The Nematode *C. elegans* as an Animal Model to Explore Toxicology In Vivo: Solid and Axenic Growth Culture Conditions and Compound Exposure Parameters

Caenorhabditis elegans is a powerful genetic model to explore toxicology and human biological mechanisms. Its genome, and biosynthetic and metabolic pathways are highly conserved with vertebrates, including most of the known components involved in cellular development, the nervous system, and cell death genes (Riddle et al., 1997; The *C. elegans* Sequencing Consortium, 1998; Nass and Blakely, 2003). The worm is sensitive to a number of charged and uncharged molecules that can easily penetrate its brain, including heavy metals, organic toxins, and a wide range of human neuroactive drugs (Rand and Johnson, 1995; Link et al., 2000; Nass and Blakely, 2003). The worm has also been utilized to screen for mutant proteins that have altered function in the presence of the drugs (Riddle et al., 1997; Link et al., 2000). Its small size (~1 mm), large number of progeny (300 to 1000 animals from a single hermaphrodite), quick generation time (~3.5 days), and ease of growth in 96- or 384-well tissue culture plates, allow for rapid growth and ease of cultivation in a laboratory (Riddle et al., 1997; Nass and Blakely, 2003). Because worms are transparent, reporter gene fusions permit direct visualization of cellular morphology and protein expression patterns. Knockout and genetic mutants can be identified in a matter of days to weeks, and gene knockdowns are readily generated via RNA-mediated interference (Fire et al., 1998; Wicks et al., 2001). Thousands of mutant animals have been identified using these techniques and are available from a federally funded institution for free or at a nominal cost (see *Caenorhabditis elegans* WWW Server: *http://elegans.swmed.edu/*).

C. elegans also provides facile approaches to determining endpoints of toxicity. If a specific behavior is dependent on a particular cell type or molecular pathway, abnormal movement of the animals as observed under a dissecting microscope would be sufficient to determine toxicity (Riddle et al., 1997). Conversely if animal death is the endpoint, simple lack of movement of the animals following touching with a hair or a metal pick under the microscope could be sufficient. Fluorescent dyes also exist that can facilitate identification of dead animals, and these animals can be evaluated under a fluorescence dissecting microscope or in a high-throughput format such as a fluorescence plate reader (Gill et al., 2003). Transparency of the animals also allows the evaluation of potential toxins on cellular morphology or viability (Riddle et al., 1997). Cells can be observed using Nomarski/DIC optics or any of the fluorescent methodologies mentioned above. There are also a number of transcriptional fusions of stress-responsive promoters with GFP that are available through the Caenorhabditis Genetics Center (CGC), and these are useful to examine the toxicity of a compound to specific cell lineages (see *Caenorhabditis elegans* WWW Server: *http://elegans.swmed.edu/*; David et al., 2003).

C. elegans is maintained in the laboratory either on solid or in liquid growth medium. They can be grown on agar plates using *E. coli* as a food source, or in liquid cultures with or without bacteria (Hope, 1999; Link et al., 2000). Worms grown on agar plates generally utilize one of two types of bacteria and media, depending on the experimental goal. Animals grown on bacteria OP50, a uracil auxotroph that forms a thin bacterial lawn

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when maintained on NGM minimal medium, are often used when examining particular phenotypes such as movement or cellular fluorescence. This food source is also used when generating mating assays. Rich medium plates, such as the high peptone medium 8P, which is overlayed with a thick lawn of growing bacteria such as NA22 or DH5α, are used when it is desirable to grow a large number of worms for biochemical, nutritional, toxicological assays, or to obtain synchronous cultures.

Growth of *C. elegans* in liquid, using bacteria as a food source, can pose a number of problems, including difficulty in maintaining synchronicity of the worm culture and unpredictable growth rates and culture saturation. Some of these difficulties could be due to the continuously changing culture conditions during worm growth resulting from the effects of both worms and bacteria on the medium such as ion, pH, and oxygen concentrations. Also, absolute content of specific organics and metals can be difficult to maintain in the medium during nutritional and toxicological assays because bacteria could sequester or metabolize these compounds (Rand and Johnson, 1995; Rao et al., 2005). Furthermore, varied concentrations of unconsumed bacteria can quench fluorescence, resulting in erroneous data interpretations when worms are grown in microtiter plates and queried for altered fluorescence or worm density using a fluorescence microtiter plate reader.

Growth of *C. elegans* in axenic medium can greatly facilitate toxicological analysis. One significant advantage is that this medium does not require any foreign organisms for growth (e.g., *E. coli*), thus minimizing the ambiguity of data interpretation that can be caused by food source metabolism or interference when measuring growth or cell integrity in a fluorescence microtiter plate reader. Other benefits of growth in this medium is that the worms grow to high densities, making them ideal for biochemical and toxicological analysis, and that individual components in the growth medium can be fine-tuned (Rao et al., 2005). Finally growth in this medium facilitates assays that can be performed in small manageable volumes allowing rapid biochemical analysis.

Below, *C. elegans* culture conditions both on agar (bacteria as a food source) and in liquid (axenic medium) are described. As examples of the utility of *C. elegans* for evaluating particular toxins or nutritional components, the authors describe an acute toxin exposure assay in which the Parkinson's disease–associated neurotoxin 6-OHDA specifically causes dopamine neuron cell death in the worm (Nass et al., 2002). They also outline a chronic toxin exposure assay on agar, as well as methods to evaluate the integrity of the DA neurons under a fluorescence microscope. Finally, they describe several liquid axenic medium assays that can be used for nutritional and toxicity studies, with hemin chloride as an example (Rao et al., 2005).

NOTE: All reagents and equipment coming into contact with worms must be sterile and proper aseptic techniques should be used within a laminar flow hood.

BASIC PROTOCOL 1

GROWTH, MAINTANANCE, AND ASSAY OF *C. elegans* FOR ACUTE AND CHRONIC EXPOSURE TO NEUROTOXINS ON SOLID MEDIA

This protocol describes methods for preparing agar plates for acute exposure to the Parkinson's disease–associated neurotoxin (agar plate protocols are largely modified from Stiernagle, 2006). Alternate Protocol 1 involves chronic exposure to a heavy metal (copper) during growth in 24-well microtiter plate. Worm strains can be obtained from the Caenorhabditis Genetics Center (*http://www.cbs.Umn.edu/CGC/*) or by contacting the authors, and basic worm maintenance methods can be found in Hope (1999).

Materials

NaCl
Bactoagar (Becton Dickinson)
Bactopeptone (Becton Dickinson)
0.5 M potassium phosphate buffer, pH 6.0 (*APPENDIX 2A*)
1 M $MgSO_4$
1 M $CaCl_2$
Cholesterol solution (see recipe)
NA22 bacteria (see recipe)
Streptomycin
Nystatin solution (see recipe)
OP50 bacteria culture (see recipe)
Synchronization solution (see recipe)
M9 buffer (see recipe)
6-OHDA stock solution (see recipe)
Agar pad solution (2% w/v agar)
2% (w/v) sodium azide
C. elegans (Bristol N2, fluorescently labeled, or other strain)

2-liter autoclavable bottles (containing a stir bar) with caps
90-mm plastic bacterial petri dishes
Bent glass rod or Pasteur pipet ("hockey stick") for spreading culture
Laminar flow hood, optional
60-mm petri dishes or 24-well plates
15-ml conical tubes, sterile
Beckman CS-6R centrifuge with GH3.7 horizontal rotor (or equivalent centrifuge or rotor)
Phase-contrast or fluorescence microscope (inverted, confocal, or dissecting)
Nutator (Becton Dickinson)
1.6-ml silicon-coated microcentrifuge tube
90-mm NGM agar plate
20°C refrigerated incubator with platform rockers
Pasteur pipet or a worm pick
Stereo fluorescence microscope with bottom illumination (e.g., Leica MZ16 FA)

Prepare 8P plates

1. Mix 3 g NaCl, 25 g Bactoagar, 20 g Bactopeptone, and 1 liter of water in a 2-liter autoclavable bottle containing a stir bar. Screw cap loosely on top, and cover bottle cap with aluminum foil. Autoclave for 30 min.

2. Cool at room temperature by stirring on stir plate for 30 min, or until bottle can be touched by hand (still warm to the touch).

3. Add the following ingredients in order to the mixture while stirring on a warm stir plate:

   25.0 ml 0.5 M potassium phosphate buffer, pH 6.0
   1.0 ml 1 M $MgSO_4$
   1.0 ml 1 M $CaCl_2$
   1.0 ml cholesterol solution.

   Some light precipitate may form following the addition of the $CaCl_2$ or cholesterol. This does not affect the medium integrity.

4. Pour ~25 ml of the above medium mixture into sterile 90-mm bacterial petri dishes.

5. After agar has hardened and cooled, add three to four drops of the NA22 bacteria culture on the agar. Spread on plate with a hockey stick. Allow medium to absorb into agar and incubate overnight at 37°C.

   If plates are still wet after 1 hr, the plates can be placed in a laminar flow hood, with the top of the plate partially removed. Leave for 20 min or until dry.

Prepare NGM plates

6. Mix 3 g NaCl, 17 g agar, 2.5 g Bactopeptone, 0.2 g streptomycin, and 1 liter of water in a 2 -liter autoclavable bottle containing a stir bar. Screw cap loosely on top, and cover bottle cap with aluminum foil. Autoclave for 30 min.

7. Cool at room temperature by stirring on stir plate for 30 min, or until bottle can be touched by hand (still warm to the touch).

8. Add the following ingredients in order to the mixture while stirring on a warm stir plate:

   25.0 ml 0.5 M potassium phosphate buffer (pH 6.0)
   1.0 ml 1 M $MgSO_4$
   1.0 ml 1 M $CaCl_2$
   1.0 ml cholesterol solution
   1.25 ml nystatin solution.

   Some light precipitate may form following the addition of $CaCl_2$, cholesterol, and/or nystatin. This does not affect the medium integrity. Nystatin is not necessary, but decreases the likelihood of fungal contamination.

9. Pour ~10 ml of the medium mixture per sterile 60-mm petri dish, or pipet ~1.5 ml in each well in a 24-well plate.

10. After agar has hardened and cooled, add one to three drops of the OP50 bacteria culture to the plates or wells and spread with a hockey stick. Allow culture to absorb into agar and incubate overnight at 37°C.

    Do not allow the bacterial lawn to touch the sides of the plate, or the worms may crawl up the side. If plates are still wet after 1 hr, the plates can be placed in a laminar flow hood, with the top of the plate partially removed. Leave for 20 min or until dry.

Synchronize worms

11. Wash one to two 8P plates full of gravid (full of embryos) adults with water into a 15-ml conical tube.

    A plate of gravid adults can be generated by inoculating an agar plate containing bacteria with dozens of worms, and allowing the animals to ingest most of the bacteria on the plate. The resultant worm population will contain many gravid adults, i.e., animals that contain many eggs. Wild-type animals can be incubated between 16° to 24°C until the bacteria have almost completely been ingested by the worms.

    There are several methods for transferring worms from one plate to another. Each can be utilized to inoculate a plate of worms that can be used for synchronization. A very quick and efficient method for transferring hundreds of worms is by "chunking." This is a process in which a spatula or sterile pipet tip is used to carve out a small piece (a centimeter, more or less) of agar from a plate of worms and using the spatula or tip to transfer the animals to a fresh worm plate. This method works well for transferring worms that have burrowed into the agar or are difficult to pick individually. The worms will crawl away from the old agar to the new bacterial lawn.

    A large number of worms can also be transferred from one plate to another by adding ~1 ml of sterile water to a plate containing worms with a plastic disposable Pasteur pipet, and tilting the plate until the liquid has covered the entire surface. The worms will

swim off the surface of the plate into the water and worms can be sucked up into the pipet and deposited on a fresh plate. The inoculated worm plate should then be allowed to sit for an hour or so until the liquid has absorbed into the agar. Individual worms can be transferred to a new plate by using a flattened 2-cm piece of 32-G platinum wire mounted on a glass Pasteur pipet tip. This method is not recommended if the sole purpose is to generate a plate of gravid adults for synchronization, unless working with heterozygous stocks of worms or the worms require mating, since it is much more labor intensive. Further information regarding this and other transfer methods can be found in Stiernagle (2006).

This synchronization method is modified from Hope (1999), Stiernagle (2006), and references therein.

12. Mix gently, and centrifuge for 2 min at 700 × *g* (2000 rpm), room temperature.

13. Discard bacteria and water, leaving the worm pellet at the bottom. Refill with water, centrifuge for 2 min at 700 × *g*, room temperature, and discard water. Repeat until water is clear.

14. Discard water, and add 5 ml synchronization solution.

    CAUTION: *The synchronization solution contains NaOH and bleach, which are toxic and caustic. Care should be used when working with this solution.*

    The bleach and NaOH in the synchronization solution are toxic to the worms, causing the hermaphrodites to lyse and release their eggs. The eggshells protect the eggs from this caustic solution, but prolonged exposure (>10 min) will cause a dramatic decrease in egg viability.

15. Gently mix tube every minute for 5 to 7 min. Examine a drop under a light microscope to make sure that most of the eggs are free in solution.

16. Immediately add 10 ml M9 buffer, mix, and centrifuge for 2 min at 700 × *g*, room temperature.

17. Repeat the M9 rinse three times, and place tube on the Nutator for mixing. Mix for 18 hr.

    Some protocols suggest that the animals can be mixed for 24 to 30 hr or more. In the experience of the authors, mixing for >18 hr can cause the worm DA neurons to be more resistant to toxins. This could be due to an increase in oxidative stress and thus a greater induction of stress response genes.

    The eggs will develop and hatch in the M9 solution but the worms will be held up at the growth stage of L1 due to the lack of food in the medium. The starved synchronized animals will resume development following exposure to food.

18. Centrifuge the synchronized L1s for 2 min at 700 × *g* (2000 rpm), room temperature. Discard supernatant. Add 10 ml distilled water, mix gently, centrifuge for 2 min at 700 × *g*, room temperature, and discard supernatant.

    L1 animals are ∼250 μm in length, and are the first of four larval stages following egg hatching. If the embryos hatch in the absence of food, the embryos will arrest at this stage until food is introduced into the medium. The embryos can survive at this stage up to a week or more in the absence of food.

19. Add ∼1 ml distilled water. To estimate the number of worms per volume pipet 2 μl on a slide and count under a light dissecting microscope.

    L1 concentration can also be determined using a spectrophotometer. OD_{600} = 0.15 is ∼130 worms in water. Other developmental stages of worms can also be utilized, but often require a higher concentrations of toxins.

Expose worms to 6-OHDA

20. Calculate the amount of worms for a 600-µl volume that will contain 10,000 worms/ml.

21. Add 30 µl of the 6-OHDA stock solution to a 1.6-ml silicon-coated microcentrifuge tube to make a final experimental concentration of 5 mM 6-OHDA in 1% DMSO.

    CAUTION: *6-OHDA is a potent neurotoxin. Care should be used when working with solutions containing this compound.*

22. Add water and then worm volume (in that order; as calculated above) to bring to 600 µl.

23. Leave in the dark for 30 min, mixing gently every 10 min.

24. Add 900 µl water. Centrifuge 2 min at 700 × *g*, room temperature. Discard supernatant and repeat. Place the worms on a large size (90-mm) NGM plate and incubate at 20°C. Examine 2 to 3 days later under a fluorescence dissecting microscope as described below.

    The presence and choice of the antioxidant is important regarding toxin efficacy. Depending on the antioxidant used (in this case DMSO), neurotoxicity of 6-OHDA can vary 10-fold or more (see below).

Quantify DA neuron toxicity

25. Add one drop of melted agar pad solution in the middle of a microscope slide to prepare the agar pad. Immediately place another slide on top of the drop, perpendicular and in the middle of the slide, and press with fingers to make a very thin layer of agar. Keep pressed for 20 sec and carefully remove the top slide so as not to disrupt the thin agar pad.

26. Add one drop of the 2% sodium azide to the center of the agar pad.

    CAUTION: *Sodium azide is very toxic. Care should be used when working with this solution.*

27. Following the worm incubation described above, wash the worms once in water (leave a little of the water with the worms), and either add one drop of concentrated worms using a Pasteur pipet or use a worm pick to pick individual worms onto the sodium azide solution.

28. Place a coverslip on top of the worms and examine under a Leica MZ16 FA stereo fluorescence dissecting microscope or equivalent.

29. For dopamine neuron analysis, examine all four cephalic sensilla (CEP) dendrites (see Nass et al., 2002). Follow the GFP fluorescence from the nerve ring to the tip of the nose.

    If any part of the dendrite is absent, the worm has degenerating dopamine neurons.

    The dendrites can also be examined on the agar plate by slowing down the animals' movement. The authors typically place plastic wrap over ice in an ice bucket, and place the plate on the ice for 10 to 20 min. This slows the movement of the worms enough to visualize the DA neurons directly under the fluorescence microscope.

ALTERNATE PROTOCOL 1

CHRONIC EXPOSURE OF *C. elegans* TO COPPER ON AGAR IN MICROTITER PLATES

C. elegans can also be grown in liquid- or solid-based medium in multiwell plates, using formats anywhere from 6- to 384-well or higher (Link et al., 2000). These small cultures can be useful when determining toxin exposure parameters by examining a number of different toxin concentrations, determining the most sensitive age of exposure, or

if there are limited amounts of toxin that can be used due to compound availability, expense, or toxicity. Growth in microtiter (multiwell) plates also has the advantage in that the toxicity assay can be analyzed quickly using optical density or fluorescence in an automated platform such as a fluorescence plate reader, and screen for drugs in a high-throughput screening platform. In this protocol, worms are exposed chronically to a soluble toxin such as copper (Cu).

Additional Materials *(see Basic Protocol 1)*

300 mg/liter $CuCl_2$
L1 worms (see Basic Protocol 1)

1. Add 500 μl of warm NGM agar (see Basic Protocol 1) to each well of a 24-well plate.
2. When agar has hardened, add ~10 μl of OP50 bacteria culture to each well as a drop. Allow liquid to absorb into the agar, cover the plate, and incubate overnight at 37°C.
3. For each well add various volumes of the copper solution to obtain the desired final concentration of the toxin in the well.
4. Allow the toxin to absorb into the agar.

   If plates are needed immediately, dry plates in a laminar flow hood with the top slightly open for 20 min.
5. Place ~50 to 80 L1s in each well, and incubate at 20°C for 2 days.
6. Score animals for viability by touching a pick to their heads and scoring for movement, or for DA neuron degeneration as described in Basic Protocol 1.

   The toxin can be added directly to the medium prior to autoclaving or immediately following cooling before the plates are poured. Although this can save time and the compound may be more evenly distributed, a disadvantage of this method is that the solute could precipitate in the hot or warm medium, or the subsequent overlay of growing bacteria could sequester or modify the compound. Rand and Johnson (1995) elegantly address these concerns.

MAINTENANCE OF *C. elegans* IN LIQUID AXENIC MEDIUM FOR BIOCHEMICAL STUDIES

BASIC PROTOCOL 2

This section presents growth conditions for axenic (not contaminated by or associated with any other living organisms) liquid medium. The protocol is a modification of the original CeHR medium developed by Eric Clegg's group (*http://www.usacehr.org/cehr_medium.htm*). There are two alternate protocols: The first one (Alternate Protocol 2) is with reduced metals for studies with metalloproteins and gene regulation, and the second protocol (Alternate Protocol 3) eliminates all cations by metal chelation followed by addition of known quantities of metal ions. Cultures are initiated and/or developmentally synchronized using bleach and maintained by subculturing. Aliquots of these cultures can be frozen at −80°C or in liquid nitrogen for long-term storage.

Materials

C. elegans (Bristol N2, fluorescently labeled, or other strain)
60-mm NGM agar plates spotted with OP50 *E. coli* strain (see Basic Protocol 1, steps 6 to 10)
M9 buffer (see recipe)
0.1 N NaCl
mCeHR growth medium (see recipe), supplemented with 100 μg/ml tetracycline
5 N NaOH

5% (v/v) bleach
mCeHR growth medium (see recipe)
S buffer (see recipe)
Freezing solution (see recipe)
5 mM and 10 mM sodium azide (NaN_3)
0.3 M NH_4OH, pH 8.0
Hemin chloride

15- and 50-ml conical tubes, sterile
25- or 75-cm^2 tissue culture flasks (Nunc)
20°C refrigerated incubator equipped with platform shaker or rocker (Labline Orbit Shaker or Hoefer Rocker)
Beckman CS-6R centrifuge with GH3.7 horizontal rotor (or equivalent rotor)
Vortex Genie-2
Phase-contrast inverted microscope with 10× objective
2-ml screw cap cryostat freezing vials
Glass slides
24-well microtiter plate

Prepare worms for culture in liquid medium

1. Grow worms on ten 60-mm NGM agar plates spotted with OP50 *E. coli* strain until there are large number of gravid worms with very little bacteria on the plates (freshly or nearly starved).

   Worms can be obtained from the CGC (Caenorhabditis Genetics Center) stock center, and are usually provided on NGM plates. Worms can be transferred to fresh NGM plates by "chunking." See Basic Protocol 1, step 11 for details.

2. Rinse the plates with 5 ml M9 buffer and transfer the worms to a 50-ml conical tube.

   If there are numerous eggs remaining on the agar they can be loosened and collected by swirling and tapping the plates. Alternatively, a gloved finger or rubber policeman can be used to quickly liberate embryos from the agar surface.

3. Let the tube stand for 5 to 10 min and slowly remove the buffer from the top.

   This step will remove a significant amount of bacteria because the worms are denser than E. coli and tend to sink faster to the bottom of the tube.

4. Resuspend the worm pellet in 5 ml M9 buffer and repeat step 3 two times.

5. Resuspend the worms in 0.1 N NaCl in a volume appropriate for bleaching (see below) and bleach (Basic Protocol 1, steps 14 to 17).

6. After bleaching, use a Pasteur pipet to transfer the eggs into a 25-cm^2 tissue culture flask containing mCeHR growth medium supplemented with 100 μg/ml of tetracycline; use sterile techniques.

   To ensure that the worm cultures are established and to prevent any bacterial carryover during the initial period, the growth medium can also be supplemented with a triple antibiotic cocktail of tetracycline (100 μg/ml), streptomycin (250 μg/ml), and nalidixic acid (250 μg/ml). The authors use this cocktail very sparingly.

7. Incubate at 20°C in a refrigerated incubator on a rocker platform set at ~70 rpm.

   The growth rate of worms in the first round will be slow (typically ~7 to 10 days) as they become established for growth in liquid medium, but successive generations will be closer to 4 days. Some mutant strains (e.g., rol) will take longer to grow (5 to 6 days).

8. Let worms grow to an appreciable density such that bleaching gravid worms will yield sufficient number of larvae to start a subculture.

Prepare egg cultures and synchronize for liquid culture

9. Pipet nematode suspension into 15-ml sterile conical tubes and centrifuge for 5 min at 800 × *g*, 4°C, in a GH-3.7 swinging-bucket rotor.

10. Aspirate the supernatant using a Pasteur pipet and resuspend the worm pellet in 10 ml of 0.1 M NaCl by gently pipetting. Incubate the nematodes on ice for 5 min.

11. Aspirate supernatant using a Pasteur pipet, including worms that have not settled to the bottom of the tube. Resuspend the pellet in 0.1 M NaCl in a volume that is a multiple of three. For example, 1.5 ml, 3 ml, 6 ml, and 9 ml.

    Individual worms and not clumps should be visible in the liquid suspension by eye when the worm suspension is swirled in the conical tube.

12. Add 5 N NaOH and 5% bleach to the worm suspension in a 1:2:6 (v/v/v) ratio.

    For example: If the worms were resuspended in 6 ml of 0.1 M NaCl, then add 3 ml of NaOH/bleach mix (2 ml 5% bleach: 1 ml 5 N NaOH).

13. Mix by briefly vortexing (number 10 setting on a Vortex Genie-2). Continually monitor the worms under a phase-contrast inverted microscope vortexing periodically.

    It should take <10 min for the worms to burst open.

    Worms should be constantly monitored under a phase-contrast microscope with a 10× objective.

14. Let the worms remain in bleach for an additional 30 to 40 sec to further dissolve the worms and immediately centrifuge them for 45 sec at 800 × *g*, 4°C. Aspirate the supernatant with a Pasteur pipet.

15. Add 10 ml of sterile water to egg pellet and vortex tube for 5 sec. Centrifuge to pellet the eggs for 45 sec at 800 × *g*, 4°C. Aspirate the supernatant using a Pasteur pipet. Wash the egg pellet once more with sterile water.

16. Add 10 ml M9 buffer to the embryos and transfer them to a sterile 25-cm^2 tissue culture flask. Incubate the flask overnight at 20°C on a platform rocker at 70 rpm.

    Use the larvae from the hatched embryos within 2 days.

    L1 larvae stored in M9 buffer for longer time periods tend to have a greater lag time and become asynchronized when reintroduced to mCeHR medium. Changes to fresh M9 buffer every third day may help alleviate this problem.

Maintain and subculture C. elegans

17. To maintain a continuous subculture of the worms, transfer L1 larvae from M9 buffer to 50-ml conical tubes and centrifuge the worms for 5 min at 800 × *g*, 4°C. Resuspend the larvae in mCeHR medium and transfer them to a 25-cm^2 tissue culture flask. Incubate flask at 20°C on a rocker platform at 70 rpm.

    Antibiotics are no longer needed if proper sterile techniques are used. With two successive rounds of bleaching, contaminating bacterial carryovers are usually eliminated. A density of 3000 worms/ml/cm^2 ensures that adequate nutrients are available to the worms. Growth rates should be carefully recorded at this point (L1 to gravid adults) and are easy to monitor in a synchronized population.

Freeze worms for axenic medium cultures

18. Pellet mixed population of worms (asynchronous) by centrifuging for 5 min at 800 × *g*, 4°C, and resuspend them in S buffer. Divide worms into 0.5-ml aliquots and transfer into a 2-ml freezing vial and add equal volume of freezing solution. Vortex briefly. Freeze samples at −80°C.

19. To thaw worms place them directly in a 37°C water bath until the ice has mostly melted (<2 min). Gently pipet worms with a Pasteur pipet into mCeHR medium.

Worms freeze well at a concentration of ~1 million worms in 1 ml per vial. Although a pure synchronized L1 population can be frozen if available, it is convenient to freeze a mixed culture. Because early larval stages tend to survive better when thawed, a mixed culture ensures that there are sufficient quantities of young larvae.

Expose worms to hemin in liquid axenic medium

20. Pellet synchronized L1 larvae by centrifuging 5 min at 800 × *g*, 4°C. Resuspend the pellet in ~2 ml of M9 buffer.

The volume of buffer can be increased if the resuspended larvae appear dense.

21. Vortex or shake the tube vigorously and immediately pipet 100 µl of the worms into a microcentrifuge tube. Add equal volume of 10 mM sodium azide (NaN_3) to anesthetize and immobilize the worms. Mix and incubate for ~5 to 10 min at room temperature.

22. Vortex and spread 40 to 50 µl (50 to 150 worms) of the solution in an S-shape on a glass slide. Dilute concentrated worms with 5 mM sodium azide.

Counting <20 worms often leads to errors.

23. Calculate the number of worms/ml in the original 2-ml solution and dilute to ~1 worm/µl. Seed ~50 worms/cm^2/0.5 ml.

For example, a single well of a 24-well (2 cm^2/well) plate should contain ~100 worms in 1 ml growth medium.

24. Prepare a fresh solution of 10 mM hemin in 0.3 M NH_4OH, pH 8.0.

For lower final concentrations of hemin, make additional stocks of 1 mM and 0.1 mM.

25. Add 800 µl of mCeHR growth medium into individual wells of a 24-well microtiter plate. Aliquot 100 µl of 0.3 M NH_4OH, pH 8.0 containing hemin at the desired final concentration.

The total volume of 0.3 M NH_4OH, pH 8.0, in each well should be the same to ensure consistency.

Hemin concentrations for a dose response curve typically include 0, 1.5, 4, 20, 100, 500, 800, and 1000 µM. At 1.5 µM hemin the worms develop slowly (>9 days) with smaller brood size, while at concentrations ≥800 µM there is growth arrest. No gravid worms should be observed at 0 µM hemin.

26. Add 100 µl of M9 buffer containing ~100 L1 larvae (mix tube well prior to the addition because worms tend to sink to the bottom of the tube) to the culture medium in each well.

The final volume of the growth medium plus worms in each 2-cm^2 well is 1 ml. If 12-well (4-cm^2) or 6-well (10-cm^2) plates are used, then the number of worms and volume of the growth medium is proportionately changed to accommodate the difference in surface area.

27. Examine the plate under the microscope to ensure that all the wells have equal numbers of larvae. Incubate plates at 20°C on a rocker platform at 70 rpm. Cover the plates with aluminum foil because hemin is light sensitive.

The day the worms are added to the culture medium is day 0. Worms are thereafter monitored each day for growth and are usually counted on days 3, 6, and 9.

28. Swirl the plate gently so that all worms are in the center of the well and not in the corners. Pipet the entire contents (~1 ml) of each well into a microcentrifuge tube.

Occasionally, the volumes will be slightly lower due to evaporation in the incubator. If this is the case, bring the volume to 1 ml with sterile M9 buffer.

29. Vortex vigorously and pipet 100 µl into another microcentrifuge tube. Add 100 µl of 10 mM NaN_3 and incubate for 5 to 10 min. Count the worms to determine worm viability as a function of hemin concentration in either 20 or 40 µl as in steps 21 and 22.

    At low and high hemin concentrations worms are usually dead or the growth is severely retarded. If this is the case, count the entire contents of that well by centrifuging 5 min at 800 × g, room temperature, and resuspending the pellet in 50 µl of 5 mM NaN_3.

GROWING *C. elegans* IN REDUCED METAL MEDIUM (mCeHR-2)

ALTERNATE PROTOCOL 2

Inductively coupled plasma–mass spectrometry and X-ray spectroscopy has revealed that worms grown in axenic mCeHR growth medium contain significantly higher concentrations of copper and manganese compared to worms fed *E. coli* strain OP50. Even though there are no apparent differences in growth rates or other parameters, it is likely that exposure to higher concentrations of metals will alter genetic responses in nutritional and toxicological assays. To circumvent this issue, dose-response curves were performed to analyze optimal worm growth at the lowest concentrations of copper, manganese, iron, and zinc. These studies led the authors to significantly lower the concentration of copper from 45 µM to 10 µM, and manganese from 100 µM to 5 µM. These modifications can be important depending on the heavy metal evaluated. For example, results from model organisms (*S. cerevisiae*, *C. reinhardtii*, and mice) and human diseases reveal that perturbation of one metal directly affects the homeostasis of another (e.g., copper deficiency alters iron metabolism and elevated zinc decreases intestinal copper uptake).

Use mCeHR-2 in place of mCeHR-1 above, and follow procedure as outlined in Basic Protocol 2 for liquid culture.

GROWING *C. elegans* IN METAL-CHELATED MEDIUM (mCeHR-3)

ALTERNATE PROTOCOL 3

This growth medium was created as an alternative to mCeHR-2 in order to eliminate trace levels of contaminating metals from milk and lactalbumin. The mCeHR-3 growth medium can therefore be used in studies where accurate knowledge of metals is critical, especially at lower concentrations.

Additional Materials *(also see Basic Protocol 2)*

50× chelation mix
Lactalbumin (see recipe)
UHT skim milk, sterile (see recipe)

1-kDa MWCO dialysis tubing
0.2-µm filter

Additional reagents and equipment for dialysis (*APPENDIX 3H*)

NOTE: Wash all glassware with 6 N HCl followed by copious rinsing with distilled deionized water to remove any contaminating metals bound to the glassware.

1. Dilute 50× chelation mix to 1× in 500 ml lactalbumin and 4× in 1 liter UHT skim milk in two separate beakers. Stir overnight at 4°C.
2. Remove the metal-chelate complexes by dialyzing (*APPENDIX 3H*) each of these solutions against 2 liters of sterile water overnight at 4°C.

    The volume will increase 2- to 3-fold during dialysis.

3. Recalculate the volumes of lactalbumin and milk, filter sterilize through a 0.2-μm filter unit, divide into 50-ml aliquots, and freeze at −80°C.

Although milk cannot be filter sterilized because of the casein micelles and other particulates, EDTA/EGTA treatment (provided in the chelation mix) clarifies milk due to disruption of these micelles and the milk can thus be easily filtered.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

6-OHDA stock solution (100 mM 6-OHDA/20% DMSO)

Add in the following order: 32 ml water, 8 ml DMSO and 1.0 g of 6-OHDA (Sigma no. H8523) for a final concentration of 100 mM 6-OHDA in 20% DMSO. Mix gently. Divide into 5-ml aliquots, cover with aluminum foil, and store up to 6 months at –20°C.

Tubes should be thawed no more than twice. Efficacy of the toxin decreases as tube is repeatedly thawed.

CeHR medium, 1 liter

Filter the following volumes of stock solutions in the order described with a 1-liter, 0.2-μm filter unit:
10 ml 2 mM choline diacid citrate
10 ml vitamin mix (see recipe)
10 ml 2.4 mM i-Inositol
10 ml 2 mM hemin (see recipe)
250 ml H_2O

Apply suction and allow solution to filter, then add the following in the order described:
20 ml nucleic acid mix (see recipe)
100 ml mineral mix (see recipe)
20 ml lactalbumin (see recipe)
20 ml essential amino acid mix (Invitrogen no. 11130-051)
10 ml non-essential amino acid mix (Invitrogen no. 11140-050)
20 ml 450 mM KH_2PO_4
50 ml 1.46 M D-glucose
10 ml 1 M HEPES, Na salt
250 ml H_2O
1 ml cholesterol (see recipe)

Apply suction and allow solution to filter, then add the following in the order described.
Store up to 1 year at −80°C
Add 20% (v/v) UHT skim milk just prior to use.

Milk cannot be filtered through a filter unit.

Make medium fresh in a laminar flow hood with sterile techniques. The pH without milk is 6.0 to 6.5.

Chelation mix, 50×

0.5 M EDTA
0.1 M EGTA, pH 8.0
Store up to 1 year at room temperature

Cholesterol, 5 mg/ml

Dissolve 200 mg cholesterol in 40 ml 95% ethanol. Store up to 1 year at 4°C.

Freezing solution

S buffer (see recipe) plus 30% (v/v) glycerol. Store up to 1 year at room temperature.

Hemin

2 mM hemin chloride (Frontier Scientific) in 0.1 N NaOH. Adjust pH to 8.0 with concentrated HCl. Store up to 2 weeks at −20°C.

If the pH falls below 6.0 (acidic), hemin will precipitate. Higher concentrations of hemin and other metal substituted porphyrins can be made in 0.3 M NH_4OH.

Lactalbumin

Combine 170 mg of lactalbumin hydrolysate (Invitrogen) in 1 ml water. Store up to 6 months at 4°C.

M9 buffer

3 g KH_2PO_4
6 g Na_2HPO_4
5 g NaCl
1 liter H_2O
Autoclave 30 min
Add 1 ml 1 M $MgSO_4$ (sterile)
Store up to 1 year at 4°C

Mineral mix, 1 liter

4.1 g $MgCl_2 \cdot 6H_2O$
2.9 g sodium citrate
4.9 g potassium citrate$\cdot H_2O$
0.07 g $CuCl_2 \cdot 2H_2O$
0.2 g $MnCl_2 \cdot 4H_2O$
0.1 g $ZnCl_2$
0.6 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$
0.2 g $CaCl_2 \cdot 2H_2O$ (always add last)

Make small volumes of stocks for this mix so it is used quickly and are not stored for extended periods.

NA22 bacteria culture

Add 8 g Tryptone, 5 g yeast extract, 2.5 g NaCl to 500 ml water. Divide into four 125-ml aliquots. Autoclave for 30 min. Inoculate NA22 bacteria culture (~20 µl from another NA22 bacteria culture) or from a single colony from an 8P plate containing NA22 bacteria. Incubate overnight at 37°C. Store culture at 4°C.

Cultures are generally viable for at least 2 to 3 weeks at 4°C.

Nucleic acid mix, 100 ml

To 60 ml of water add:
1.74 g adenosine 5′-monophosphate, sodium salt
1.84 g cytidine 5′-phosphate
1.82 g guanosine 2′- and 3′-monophosphate *or* 2.04 g guanosine 5′-phosphate
1.84 g uridine 5′-phosphate, disodium salt
0.63 g thymine (add last)

continued

Bring stock solution to 100 ml and store in the dark at 4°C or freeze aliquots at −20°C.

Make small volumes of stocks for this mix so it is used quickly and are not stored for extended periods.

Nystatin, 10 mg/ml

Add 400 mg nystatin to 40 ml 70% ethanol. Mix.

Nystatin will not completely dissolve and will form micells. Therefore it must be mixed thoroughly each time before adding to medium.

OP50 bacteria culture

Add 12.5 g Luria broth (*UNIT 4.16*) and 100 mg streptomycin to 500 ml water. Divide into four 125-ml aliquots. Autoclave 30 min. Inoculate with OP50 bacteria culture (∼20 μl) or from a single colony from a NGM plate containing OP50 bacteria. Incubate overnight at 37°C. Store culture at 4°C.

Cultures are generally viable for at least 2 to 3 weeks at 4°C.

S Buffer, 1 liter

129 ml 0.05 M K_2HPO_4
871 ml 0.05 M KH_2PO_4
5.85 g NaCl
Sterilize by autoclaving.
Store up to 1 year at room temperature

pH of this solution is ∼6.0

Synchronization solution

Add 5 ml Chlorox bleach (fresh) and 1.25 ml 10 M NaOH to 18.75 ml water. Use immediately.

UHT skim milk

High-temperature ultra-pasteurized (UHT) skim milk can be obtained in a grocery store (e.g., Horizon's Organic UHT skim milk). Using sterile techniques open the container of milk and streak on Brain Heart Infusion (Difco no. 237500) agar plates. Incubate plates at 37°C and 30°C to test for growth of any microbes. Transfer milk to 50-ml sterile conical tubes and freeze at −80°C.

The authors find that 10% (v/v) milk is usually sufficient with no discernable differences in worm growth compared to mCeHR-1 medium supplemented with 20% (v/v) milk.

Vitamins and growth factor mix, 100 ml

Solution 1: To 60 ml of water add:
0.15 g N-acetyl-α-D-glucosamine (Calbiochem)
0.15 g DL-alanine (Calbiochem)
0.075 g nicotinamide
0.0375 g D-pantethine
0.075 g DL-pantothenic acid, hemi calcium salt
0.075 g pteroylglutamic acid (folic acid; ACROS/Fisher)
0.0375 g pyridoxamine·2HCl
0.075 g pyridoxine·HCl
0.075 g flavin mononucleotide, sodium salt
0.075 g thiamine hydrochloride

continued

Solution 2: Prepare each of the following chemicals in ~5 ml 1 N KOH:
0.075 g *p*-aminobenzoic acid
0.0375 g D-biotin
0.0375 g cyanocobalamine (B_{12})
0.0375 g folinic acid, calcium salt
0.075 g nicotinic acid
0.0375 g pyridoxal 5′-phosphate

Solution 3: 0.0375 g (±) α-L-lipoic acid, oxidized form in 1 ml ethanol:
Mix solutions 1, 2, and 3 and bring final volume to 100 ml. Store in dark at 4°C or freeze aliquots at −20°C.

Make small volumes of stocks for this mix so it is used quickly and are not stored for extended periods. All chemicals are available from Sigma unless noted otherwise.

COMMENTARY

Background Information

It is now remarkably clear from the seminal *C. elegans* sequencing project that was largely completed in 1998 to the explosive growth in worm studies in the last few years that many of the known biological components in *C. elegans* are conserved in humans (The *C. elegans* Sequencing Consortium, 1998). This tiny nematode is sensitive to a number of human toxins and pharmaceuticals that interact and activate many of the same molecules and signaling pathways found in mammals (Rand and Johnson, 1995; Nass and Blakely, 2003). Both species also contain similar cell death pathways, and these compounds can induce similar pathological and morphological changes. These similarities between the worm and man, along with the powerful genetics and rapidity in analysis that this invertebrate provides, suggests that *C. elegans* can significantly assist in elucidating the molecular basis of human cellular toxicity.

C. elegans has been studied for over 40 years, and much of this work has focused on cellular and developmental biology. Only more recently has the worm been utilized to explore toxin or drug actions. These new uses carry with them new challenges that often require different considerations, as well as modifications of existing protocols. Basic *C. elegans* husbandry and maintenance has been described in several books and papers, and these are useful resources if the researcher would like to work with the worm in the laboratory (Lewis and Fleming, 1995; Riddle et al., 1997; Hope, 1999; Stiernagle, 2006). There are also very good reviews on the usefulness and considerations in using *C. elegans* in pharmacological research (Rand and Johnson, 1995; Link et al., 2000).

C. elegans develop from L1 larvae to egg-laying adults in ~3.5 days at 20°C. After the egg hatches, the larvae matures through four stages (called L1, L2, L3, and L4). At the end of each developmental stage they shed their cuticle (a layer of carbohydrates and protein that cover the hypodermis) by molting. After the last molt, they develop into mature adults that are capable of reproducing. Development to adulthood is temperature dependent ranging from <2.5 days at 25°C to 6 days at 15°C. The average lifespan of a wild-type worm is ~18 days at 20°C. In response to crowding or starvation, *C. elegans* can arrest development at the end of the second larval stage, and form a special larval stage called dauer ("enduring") larvae. Growth is resumed when dauers are exposed to a food source.

In this unit, the authors describe their agar-based methods for acute exposure to the Parkinson's disease–associated neurotoxin 6-OHDA. They also outline their general assay for chronic exposures to metals such as copper. They provide details and considerations for each type of assay that should assist the reader in setting up worm-based toxicity experiments.

The authors also provide details for worm-based growth in liquid axenic media. Although large quantities of *C. elegans* can be grown in liquid culture using concentrated *E. coli* for food (Lewis and Fleming, 1995; Stiernagle, 2006), there are several caveats using this method. The foremost is the use of another complex organism as a food source that could confound results because nutrients could be assimilated and modified by *E. coli*, prior to ingestion by worms.

To circumvent these problems, defined growth medium (CbMM) for worms was

developed more than 30 years ago (Vanfleteren, 1978). More recently a chemically defined medium of CbMM, *C. elegans* Maintenance Medium (CeMM), was developed that would allow for axenic growth of *C. elegans* in liquid. However, worms grown in CeMM medium take 2 to 3 times longer to develop compared to worms grown on standard NGM agar plates, and these worms appear to be nutrient starved (Szewczyk et al., 2003). Thus, it becomes imperative that experimental results derived from animals grown in liquid medium are comparable to results obtained from animals grown on NGM plates.

In the authors' attempt to control nutrient levels and closely mimic the growth rate of *C. elegans* on NGM plates, the authors reformulated the original CeHR medium that was developed for toxicological studies at the U.S. Army Center for Environmental Health Research (Clegg et al., 2002). In the past three years they have systemically tested different components in the growth medium and fine-tuned the concentrations of individual chemicals to achieve maximal growth rates for *C. elegans* that is comparable to NGM plates (Rao et al., 2005). The mCeHR-1 medium has now also been used to culture other nematode species including *Panagrellus redivivus*, *Oscheius myriophila*, and *C. remanei*.

Critical Parameters and Troubleshooting

Although treatment with the synchronization solution will yield a large number of synchronized worms (often >95%), it can be difficult to achieve complete synchronization. This is especially apparent if the animals are examined after they become adults. The presence of a large number of dead embryos the day after bleaching is usually indicative that the bleaching procedure was sub-optimal. It is therefore important to make sure that the bleach is no more than 1 month old and kept in an airtight container, and that the synchronization solution is made fresh immediately before use. The authors have also found that some mutant strains can be more sensitive to the synchronization solution (e.g., some dpy strains have significantly lower embryo viability following exposure due to a compromised cuticle; Rand and Johnson, 1995). It is therefore recommended for a new strain that has not been synchronized before by the user, that the synchronization should be closely monitored to determine the yield of viable L1 larvae. If there is a high mortality rate, then lower exposure times or concentrations may be needed, or a greater number of gravid adults may be required for synchronization.

The 6-OHDA *C. elegans* assay is sensitive to a number of variables. Long incubation times (>18 hr) in M9 buffer following exposure to the synchronization solution often results in increases in DA neuron resistance to 6-OHDA. The cause could be due to an increase in production of stress response–related proteins. Therefore it is recommended not to prolong the incubation in the M9 buffer. Significant variability in the efficacy of the 6-OHDA purchased from Sigma Chemicals has been found. Some batches appear much more potent relative to others. The range of color of the bromide salt varies from off-white to tan to brown or reddish brown. The authors find that the darker colored 6-OHDA compound is less potent, which could also be due to oxidation of the salt.

Because 6-OHDA is easily oxidized, it is important to solubilize the salt in the presence of an anti-oxidant. Not only will this protect against short-term oxidation, it will also likely increase the efficacy of the neurotoxin (Roginsky et al., 1997). The solution should also be protected from light, and made fresh for each experiment, or avoid repeated freeze thaw. The authors' previously published method uses a 6-OHDA assay mix of 50 mM 6-OHDA with 10 mM ascorbic acid, and exposes L3 larvae to the toxin (Nass et al., 2002). Although this method is efficient, a significant percentage of the worms die due to exposure to absorbic acid alone (often >20%). The authors now typically expose L1 animals to 5 mM 6-OHDA in 1 % DMSO (worms can survive in a number of solvents, including upwards of 2% DMSO; Rand and Johnson, 1995). DMSO is also a potent anti-oxidant, and it is less toxic to the worms resulting in a much greater number of animals surviving the 6-OHDA treatment. The younger animals are also more sensitive to the neurotoxin and exposure of the animals at this stage simplifies the assay protocol.

It is important to maintain the pH of the axenic medium during worm culture. Many of the components will precipitate if the pH changes significantly (hemin is especially sensitive). The use of HEPES sodium salt is very important for maintaining the pH. Increasing the concentration of HEPES from 10 mM to 30 mM will permit greater buffering capacity; however long term exposures of worms to this concentration of HEPES has not been tested. Nucleic acid stock solutions also tend to

precipitate at room temperature over time, but this does not appear to hinder worm growth as individual components are already in excess.

If worms are grown for prolonged lengths of time in the same growth medium, dauer worms (see Background Information) appear in the culture. This alternate life stage can be problematic as dauer larvae are more resistant to bleach than their nondauer counterparts, making them difficult to eliminate from the culture once they take hold. The growth medium should be changed every 5 days to avoid dauer formation.

Growth rate is also sensitive to hemin concentrations. A hemin concentration of 20 μM is optimal, but concentrations of up to 50 μM can be used without any apparent effect. The authors have observed though that several heme-responsive genes are already significantly altered by 10 μM hemin.

Anticipated Results

More than 80% of the worms exposed to 6-OHDA generally have significant DA neuron degeneration. If worms that have developed PDEs (posterior deirids) are exposed to the toxin (i.e., L3 or older), the CEPs (cephalic cells) and the ADEs (anterior deirids) appear to have much greater neurodegeneration relative to the PDEs. Worms grown in the mCeHR growth medium at 20°C are robust and healthy. They should be moving/swimming most of the time, and at the adult gravid stage contain 6 to 8 healthy embryos. If the worms are not developing and have been static for more than 3 days, this is usually a sign of sub-optimal medium.

Time Considerations

The 6-OHDA assay should take less than 1 hour on the day of the toxin exposure. Preparation of slides for microscopy and analysis of DA neuron cell death should take <20 min per sample. Assembling mCeHR medium from pre-existing stock solutions takes <1 hr. Making *C. elegans* axenic in CeHR medium will take 8 to 10 days. However, it is important to monitor worm growth and development daily. Once axenic worms have been established, it should take no more than 4 days for wild-type N2 worms to become gravid adults.

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