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Comprehensive Assessment of Germline Chemical Toxicity Using the Nematode *Caenorhabditis elegans*

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Abstract

Identifying the reproductive toxicity of the thousands of chemicals present in our environment has been one of the most tantalizing challenges in the field of environmental health. This is due in part to the paucity of model systems that can (1) accurately recapitulate keys features of reproductive processes and (2) do so in a medium- to high-throughput fashion, without the need for a high number of vertebrate animals.

We describe here an assay in the nematode *C. elegans* that allows the rapid identification of germline toxicants by monitoring the induction of aneuploid embryos. By making use of a GFP reporter line, errors in chromosome segregation resulting from germline disruption are easily visualized and quantified by automated fluorescence microscopy. Thus the screening of a particular set of compounds for its toxicity can be performed in a 96- to 384-well plate format in a matter of days. Secondary analysis of positive hits can be performed to determine whether the chromosome abnormalities originated from meiotic disruption or from early embryonic chromosome segregation errors. Altogether, this assay represents a fast first-pass strategy for the rapid assessment of germline dysfunction following chemical exposure.

Video Link

The video component of this article can be found at http://www.jove.com/video/52445/

Introduction

There are approximately 87,000 chemicals registered for commerce in the United States, yet only a small number of these have been tested for potential health effects[1]. Of those that have been tested, only a portion has been assessed for reproductive health effects due in part to the difficulty in determining alteration of the early reproductive events in mammals, especially during female germ cell development and differentiation. Indeed, the first meiotic events take place during the early stages of embryonic development in female mammals and are therefore difficult to access and collect in numbers suitable for screening purposes.

The germline provides the crucial link between generations, and its appropriate function is dependent upon the precise execution of the intricate program of cellular and chromosomal division termed meiosis. Dysregulation of the meiotic process may result in reduced fertility and the production of gametes and embryos with an abnormal number of chromosomes, a condition termed aneuploidy. Chromosome segregation errors in meiosis are highly relevant to human health. Chromosome abnormalities are common, with a frequency of 1 in 150 live births, Trisomy 21, 18 and 13 as well as X and Y chromosome errors being the most prevalent types[2,3]. Furthermore, congenital malformations, including those of chromosomal origins, are the leading cause of infant death in the U.S.[4] The idea that environmental influences can affect chromosomal segregation and behavior is not new[5], but is still poorly understood. It is therefore crucial to investigate which of the chemicals introduced into our environment are interfering with human fertility, early development and overall reproductive health.

In light of these limitations of the mammalian models, we have developed a high-throughput screen assay to test reproductive toxicity in the roundworm *C. elegans.* We have mobilized several important features offered by this commonly used genetic model system such as its small size, low cost, short reproduction cycle, high proportion of germ cells and ease of manipulation[6]. Worms can be grown in 96-well plates or in high volume liquid cultures and because of their transparency can be directly imaged on plates for detection of fluorescent reporters. The assay described below takes advantage of these characteristics and takes advantage of a worm strain containing the fluorescent reporter *Pxol-1::GFP* to detect germline disruption and induction of embryonic aneuploidy.

The use of this reporter strain is based on the generally rare occurrence of males in a mainly hermaphroditic worm population. These males (<0.2%) naturally originate from error in the segregation of the X chromosome[7]. However, as germline disruption frequently leads to errors in segregation of autosomes and of heterochromosomes, it correlates both with an elevated incidence of males phenotype (X missegregation) as well as embryonic lethality (autosome missegregation). To easily detect the induction of males while circumventing the issue of embryonic lethality, a male-specific promoter (*xol-1*) is used to drive expression of GFP in early embryos still contained within the worm's uterus. As such,

the appearance of GFP-expressing embryos is used as a proxy for the presence of aneuploid embryos. This method has been previously used to identify genes implicated in germline maintenance and meiosis[8,9]. Adapted to chemical screening, this strain is employed in a medium to high-throughput screen. Importantly, the strain faithfully reports the aneugenicity of chemicals and is therefore relevant to mammalian reproductive endpoints[10]. The assay described here will be particularly useful to toxicologists in pharmaceutical and chemical industry settings looking to rapidly assess the toxicity of chemicals towards reproductive endpoints. Furthermore, this assay fully aligns with the governmental priorities highlighted in the Toxicity in the 21st century report[11].

Protocol

1. Preparation of Feeding Bacteria

NOTE: This section describes the preparation of feeding bacteria (*E. coli* strain OP50).

1. Isolate a single colony of *E. coli* strain OP50 from a lysogeny broth (LB) agar plate and aseptically inoculate into 300 ml of autoclaved LB broth.
2. Allow the inoculated culture to grow overnight in a shaker at 200 rpm and 37 °C, until saturation is reached.
3. Transfer the OP50 into 6 sterile, pre-weighed 50 ml conical tubes. Pellet the bacteria by centrifuging for 5 min at 6,000 rpm (5,000 x g).
4. Discard the supernatant and wash the bacteria with 50 ml of sterile M9. Repeat twice.
5. After the second wash remove the remaining M9, ensuring that no M9 is left in the tube.
6. Determine the weight of the pellet by weighing the tube containing the bacteria pellet and subtract the weight of the 50 ml conical tube.
7. Resuspend the pellet in M9 at a concentration of 100 mg/ml.
8. Store the OP50 solution at 4 °C until it is used for the worm culture.

2. Preparation of Nematode Growth Medium (NGM) Petri Plates

NOTE: This section describes the preparation of NGM Petri plates, which are the plates where the *C. elegans* worms are routinely maintained in the laboratory.

1. Autoclave the NGM agar medium.
2. Using sterile procedures, dispense 17 ml of NGM agar solution into 6 cm Petri plates using a peristaltic pump.
   NOTE: A constant amount of agar in the plates reduces the need for refocusing the microscope when switching from one plate to another.
3. Leave the plates at room temperature for 2-3 days before use to let the agar solidify and allow the excess moisture to evaporate.
4. Seed the NGM plates using a sterile technique. Apply 1-2 drops of OP50 liquid culture and spread using a glass rod. Make sure not to spread the bacteria lawn all the way to the edges of the plates.
5. Allow the OP50 lawn to grow for 1-2 days at room temperature before using the plates to grow worms.

3. Preparation of a Synchronous Worm Population

NOTE: The life cycle of *C. elegans* is comprised of the embryonic stage, four larval stages (L1-L4) and adulthood. This section describes the preparation of an age-synchronous population of worms. All materials coming in contact with *C.elegans* after the bleach treatment must be sterile.

1. **Day 1 – Preparation of Gravid Worm Population**
   1. Use NGM plates in which the majority of the worm population consists of starved L1 larvae.
   2. Collect the L1 larvae with a sterilized worm pick and transfer them to fresh 6 cm NGM plates seeded with OP50. Incubate for approximately 65 hr at 20 °C until the majority of the worms are gravid adults. To avoid plate overcrowding and allow the worms to grow without depleting the bacteria, do not transfer more than one or two clusters of L1 larvae to each fresh NGM plate.

2. **Day 4 – Establishment of a Synchronized L1 Larvae Population**
   1. Collect the gravid worms from the NGM plates by washing them with 1-2 ml of M9 and transfer them to a conical 15 ml tube.
   2. Let the gravid worms sediment to the bottom of the 15 ml conical tube for approximately 5-10 min. Remove the supernatant without disturbing the worm pellet.
   3. Transfer the worm pellet to 2-4 microcentrifuge tubes and add 1 ml of bleach/NaOH solution. Incubate for 2-3 min at RT. Monitor the progress of the reaction under the stereomicroscope to confirm all the worms are dead. Do not bleach longer than 5 min as the embryos could die.
   4. Collect the worms by centrifuging for 1 min at 3,000 rpm (900 x g). Remove the supernatant and add 1 ml of sterile M9 to neutralize the reaction.
   5. Wash the worms twice by centrifuging for 1 min at 3,000 rpm (900 x g).
   6. Remove the supernatant and add M9 to up to 100-200 µl.
   7. Using a glass Pasteur pipette add a drop of the worm/M9 mix to clear NGM plates and incubate them for at least 24 hr at 20 °C to allow the embryos to hatch.
      NOTE: Without food the larvae's growth will be halted at the L1 stage.

3. **Day 5 – Establishment of a Synchronized L4 Larvae Population**
   1. Collect the L1 larvae from the clear NGM plates by washing the plates with 1-2 ml of M9 and transfer them to a conical 15 ml tube.
   2. Let the dead adult worms sediment to the bottom of the 15 ml conical tube for approximately 5 min.
   3. Collect the supernatant, where the L1 worms are, in a new conical 15 ml tube, and precipitate the worms by centrifuging for 2 min at 2,600 rpm (1,000 x g).

4. Remove the supernatant leaving approximately 500 µl.
5. Determine the concentration of worms in the M9 solution by counting the number of worms in 10 µl drops using a stereomicroscope. Count at least 5 drops.
6. Adjust the concentration of the worms to 20-25 worms/µl and add 50 µl of the worm/M9 mix to NGM plates seeded with OP50.
7. Incubate for 65 hr at 15 °C (for incubation through the week-end) to allow the L1 synchronized population to grow until they reach the L4 stage.

4. Exposure of Worms to Chemicals in 96-well Plates

NOTE: This section describes the use of *Pxol1::gfp* transcriptional reporter containing *C. elegans* strain to screen for the induction of aneuploidy.

1. Collect the L4 larvae from the clear NGM plates by washing the plates with 1-2 ml of M9 and transfer them to a conical 15 ml tube.
2. Let the L4 worms sediment to the bottom of the 15 ml conical tube for approximately 5-10 min. Remove the supernatant without disturbing the worm pellet.
3. Add 3-5 ml of M9 and determine the concentration of worms in the M9 solution by counting the number of worms in 10 µl drops using a stereomicroscope. Count at least 5 drops for each sample.
4. Resuspend the worms at a concentration of 1,000 worms/ml in M9.
5. Dilute the OP50 bacteria from section 1 10-fold with M9. Make sure the resuspended bacteria reach room temperature because lower temperature affects worm development.
6. Using a multichannel pipette, first add 100 µl of the worm/M9 mix (from step 4.4) and then add 400 µl of the diluted OP50 bacteria (from **step 4.5**) to each well of a 2 ml deep round bottom 96-well plate. At the end, each well will have 100 worms in 500 µl.
7. Add 0.5 µl of either the test chemical or the appropriate control to the desired wells, to achieve a suggested final concentration of 100 µM, a concentration commonly used in chemical screens in *C. elegans*[12]. Expose ethanol or DMSO solvent controls at a final concentration of 0.1%.
   NOTE: We recommend the use of nocodazole (100 µM) as positive control.
8. Seal the plate using adhesive film. Be sure to seal the plate well to prevent cross contamination between wells.
9. Wrap the plate with aluminum foil.
10. Transfer the plate to a shaker (170-180 rpm) of the appropriate temperature for the suitable length of time (24 or 65 hr). The temperature of the room affects the growth of the worms; maintain the temperature around 20 °C.

5. Image Acquisition of Gravid Worms in a 384-well Plate

NOTE: This section describes the use of a high content microscope to image the exposed *Pxol1::gfp* worms, to visualize the expression of GFP in the embryos within the uterus of adult hermaphrodites. For each 384-well plate to be screened, use worms from 4 x 96-well plates.

1. After chemical exposure in the shaker, let the 96-well plate rest for 10-15 min to allow the adult worms to sediment to the bottom of the plate.
2. Using a multichannel pipette, remove 350 µl of the M9, being very careful not to disturb the worms in the bottom of the plate.
3. Wash the worms with 1 ml of M9 and repeat step 5.1.
4. Using a multichannel pipette, remove 1 ml of the M9, being vey careful not to disturb the worms in the bottom of the plate.
5. Using a multichannel pipette, resuspend the worms in the remaining M9 and collect 100 µl of the worm/M9 mix from the 96-well plates and load it into the wells of a black walls/clear bottom 384-well plate. Repeat the process using worms from 4 x 96-well plates, until all the wells of the 384-well plate have been loaded.
   NOTE: It is important for all the wells to have the same volume in order to increase the efficiency and speed of the autofocus during the image acquisition process. Each well should contain between 80 to 100 worms.
6. Add 1 µl of levamisole (100 µM) to each well and let the worms incubate for approximately 30 min.
   NOTE: Levamisole acts as an acetylcholine receptor agonist and immobilizes the worms.
7. Transfer the plate to a widefield high content microscope capable of providing automated imaging.
8. For image acquisition, use a high content image acquisition and analysis software according to the manufacturer's guidelines. Select the 4X objective to acquire 1 image per well.
9. Before starting the image acquisition, adjust the GFP imaging parameters to 45 msec of exposure and image resolution to 2,160 x 2,160.
10. Collect the data as the number of GFP positive worms divided by the total number of worms in the well, the latter measure being relatively consistent between wells.

Representative Results

Exposure of the *Pxol-1::GFP* reporter strain to chemical agents such as the microtubule poison Nocodazole (**Figure 1**) leads to the induction of a high proportion of GFP-expressing embryos in the uterus of exposed adult hermaphrodites compared to DMSO control. The GFP-positive embryos are significantly brighter than the weak background fluorescence observed in other embryos as well as the auto-fluorescence observed in the gut of the animals. Exposed worms are directly imaged on 384-well plates and the number of worms containing at least one GFP-positive embryo is counted for each well and normalized by the total number of worms in that well. A positive hit from the chemical screen means a compound induced a proportion of GFP-positive embryos in a worm population at a frequency 1.7x higher than DMSO[10]. Following threshold optimization, the high content image analysis (see **Materials** file) allows the automated calculation of the number of positive objects (*i.e.,* embryos) divided by the total number of worms in the well and is the method of choice for the large scale adaptation of the assay.

Start with starved worm population to generate a gravid adult population
↓ (Culture 3 days)
Bleach the gravid adult population
↓
Transfer bleached worms to clear NGM plates
↓ (Culture 1 day)
Collect synchronized L1 population and transfer to NGM plates with OP50
↓ (Incubate 65 hr at 15 ºC)
Collect the synchronized L4 population
↓
Dispense 100 L4 worms to each well of the 96-well plates in 0.5 ml of M9/OP50
↓
Add chemicals (100 µM) to each well
↓ (Incubate for 24 or 65 hr)
Transfer 100 µl of the synchronized gravid adult population (80 worms) from the 96 well plates to a 384-well plate.
↓
Add 1 µl of levamisole to each well (1 µM final concentration)
↓ (Incubate 30 to 45 min)
Capture and analyze images of each well with high content microscope

Table 1. Experimental workflow. Day 1, use starved worm population to generate a gravid adult population. Day 4, bleach the gravid adult population to generate a synchronized L1 population. Day 5, transfer the L1 population from clear NGM plates to OP50 seeded NGM plates to generate a synchronized L4 population. Day 8, transfer the synchronized L4 population to 96-well plate and expose them to the different chemicals. Day 9 and Day 11, transfer the exposed gravid adults to a 384-well plate to be imaged with a high-content microscope.

M9 Buffer - 1 L
1. Combine the following ingredients in large beaker or graduated cylinder using magnetic stir bar
3 g KH_2PO_4
6 g Na_2HPO_4
5 g NaCl
1 ml 1M $MgSO_4$
H_2O to 1 L.
2. Aliquot into appropriate sized bottles (300 ml in 500 ml sized bottles)
3. Sterilize by autoclaving for 30-60 min
NGM media for plates – 4 L
1. Add the following in an Erlenmeyer flask, then fill to 4 L:
12 g NaCl
10 g Bactopeptone
80 g Agar
2. Add and mix well:
4 ml of Cholesterol (5 mg/ml)
4 ml $CaCl_2$ (1 M stock solution)
4 ml $MgSO_4$ (1 M stock solution)
3. Autoclave for 30-60 min
4. Let cool slightly and add 100 ml of KH_2PO_4 (1 M, pH 6 stock solution), then pour plates
LB media - 1 L
1. Add the following to 800 ml H_2O
10 g Bacto-tryptone.
5 g yeast extract.
10 g NaCl.
2. Adjust pH to 7.5 with NaOH.
3. Adjust volume to 1 L with dH_2O
4. Sterilize by autoclaving for 30 - 60 min
Bleach solution for synchronizing populations – 50 ml
7.5 ml 10 N NaOH
6 ml bleach (regular)
36.5 ml dH_2O

Table 2. Solutions.

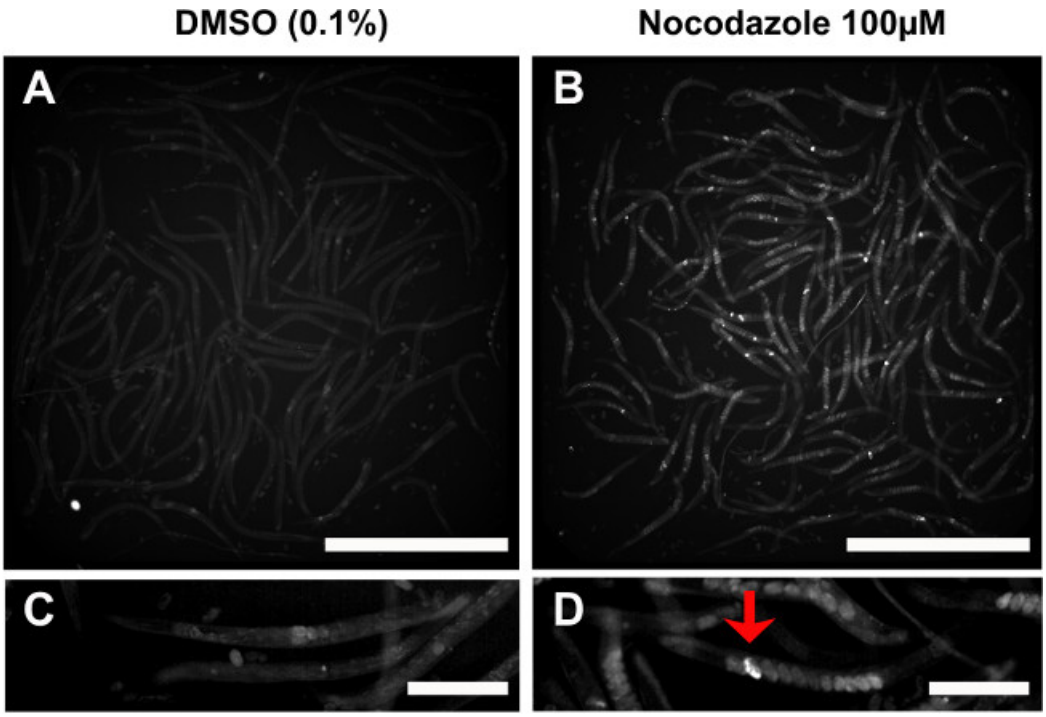

Figure 1: Examples of images obtained from exposure of the *Pxol-1::GFP* reporter strain to control DMSO and positive control nocodazole. In this example, worms were exposed for 24 hr to nocodazole or DMSO. *Pxol-1::GFP* worms were exposed to **(A)** 0.1% DMSO (negative control) or **(B)** 100 µM nocodazole (positive control) and imaged in 384-well plates. Red arrow: GFP positive embryos clearly visible within one nocodazole treated worm's uterus. Scale bar = 1 mm. (**C**) and **(D)** are magnified portions of **(A)** and **(B)** respectively. Scale bar = 0.33 mm.

Discussion

The method described here constitutes the first large scale strategy for the identification of germline toxicants. It requires the use of a GFP transgenic *Pxol-1::GFP* containing strain that faithfully reports the induction of aneuploidy in early embryos which is used as a proxy for germline dysfunction. The method involves the careful synchronization of a *C. elegans* worm population and worms' exposure to chemicals in 96-well format followed by imaging of the GFP positive worms by automated high-content microscopy.

Several steps of this protocol are crucial to the consistency of the results. First, the worm populations should be highly synchronous to as maximize the number of properly staged L4 larvae that will be used for the exposure. For this reason, the methodology described here includes two synchronization steps, first by bleaching of the worm population and then by L1 starvation. The second important parameter of this method is the length of exposure. The worms are routinely exposed for either 24 hr or 65 hr. As meiosis is a continuous process in *C. elegans*, these two exposure windows allow the capture of either altered late meiotic events (24 hr) or both early and late meiotic events (65 hr). It is important to note however that the longer exposure seems to better predict mammalian reproductive toxicity [10].

One particularly attractive aspect of the method is its flexibility. While the protocol is designed for high throughput screening, a scaled down version of the assay can be performed for the screening of a smaller number of samples using 24-well plates or 1.5 ml tubes. Lowering the scale of the screen enables the test of a higher number of worms per sample which could increase the sensitivity of the assay. In the present format, screening compounds in quadruplicates is recommended to provide higher statistical confidence in the aneuploidy rates measured. A potential hurdle in this large scale methodology comes from the need for automated detection of GFP-positive embryos. Although this task can easily be performed by eye, the careful establishment of appropriate parameters on the image analysis software, ideally with an imaging specialist, is crucial for the automation of the procedure. Furthermore, beyond the detection of GFP-positive embryos, secondary validation of the hits should be performed to determine if chromosomal errors are of meiotic or embryonic origin. We have previously shown that such secondary assays are easily performed in *C. elegans* and include the DAPI-staining of germline nuclei as well as acridine orange staining of the worms for measurement of apoptosis[10]. From these two simple assays, abnormal progression through meiosis, abnormal nuclear morphology and fragmentation as well as germ cell loss can all be assessed.

Taken together, we have described here the steps required for the rapid assessment of germline toxicity using the model system *C. elegans*. Importantly, the use of aneuploidy in the worm as a marker for germline toxicity is a relevant assay as it is predictive of mammalian reproductive toxicity endpoints including decreased litter size and ovarian defects[10]. This is of particular significance as toxic effects on the female germline are particularly arduous to investigate. Thus, this fast assay provides an alternative to large animal tests as a first pass toxicity screen and sheds light on the effect of environmental chemical exposures on various aspects of the complex meiotic program.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Office, U. G. A. Report No. GAO-06-1032T. *Actions are needed to improve the effectiveness of EPA's chemical review program. Testimony before the Committee on Environment and Public Works. US Senate*. Available from: http://www.gao.gov/cgi-bin/getrpt?GAO-06-1032T (2009).
2. Fragouli, E., Wells, D., Delhanty, J. D. Chromosome abnormalities in the human oocyte. *Cytogenet Genome Res.* **133**, (2-4), 107-118 (2011).
3. Hassold, T., Hunt, P. A., Sherman, S. Trisomy in humans: incidence, origin and etiology. *Curr Opin Genet Dev.* **3**, (3), 398-403 (1993).
4. Heron, M., Hoyert, D. L., Murphy, S. L., Xu, J., Kochanek, K. D., Tejada-Vera, B. Deaths: final data for 2006. *Natl Vital Stat Rep.* **57**, (14), 1-134 (2009).
5. Hunt, P. A. Meiosis in mammals: recombination, non-disjunction and the environment. *Biochem Soc Trans.* **34**, (Pt 4), 574-577 (2006).
6. Brenner, S. The genetics of Caenorhabditis elegans. *Genetics.* **77**, (1), 71-94 (1974).
7. Hodgkin, J., Horvitz, H. R., Brenner, S. *Nondisjunction Mutants of the Nematode CAENORHABDITIS ELEGANS.Genetics.* **91**, (1), 67-94 (1979).
8. Nicoll, M., Akerib, C. C., Meyer, B. J. X-chromosome-counting mechanisms that determine nematode sex. *Nature.* **388**, (6638), 200-204 (1997).
9. Kelly, K. O., Dernburg, A. F., Stanfield, G. M., Villeneuve, A. M. Caenorhabditis elegans msh-5 is required for both normal and radiation-induced meiotic crossing over but not for completion of meiosis. *Genetics.* **156**, (2), 617-630 (2000).
10. Allard, P., Kleinstreuer, N. C., Knudsen, T. B., Colaiacovo, M. P. A Screening Platform for the Rapid Assessment of Chemical Disruption of Germline Function. *Environ Health Perspect.* **121**, (6), 717-724 (2013).
11. National Research Council. *Toxicity Testing in the 21st Century: A Vision and a Strategy.* The National Academies Press (2007).
12. Boyd, W. A., Smith, M. V., Kissling, G. E., Freedman, J. H. Medium- and high-throughput screening of neurotoxicants using C. elegans. *Neurotoxicol Teratol.* **32**, (1), 68-73 (2010).

