Caenorhabditis elegans **larval growth assay for nanomaterials**

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1. Introduction

1.1 Overview. This protocol describes an assay for toxic effects of nanomaterial exposure on larval *Caenorhabditis elegans*. The endpoints analyzed include lethality, growth inhibition, and teratogenesis. The basic version of the assay takes approximately 4 days. This assay was adapted by the Meyer laboratory at Duke University from a high throughput, automated assay described by the Freedman laboratory at NIEHS, with advice from Windy Boyd (Freedman laboratory). We present two different dosing solutions and two different methods for measuring growth.

This protocol assumes general familiarity with culture of *C. elegans* and toxicological principles; however, the nematode culturing requirements are not onerous and could be learned fairly quickly with the assistance of a *C. elegans* laboratory.

1.2 Test Organism. *C. elegans* is, in many ways, an ideal model organism for laboratory testing, and is being increasingly utilized for toxicity testing (Leung *et al*., 2008). *C. elegans* reproduces quickly and in large numbers allowing for relatively high throughput toxicological screening assays. *C. elegans* develops from egg through four larval stages (termed L1-L4) to gravid adult hermaphrodites in approximately 72 h at 20ºC. Because of this short life cycle and high reproductive capacity along with the ability to store stocks frozen in liquid nitrogen long-term, maintenance and culture of *C. elegans* are straightforward. In the lab, *C. elegans* is often fed the slow growing uracil auxotroph OP50 strain of *E. coli* that is also easy to maintain and quantify by measuring the optical density (OD) of the bacteria solution at 570 nm. We have recently also used UvrA (Croteau et al., 2008) bacteria as a food source to that is easy to kill using ultraviolet C (UVC) radiation (described below).

1.3 General Approach. Age-synchronized stage 1 larvae (L1s) are exposed in liquid culture to the nanomaterial of interest, and daily observations are made microscopically to quantify survival, growth, and teratogenesis. It is possible to add examination of additional endpoints, such as egg production, egg hatch, adult growth, etc. *C. elegans* is a model system with particularly strong genetic and genomic tools (Antoshechkin and Sternberg 2007). It is therefore also possible to utilize transgenic, mutagenized, or gene knockdown approaches to enhance the sensitivity of this assay (e.g.: stress-responsive gfp-linked reporter genes; mutants lacking specific defenses; strains with GFP expression in specific cells to enhance visualization).

1.4 Considerations for Optimizing Liquid Culture Conditions. It should be noted that culture conditions have a considerable impact on the larval growth of *C. elegans* from the L1 stage to adulthood. Previous work has suggested that there may be a requirement for metabolic activity in the microbial food source of *C. elegans* (Lenaerts *et al.,* 2008). Lenaerts also notes the possibility that there is a heat labile component of *E. coli* that is required by *C. elegans* and is lost during the process of heat-killing. Our own observations have demonstrated that *C. elegans* being fed heat killed OP50 *E. coli* take more than twice as long to reach adulthood as those feeding on live OP50 *E. coli*. We have also noted that even gentle orbital shaking leads to greatly reduced the rate of larval growth, perhaps due to the inability of *C. elegans* to feed efficiently on a constantly moving food source. If the level of liquid medium is kept sufficiently low in the wells of the culture plates to prevent hypoxic conditions, *C. elegans* can be cultured with no shaking. Based on these observations, we have determined that the optimal liquid culture conditions for these *C. elegans* larval growth assays are to incubate the worms in K+ medium at 20ºC with no shaking while feeding them with live OP50 *E. coli*.

2. Supplies

2.1 Nematode and bacteria For the standard assay the well-characterized N2 wildtype strain of *C. elegans* is used, and is fed OP50 bacteria. Both are obtained from the *Caenorhabditis* Genetics

Center http://www.cbs.umn.edu/CGC/, and the N2s are purchased anew each year to prevent genetic drift.

2.2 Equipment

- Microscope with digital camera and image acquisition and analysis software (or comparable)
- Cooling incubators for 20ºC nematode culture
- Tabletop centrifuge
- Balance
- 25 cm^2 cell culture flasks with vented caps
- 15 mL centrifuge tubes
- 1.5 mL microfuge tubes
- Glass Pasteur pipets
- Floor shaker set to 37° C
- Incubator set to 37° C
- 100-mm petri dishes
- Water bath set to 60° C
- Orbital Shaker
- Low-binding pipette tips (VERY IMPORTANT since larval worms tend to stick to inside of tips)
- Other standard laboratory equipment and supplies (pipettors, 24-well plates, etc.)

2.3 Chemicals and Solutions

- LB broth
- K-medium
- K-medium plus cholesterol (K+; also known as Complete K medium: Boyd et al., 2009)
- Bleaching solution (for egg preps)
- K-agar
- Sodium Azide
- Moderately Hard Reconstituted Water (a low ionic strength alternative to K or K+ medium developed by Paul Bertsch's laboratory)

3. Experimental Procedure

Note that essentially the same procedures can be carried out using K+, moderately hard reconstituted water, or other media. The procedure is described here for K+.

3.1 Prepare nematodes and supplies

1. At Day -1, Egg prep (see Appendix) from ~3 day-old plates containing gravid adults and eggs. This should be done ~24 h prior to dosing. Place rinsed eggs in K+ medium overnight in 25 cm^2 cell culture flasks with vented caps to get synchronized L1s (Lewis and Fleming 1995).
2. Every 24 hrs prepare dosing solutions:
    - Dosing solutions are 500uL total in K+ for 24 well plates

- Determine volumes of nanoparticle stocks necessary to achieve target dosing concentrations in 500uL total volume; if volume of stock needed is large enough to cause concern over dilution of K+, consider making more concentrated K+ such that when nanoparticle stocks are added (usually suspended in water), the final K+ concentration is ~1X
- A good amount of OP50 can be achieved in dosing solutions by spinning down (15000rpm for 6 min) 50-100uL of the ~16hr OP50 culture in LB-broth for every 1mL of dosing solution (for day 1 of the experiment, you can put 10uL of the ~16hr OP50 culture in LB-broth for every 1mL of dosing solution since L1 and L2 larvae do not eat as much). Once spun down, aspirate off LB-broth and resuspend pellet in the appropriate volume of K+ medium (will need to make more concentrated to add to wells with a higher concentration of nanoparticles since less K+ medium will be added to these wells).
- Pipette appropriate volumes of K+ (or other) medium with OP50 into wells.
- Lastly, add nanoparticles from stocks as calculated to achieve target dosing concentrations.
- Consider the properties of the nanomaterial under consideration to avoid agglomeration, etc.

3.2 Set up exposure

1. At Day 0, spin down synchronized L1s at 2500 x g for 4 minutes in 15 mL centrifuge tubes
2. Decrease volume of K+ medium by aspiration, resuspend worms, then pipette 2uL onto glass slide and count worms to determine worms per uL.
3. Want at least 100 worms per 5uL so if necessary, spin down and aspirate off more K+ to further concentrate worms
4. Into a 24 well plate, add appropriate volume such that you end up with ~500 L1 larvae in each well (make sure to add no more than 25uL so as not to dilute dosing solution too much), shake tube each time you pipette to ensure an evenly distributed suspension of worms
5. Incubate at 20ºC (no shaking)

3.3 Observations

Record every 24 hours (or as desired)

3.3.1 Survival

Observe nematodes in wells to check for viability. If any potential lethality (lack of motion) is observed, continue with survival assessment; otherwise, proceed to growth assessment.

1. Tilt plate and let worms settle in bottom cusp of the wells (allow ~5-10 minutes for settling)
2. Using a 1 ml pipet, pipet out larvae and dosing solution into 1.5 ml microfuge tube. Other sizes of tubes are fine; **a key point is to identify tubes that the worms do not stick to**. Otherwise, you may lose a lot of worms.
3. Wash each well with K-med and add to centrifuge tube
4. Spin at 2200 x g for 2 minutes
5. Pipet ~10 nematodes onto a small k-agar dish and gently prod with platinum wire worm picker to assess viability. Nonresponsive nematodes are defined as dead.

Note: staining-based methods for determination of live vs dead worms using a COPAS Biosort (e.g., SyTox) have been described, but in our experience tend to underestimate toxicity because only nematodes that have been dead long enough for the cuticle's integrity to be compromised stain well.

3.3.2 Growth

Assuming that some or all of the nematodes are alive, quantify the growth of the nematodes either manually or using a COPAS Biosort, as follows. The advantages of the manual method are that lengths are described in microns, rather than arbitrary units, and a COPAS is not needed. The advantage of the COPAS is that a much higher "n" can be achieved, and the measurement is far faster.

3.3.2.1 Manual measurement

1. Tilt plates @ ~45 degrees for at least 5 minutes to allow gravity to collect larvae in the bottom cusp of the wells. Using a 1 ml pipette, pipette out larvae and all dosing solution into 1.5 ml low-stick tubes.
2. Using low-binding pipette tips, pipette out larvae and dosing solution into 1.5 ml microfuge tube
3. Wash each well with K-med and add to centrifuge tube
4. Spin at 2200 x g for 2 minutes
5. Pipette between 1-5 µl out of the bottom of the vial (want at least 20 larvae) onto a slide trying not to suck up any of the *E. coli* pellet (it helps to look at bottom of the tube under a dissecting scope while pipetting)
6. Add 2-3 µl of 170 mM NaN_3 to immobilize the nematodes
7. Observe and capture image (use any magnification as long as all are consistent, 5X gets the largest number of worms in one field) then save with a name identifying the sample
8. Because slides dry out relatively quickly, do only 2 or 3 samples at a time
9. In a new 24 well plate, replenish dosing solutions (replenish every 24hrs to ensure consistent dosing with nanoparticles and enough bacteria to keep *C. elegans* well fed)
10. Spin worms down again (2200 x g for 2 minutes)
11. Pipette remaining larvae from the bottom of the tube (in as small a volume as possible [~20uL] to prevent dilution of the dosing solution) into the proper replenished well in the new 24 well plate.
12. Using imaging software (e.g. ImageJ) measure and record worm lengths
    a. Intructions for using ImageJ: Open ImageJ, File, Import, Image Sequence
    b. Click on the first image in the folder you saved images to and then click Open (all images will load in order)
    c. Click Analyze, Set Measurement, and make sure Display Label is checked
    d. Once your images are open in ImageJ, using the Segmented Line tool, draw a line down the midline of each worm in the field from tip to tip.
    e. Click Analyze, Measure or press Ctrl+M and a box will pop up with the measurement of your segmented line in arbitrary units (at 5x magnification, 746 arbitrary ImageJ units = 1mm)
    f. Periodically save the data in the measurements box to an Excel spreadsheet for later analysis.

3.3.2.2. COPAS Biosort measurement

Day 0: Dispensing worms

1. Turn on the main power of the COPAS BIOSORT system, computer and compressor.
2. Click START, wait as the laser powers up, and select DONE when laser power reaches 10 ± 1 mW.
3. When pressures have equilibrated to previously set levels, click PRESSURE OK.
4. Click Clean button and watch for bubbles and debris dislodged from the flow cell.
5. Check the sheath flow rate, and make sure it is between 9 and 10 mls per minute.
6. Run control particles: place a minimum of 10 ml – 20 ml of the control particles into the sample cup.
7. Select RUN CONTROL PARTICLES.
8. Press CLEAN several times and then click ACQUIRE.
9. Adjust the sample pressure, until system flow reads 5- 10 objects per second.
10. When a TOF mean of 45 ± 6.0 is achieved, click ERASE.
11. Rinse the sample cup twice with deionized/distilled water, then fill the sample cup to the same level and click SAMPLE VALVE to let ddH_2O flow through.
12. Align stage using ALIGN PLATE HANDLER & STAGE from the TOOLS pull down menu.
13. Set up sort values, including number of worms per well, delay and width.
14. Set gain values.
15. Place a lid from either a 24 or 96 well plate and select FILL PLATE.
16. Verify sort results by microscope; in most cases, the actual sorted worm number is only 70% of the set value. **It is important to plan your experiment with the expectation of this loss!**

Days 1, 2, 3: Measuring worms

1. Turn on the main power of the COPAS BIOSORT system, computer and compressor.
2. Click START, wait as the laser powers up, and select DONE when laser power reaches 10 ± 1 mW.
3. When pressures have equilibrated to previously set levels, click PRESSURE OK.
4. Click Clean button and watch for bubbles and debris dislodged from the flow cell.
5. Check the sheath flow rate, and make sure it is between 9 and 10 mls per minute.
6. Run control particles: place a minimum of 10 ml – 20 ml of the control particles into the sample cup.
7. Select RUN CONTROL PARTICLES.
8. Press CLEAN several times and then click ACQUIRE.
9. Adjust the sample pressure, until system flow reads 5- 10 objects per second.
10. When a TOF mean of 45 ± 6.0 is achieved, click ERASE.
11. Rinse the sample cup twice with deionized/distilled water, then fill the sample cup to the same level and click SAMPLE VALVE to let ddH_2O flow through.
12. Set gain values.
13. Check the box ReFlex.

14. Place a 96 well plate with worms and click SAMPLE.

Data processing

1. Open the txt file and save it as Excel workbook.
2. Keep only the following categories in the excel file: row, column, stat sort, stat sel, TOF, EXT, Green, Yellow and Red.
3. Sort by row, column and TOF. We eliminate outliers for each day based on the TOF value of the previous day ± 1 standard deviation.
4. Screen for EXT outliers using the same method.
5. Plot TOF vs EXT data to screen out values not falling along the TOF vs EXT line that defines a worm.
6. Use TOF or EXT as an arbitrary unit to represent the growth of worms. TOF measurements tend to be more variable because the worms are not always completely straight when they go through the flow cell, so TOF flight data is best used with higher "n"s. EXT values are less variable but can be affected by the presence/absence of food and/or nanoparticles in the gut, so that they do not always represent size only. Young worms (up to4-5 days post-L4) tend to clear their guts in <30 minutes, however, minimizing this problem.

3.3.3 Teratogenesis/disrupted development

If desired, assess altered development and/or teratogenesis. The developmental pattern of *C. elegans* is nearly invariant and fully described at the cellular level, facilitating observation of altered development. These measurements may also be useful in that in some cases growth (size) can be only marginally altered, but significant concomitant alterations in larval stage or developmental pattern may be observed. For example, slower growth may reflect an overall delay in normal development, or a normal progression of larval stages but with smaller than normal size at the later stages.

The actual endpoints measured will vary with chemical and effect.

4. Recipes

<u>K-agar Plates</u>

2.36 g KCl

3.0 g NaCl

2.5 g Bacto-peptone

17 g Bacto-agar

1 ml cholesterol (5mg/ml undenatured ethanol, heat to dissolve)

Add dH_2O to 1 L. Autoclave. Place in 60° C water bath. After cooled, add:

1 ml 1M $CaCl_2$ (sterile)

1 ml 1M $MgSO_4$ (sterile)

Dispense appropriate aliquots (15 ml for 100-mm, 7.5 for 60-mm, and 4 for 35-mm) into Petri plates.

<u>Bleaching solution (</u>for egg preps)

1.0g NaOH

20ml Clorox (5.25%NaOCl) Must be fresh! With no additional additives such as “fresh spring scent.”

80ml dH2O

K-medium

2.36 g KCl

3 g NaCl

1000 ml dH_2O

LB broth

20 g Lennox L-Broth Base

1000 ml dH_2O

Autoclave. Cool to room temperature. Inoculate (using sterile technique) with a loop of frozen *E. coli* OP50 culture. Incubate at 37°C with shaking (250 rpm) overnight.

K-Medium Plus

To each liter of room temperature sterile K-medium, add sterile volumes of:

1 ml cholesterol (5mg/ml undenatured ethanol, heat to dissolve)

1 ml 1M $CaCl_2$

1 ml 1M $MgSO_4$

1M Potassium Phosphate, pH 6.0

136 g KH_2PO_4

Add dH_2O to 900 ml. Adjust pH to 6.0 with concentrated KOH.

Add dH_2O to 1 L. Autoclave in 100-ml aliquots.

Trace Metals Solution

1.86 g Na_2EDTA

0.69 g $FeSO4*7H_2O$

0.2 g $MnCl2*4H_2O$

0.29 g $ZnSO4*7H_2O$

0.016 g CuSO4

Add dH_2O to 1 L. Autoclave in 100-ml aliquots. Store in dark (wrap in aluminum foil) at room temperature.

Sodium Azide solution

Make a 1M stock solution. Add 170 µl of 1M stock to 830 µl of K-medium.

Moderately Hard Reconstituted Water

(Low ionic strength, low-chloride-based buffer developed by Paul Bertsch's lab)

Per 1000mL of dH2O, add the following:

96.0mg NaHCO3
60.0mg CaSO4*2H2O
60.0mg MgSO4*7H2O
4.0mg KCl

Dissolve MgSO4*7H2O before adding the other ingredients.

To acclimate the worms wash with K-media then 50% K-media and 50% recon let sit for one hour then transfer to 100% recon.

5. References

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6. Appendices

6.1 "Egg Prep"

1. Use any plate containing high concentration of eggs (>5000). This can be a 3-day old dauer plate, or 4-day old egg prep plate. Using considerable pressure on a squirt bottle, apply K-medium to loosen eggs. Swirl plate for a few seconds to loosen eggs, or rub with a bent glass bar. Pipet worm solution to 15-ml centrifuge tube with a sterile pipet. Rinse plate 3x to ensure maximum recovery.

2. Fill tube with K-medium ~12 ml.

3. Centrifuge at 2000rpm for 2 min to pellet everything.

4. Remove supernatant and resuspend pellet in about 10 ml clorox solution and let it sit for 10 min. Resuspend worms with pipet every 2-3 min. (Clorox will kill all worm stages except the eggs.) Do not let sit more than 15 min or the egg prep will yield fewer eggs.

5. Centrifuge at about 2000 rpm for 2 min to pellet and remove the supernatant. You may continue to use the same pipet because you will be diluting the clorox solution.

6. Resuspend pellet in K- medium.

6.2 Live OP50 preparation

The food source is made by inoculating LB broth with OP50, culturing ~16hrs at 37° C with vigorous shaking, centrifuging and cleaning the pellet. Use the OD at 570 nm for consistency from experiment to experiment. The amount of food to be added depends on the duration of the test and the life stage of the *C. elegans* being fed and should be optimized ahead of time. The goal is to add enough that the worms do not run out of food, but as little as possible to minimize any ability of the bacteria to dilute to nanomaterial or create hypoxic conditions.

1. Determine amount of OP50 required to feed the *C. elegans* while in dosing solutions and aliquot from the ~16hr culture in LB-broth accordingly

2. Centrifuge at 10000rpm for 10 min.

3. Remove supernatant and rinse with K medium. Vortex to resuspend pellet, and centrifuge as above. Repeat for a total of three rinses and resuspend in the volume of K+ medium needed for dosing solutions.

6.3 Heat-Killed OP50 preparation

When it is unclear whether the observed larval growth effects of a toxin are attributable to their direct effects on *C. elegans* or an indirect effect as a result of the toxin killing the *C. elegans* OP50 food source it may be appropriate to feed *C. elegans* with heat-killed OP50, even though as noted earlier, *C. elegans* fed on heat-killed OP50 grow at approximately half the rate of *C. elegans* fed on live OP50. In this manner, the direct effects of the toxin can be assessed without the confounding factor of the toxin's effect on live OP50.

1. Transfer some of a ~16hr culture of live OP50 in LB-broth to a 50mL conical; leave cap slightly loose to allow venting

2. Place 50mL conical in a boiling water bath for ~30 min.

3. Allow to cool and use as described for live OP50 above.

6.4 UV-Killed *E. coli* preparation

For the aforementioned reasons (see 6.3), it may be appropriate to feed *C. elegans* with UV-killed bacteria. We have found that *C. elegans* grows better on UV-killed *E. coli* than on heat-killed *E. coli*. Thus, using UVC radiation to kill bacteria may be a better way of avoiding the confounding factors associated with feeding live *E. coli* to the worms during these growth assays. Instead of using OP50 for this preparation, a UV-sensitive strain (WP2 UvrA; Croteau et al., 2008) of *E. coli* is used to ensure UV-killing.

1. Transfer ~6mL of a ~16hr culture of live UvrA *E. coli* in LB-broth to a 150cm petri dish (or any other container with a completely flat bottom that will allow for a very thin layer of the culture to be evenly spread out)

2. Let UV lamp warm up for 5 to 10 minutes then obtain UVC (254nm) intensity reading using radiometer (set up so that the intensity at the spot you will be dosing the bacteria is ~500-700 uW/cm2; this will decrease the time needed to kill all the bacteria in the plate).

3. Based on the UVC intensity reading, dose UvrA bacteria with at least 1000J/m2

(calculate time needed as follows: intended dose/(intensity reading in uW/cm2/100) = seconds of exposure needed to achieve intended dose; Thus if you want a dose of 1000J/m2 and the intensity reading you obtain using the radiometer is 600uW/cm2, 1000/(600/100) = 1000/6 = 166.67 seconds/60 = 2.78 minutes of exposure needed.)

4. Divide the needed time of exposure by 6 and shake up/swirl plate for a few seconds after each interval to ensure that all bacteria are exposed equally and do not shield each other from the UVC.

5. After dosing, pipette bacteria into a 15mL conical and store at 4C for no longer than a week.

6. To make sure all bacteria were killed, dilute a small volume of dosed bacteria 1:20,000 then lawn out 50uL of this diluted sample onto an LB agar plate and incubate at 37C overnight. No colonies should grow.

6.5 Additional resources

Wormbase (www.wormbase.org) and the WormBook (www.wormbook.org) are wonderful resources, and the Worm Atlas (www.wormatlas.org) will be very helpful for researches interested in altered development. There are many other useful books and online tools available.