moderate speed for at least 2 hr. 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.
 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.
5. When the temperature of the agar media reaches 40 °C, add 200 µl 1 M CaCl₂, 200 µl of 5 mg/ml cholesterol solution, 200 µl 1 M MgSO₄ and 5 ml 1 M KPO₄ buffer as the solution continues to stir to final concentrations of 1 mM CaCl₂, 5 µg/ml cholesterol, 1 mM MgSO₄, 1 mM KPO₄.
 1. (Optional) For NGT-3D lifespan assay add 80 µl of 150 mM FUdR to a final concentration of 120 µM¹².
6. Add 6 ml of the 10 ml diluted bacterial culture from step 2.4 into the flask directly.
 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.
7. Using sterile procedures, dispense the media into a sterile culture chamber. Make sure the cover of the chamber closes tightly.
 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.
 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. For NGB-3D, pour 65 ml of media into the 25 cm² clear plastic cell culture bottle. This amount should fill the body of the 25 cm² cell culture bottle.
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.
 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)

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.
2. Repeat step 4.1 and make sure that the worm is free from bacteria. Generally, two repeats of 4.1 are enough.
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. Close the cover loosely allowing some air to get into the tube, but preventing drying of the agar media.
5. Incubate for 96 hr at 20 °C.
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.
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.
8. Using a transmission stereo dissecting 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

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 min.
2. Repeat step 5.1 to make sure the worm is free from bacteria.
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.
4. Close the cover loosely allowing some air to get into the tube, while preventing drying of the agar media.
5. Image or record the worm under a transmission stereo dissecting 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 metabolite¹¹, 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.

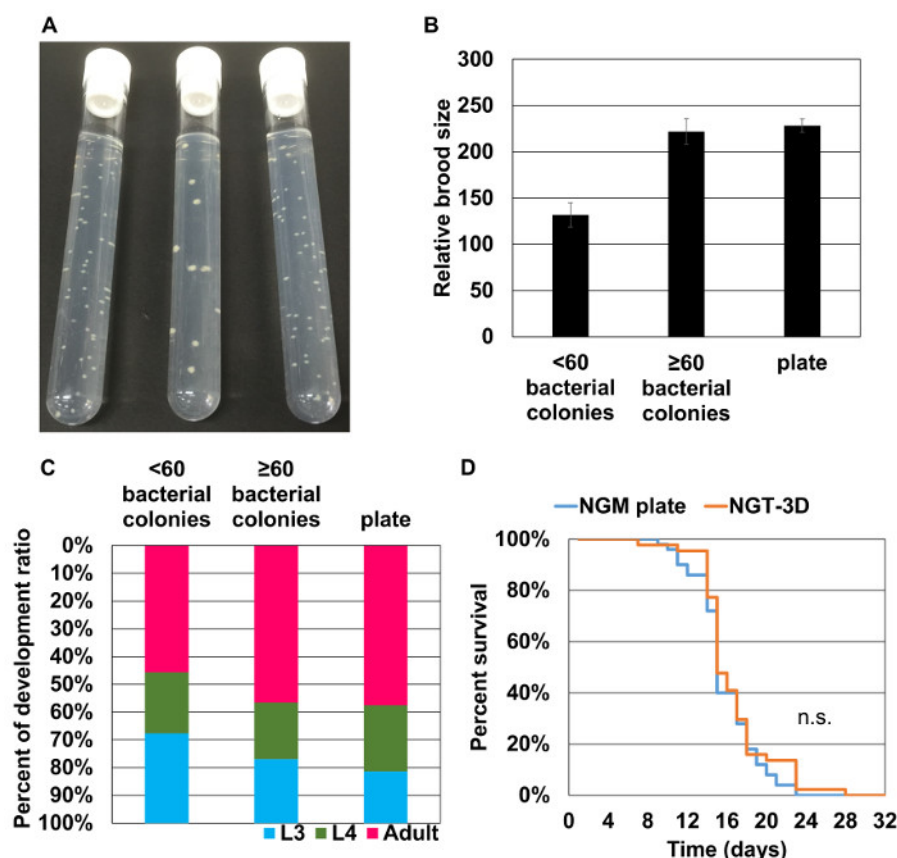


Figure 1: Development, fertility and lifespan of *C. elegans* cultivated in NGT-3D is comparable with that of the standard 2D NGM. (A) Images of NGT-3D at several dilutions of bacteria. Left and right contains a 5×10^{-7} dilution, and the middle is a 1×10^{-7} dilution. (B) Relative brood size of wild-type 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 Lee⁹. [Please click here to view a larger version of this figure.](#)

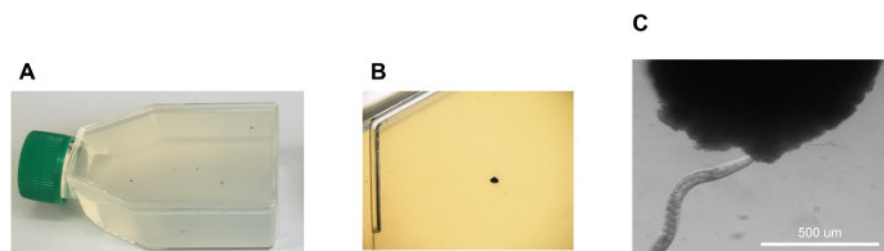


Figure 2: Imaging *C. elegans* in 3D using NGB-3D. (A,B) Images of NGB-3D; (C) Images of adult *C. elegans* near a colony of OP50 strain *E. coli* expressing the dark purple pigment deoxyviolacein. Scale bar is 500 μm . [Please click here to view a larger version of this figure.](#)



Supplementary Movie 1: Adult *C. elegans* approach an *E. coli* OP50-violace in colony in NGB-3D. [Please click here to download this file.](#)

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 3D¹³, 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 cm² 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 liquids¹⁴ 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 conditions⁹. In addition, one study showed altered movement behaviors in 3D compared to 2D¹⁵. 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.

Disclosures

The authors have nothing to disclose.

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