Worm-on-a-chip technology: An emerging 3R model in toxicity testing

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Introduction and Objectives

Thanks to its tiny size (1-mm long), the nematode *Caenorhabditis elegans* can easily fit into a microfluidic chip, offering a new alternative for animal testing and at the *in vitro* scale. *C. elegans* worms have been widely used for studying development, stress, and aging and have been recently used in environmental toxicology studies. The other advantage of this well-characterized nematode (with >40 years of genetic studies), besides its low maintenance cost and short life cycle, is the fact that many genes and signaling pathways are well-conserved between *C.elegans* and humans.

Nagi Bioscience developed a new worm-on-a-chip technology that combines high-resolution imaging and image analysis algorithms, allowing longitudinal observation at the individual level to evaluate phenotypic readouts such as worm growth, survival, and fertility.

In this collaborative study, a dose–response assay was performed with ten benchmark chemicals (such as lithium chloride, thalidomide, and bisphenol A) to evaluate their potential adverse effects by using this new "*in vitro-like*" model in two exposure scenarios. The phenotypic outcomes of each chemical exposure were compared to those of positive (30 µg/mL doxycycline and 5.15 µg/mL 5-fluorouracil) and negative controls (1% DMSO) to rank the test compounds on the basis of the severity of their adverse effects. Inter-individual variability was also assessed within this assay, by performing synchronization of the worms before injection into the chip. For each test chemical, the no-observed-adverse-effect-level (NOAEL) for each phenotypic endpoint was determined. Examples of the phenotypes observed are presented here, and potential follow-up experiments are discussed.

Methods

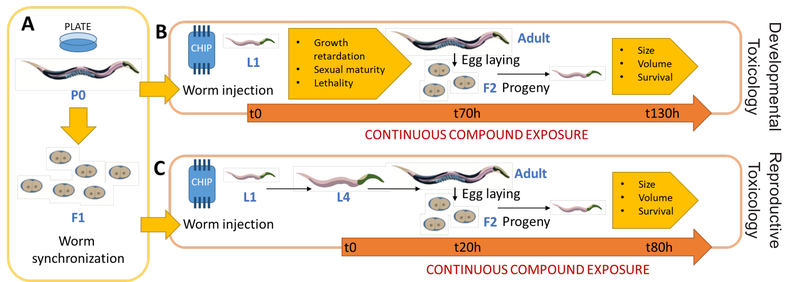

Figure 1. Study design and exposure timeline. *(A) N2 wild-type (WT) strain worms are grown on solid nematode growth medium agar plates and harvested in complete S-medium at the adult stage (P0). Eggs from the P0 population are hatched, and this L1 population is recovered via filtration for synchronization before being injected into a microfluidic chip. L1 worms are then fed with bacterial medium and continuously exposed to compounds of interest either during development from L1 to adult stage (B) or from L4 to adult stage (C). Live monitoring of each worm is performed every hour throughout the whole experiment at constant temperature.*

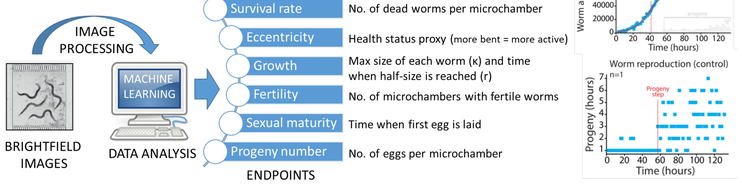

Figure 2. Overview of data analysis pipeline. *Time-lapse brightfield images of each worm in a chip channel are processed by using Nagi Bioscience's software algorithms, and quantitative data for various endpoints are collected and computed. As an example, worm area data are plotted against time (top graph), and key parameters of the curve such as the maximum area K and the half-size timing r are then extracted. The bottom graph indicates the number of worms present in each microchamber over the same period, allowing estimation of the number of progeny produced and when the worms were sexually mature.*

Table 1. List of chemicals tested *(blinded). All compounds were prepared in DMSO (except sodium chloride, which was dissolved in water) before being mixed with the bacterial medium.*

Compound name	CAS	Doses tested
Methyl acetoacetate	105-45-3	19.2 nM
Methoxyacetic acid	625-45-6	96 nM
Sodium chloride	7647-14-5	480 nM
Bisphenol A	80-05-7	2.4 µM
Hydroxyurea	127-07-1	12 µM
Acrylamide	79-06-1	60 µM
All-trans-retinoic acid	302-79-4	300 µM
Lithium chloride	7447-41-8	1.5 mM
D-(+)-Camphor	464-49-3	
Thalidomide	50-35-1	

Results

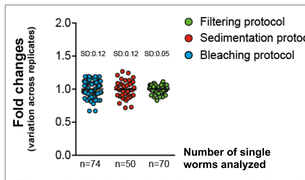

Figure 3. Variation in timing to reach the adult stage among single individuals (dots) relative to the whole population.

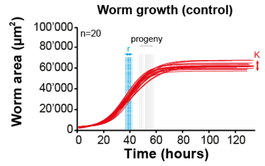

Figure 4. *Variation in the different phenotypes analyzed in the WT population. Each red curve corresponds to the fit modeling of the growth (K) of a single worm. Blue and gray dashed lines correspond to the time to reach half the max. size (r) and the timing when the progeny appears, respectively.*

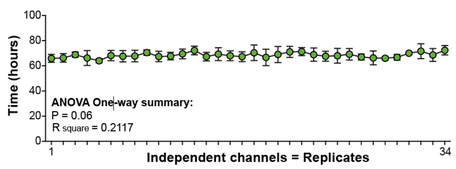

Figure 5. Reproducibility assessment of the filtering protocol for obtaining a synchronous L1 population across 34 replicates. L1 worms synchronized by filtering were injected into a Nagi Bioscience chip for a 5 days of culture. Each channel (x-axis) corresponds to 3–8 microfluidic chambers containing 1–4 worms. Each green dot corresponds to the average time when the first eggs are observed in the corresponding channel. Error bars = SD.

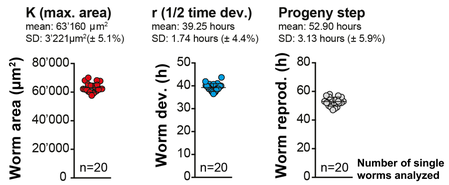

Figure 6. Variation in the different phenotypes analyzed in the control population. Each dot corresponds to the measurement performed at a single-worm level.

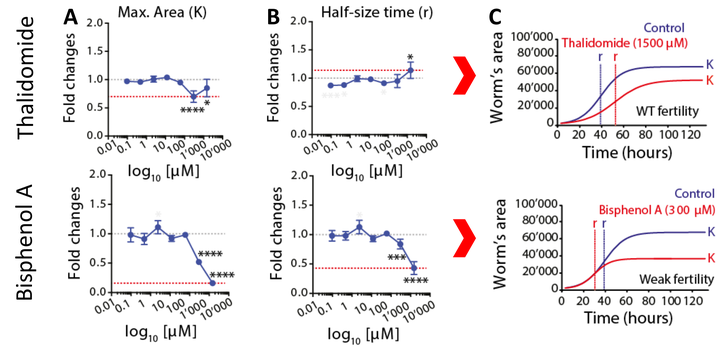

Figure 7. *Thalidomide (top row) and bisphenol A (bottom row): Dose–effect relationship in terms of (A) maximum area (K) and (B) time to reach half the max. size (r). (A,B) The dashed grey and red lines indicate the mean value of the negative control (1% DMSO) and the max. significant variation relative to the negative control, respectively. Each dot corresponds to the mean fold change relative to the negative control for each concentration tested. (C) Dynamic modeling of the toxicity profiles of thalidomide and bisphenol A, with assessment of both K and r parameters.* P<0.05, ** P< 0.01, *** P<0.001, and **** P<0.0001 by unpaired t-test.*

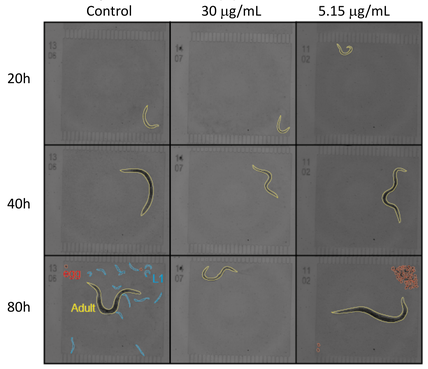

Figure 8. *Representative images of worms exposed for 20, 40, and 80 h to doxycycline (positive control used in the developmental toxicology assay[(1)]) and 5-fluorouracil (positive control used in the reproductive toxicology assay[(2)]) compared with images of worms exposed to 1% DMSO (negative control). The left panel shows a single worm (yellow outline) growing and reaching its adult stage, with appearance of eggs (red) and its L1 progeny (blue) after 80 h in culture. In the middle panel, the effect of doxycycline exposure is characterized by retarded growth and the absence of progeny. In the right panel, exposure to 5-fluorouracil does not impact worm growth or sexual maturity, as shown by the capacity of the animal to lay eggs. The impact of 5-fluorouracil is, in fact, observed at the level of the progeny, where no L1 worms are observed after 80 h of exposure.*

Results

Table 2. Summary of the developmental toxicology results (see Figure 1B)

Dev. Tox.	NOAEL (µM)		Growth parameters		Reproductive parameters			Conclusions	ECHA/*:Schenk et al. (2010) (3) reported effects
Compound name	Survival	Eccentricity	Max. size (K)	Half size timing (r)	Sexual Maturity	Fertility	Progeny number		
Methyl acetoacetate*	>1500	>1500	↓	=	Late	=	=	Small adult size (300 µM) and delay in the reproductive process (1.5 mM)	NA/No effect detected
All-trans-retinoic acid	>1500	300	↓	=	=	=	=	Low activity (300 µM) and small adult size (1.5 mM)	Might damage the unborn child
Thalidomide	>1500	300	↓	↑	Late	↓	=	Small adult size (300 µM), slow development, weak fertility, and low activity (1.5 mM)	Might damage the unborn child
Bisphenol A*	300	300	↓	↓	Late	↓	↓	Larval arrest and early death (1.5 mM), slow development and smaller size (300 µM), weak fertility and low embryo viability (300 µM)	Might damage the unborn child
Hydroxyurea	>1500	300	↓	↓	Sterile	Sterile	↓	Larval arrest and sterility (1.5 mM), low embryo viability (300 µM), and high activity (1.5 mM)	Suspected TOXIC
Acrylamide	>1500	60	↓	=	=	=	=	High activity (300 µM) and small adult size (1.5 mM)	Suspected TOXIC
Methoxy acetic acid*	>1500	>1500	↓	=	=	=	=	Small adult size (1.5 mM)	Might damage the unborn child/have effects on embryonic development
Lithium chloride	>1500	>1500	↓	↓	=	=	=	Small adult size and impairment of the developmental process (1.5 mM)	NOAEL 103 mg/kg bw/day (rat)/teratogenic compound
D-(+)-Camphor	>1500	>1500	=	=	=	=	=	No adverse effects observed (up to 1.5 mM)	NA
Sodium chloride*	>1500	300	=	=	=	=	↓	Low embryo viability and high activity (1.5 mM)	No effect reported

Abbreviations: NOAEL, No-observed-adverse-effect level; NA, Not available.

Table 3. Summary of the reproductive toxicology results (see Figure 1C)

Reprod. Tox.	NOAEL (µM)		Growth parameters		Reproductive parameters			Conclusions	ECHA/*: Schenk et al. (2010) (3) reported effects
Compound name	Survival	Eccentricity	Max. size (K)	Half size timing (r)	Sexual Maturity	Fertility	Progeny number		
Methyl acetoacetate*	>1500	>1500	=	↑	Late	=	=	Delayed sexual maturity (NOAEL: 750 µM/LOAEL: 1.06)	NOAEL 1g/kg bw/day (rat)
All-trans-retinoic acid	>1500	>1500	↓	↓	=	=	↓	Lower progeny number (NOAEL: 750 µM/LOAEL: 0.80)	Might affect fertility
Thalidomide	>1500	>1500	=	↑	Late	=	=	Delayed sexual maturity (NOAEL: 187.5 µM/LOAEL: 1.03)	Might affect fertility
Bisphenol A*	>1500	>1500	↓	↑	=	=	↓	Lower progeny number (NOAEL: 750 µM/LOAEL: 0.68)	Might affect fertility
Hydroxyurea	>1500	>1500	=	=	=	=	↓	Lower progeny number (NOAEL: 750 µM/LOAEL: 0.69)	Suspected TOXIC
Acrylamide	>1500	>1500	=	↑	=	=	↓	Lower progeny number (NOAEL: 187.5 µM/LOAEL: 0.81)	Suspected TOXIC
Methoxyacetic acid*	>1500	>1500	=	=	=	=	=	No adverse effects observed on worm's reproduction	Might affect fertility
Lithium chloride	>1500	>1500	=	=	=	=	=	No adverse effects observed on worm's reproduction	NOAEL 52 mg/kg bw/day (rat)
D-(+)-Camphor	>1500	>1500	=	=	=	=	=	No adverse effects observed on worm's reproduction	NOAEL 400 mg/kg bw/day (rabbit)
Sodium chloride*	>1500	187.5	=	=	=	=	=	No adverse effects observed on worm's reproduction	No effect reported

Abbreviations: NOAEL, No-observed-adverse-effect level; LOAEL, Lowest-observed-adverse-effect level.

Conclusions

- Our results show that synchronizing worms at the same developmental stage before chip injection improves inter-individual variability for the following phenotypic endpoints:
  - Growth endpoint: Time to reach adult stage (**Figure 3**), maximum size of the worm (K parameter) (**Figures 4** and **6**), and half-time to reach maximum size (r parameter) (**Figures 4** and **6**).
  - Fertility endpoint: Time to reach sexual maturity (time when the 1st egg is laid) (**Figure 5**).
- This study highlights the multiple advantages of this new high-throughput screening approach, including:
  - Good reproducibility and accurate results by using standardized protocols
  - Precise (automated) and dynamic dosing of compounds (up to 16 doses per chemical per chip), with low liquid consumption (<500 µL over the full course of the experiment)
  - Phenotypic readouts in real time (one picture every hour per condition over 5 days) and at single-animal resolution (Example shown in **Figure 8**)
- For each tested chemical, the NOAEL was determined in both experimental set-ups (**Table 2** and **Table 3**), and seven out of ten chemicals were predicted correctly in *C. elegans*.
- To further evaluate the predictivity of the worm-on-a-chip developmental & reproductive toxicological assays, a larger panel of compounds will have to be tested. Further insights on the modes of action of some of these chemicals (via a systems toxicology approach) could be helpful in determining key pathways sensitive to reprotoxicants.

References

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2- Kumar et al. (2010). Anticancer drug 5-fluorouracil induces reproductive and developmental defects in C. elegans. Reprod. Toxicol. 29(4): 415-20

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