Positioning the "Worm-on-Chip" technology as part of the toolbox for NAMs

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Abstract

Currently existing toxicology testing still implies an extensive experimentation in mammalian models, which is expensive and associated with important ethical concerns. The alternative methods to animal testing are typically based on cellular models. The main limitation of these *in vitro* approaches is that they often cannot predict complex responses at the level of an organism, usually involving a multi-organ crosstalk and metabolic processing of the test molecules.

Nowadays nematode *Caenorhabditis elegans* (*C. elegans*) starts to get recognition as a valuable alternative model in predictive toxicology studies, that can complement *in vitro* models to better predict the outcomes in mammals. However, experimentation in *C. elegans* is still mainly based on manual handling techniques and direct observation by the operator, hence largely limiting the potential of the worms for high-throughput and high-content screenings required for toxicology studies.

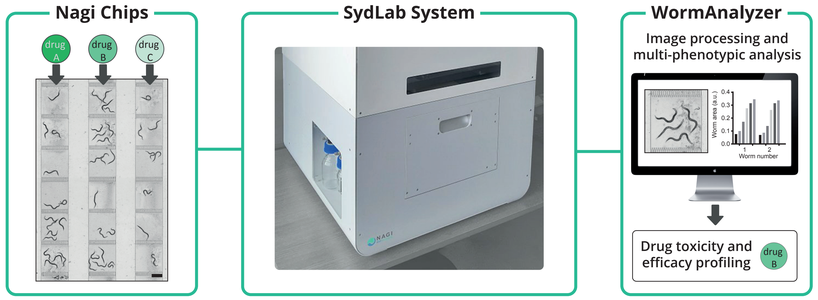
We developed a microfluidic-based robotic platform that can perform automated high-content phenotypic analysis of C. elegans and execute different types of toxicology assays, including the assessments of fertility, embryotoxicity and acute toxicity.

As an illustration, we present here the results of a study characterizing the effects on reproduction of fourteen benchmark chemicals, including 5-fluorouracil, paraquat, sodium chloride, methoxyacetate, lithium chloride, penicilin G, busulfan, dexamethasone, thalidomide, triadimenol, bisphenol A, diphenylhydantoin, benzalkonium chloride and methotrexate. Synchronized populations of worms were chronically exposed to 5 doses of these compounds starting from the last larval stage (L4) until day 3 of adulthood. The images of each worm were recorded every hour and time-resolved phenotypic readouts were then extracted from the collected images, including growth dynamics, sexual maturity, fertility, embryonic viability and progeny accumulation rate . The phenotypic outcomes were compared to those of positive (5-fluorouracil) and negative (1% DMSO) controls. Out of the tested compounds methotrexate showed the most pronounced adverse effects on embryonic viability, while bisphenol A strongly affected the development of the mothers.

In conclusion, we propose an innovative solution for rapid identification of toxic compounds and their potential mechanism of toxicity, using a biological model that perfectly bridges the gap between *in-vitro* and *in-vivo* assays.

Microfluidic platform overview

Our microfluidic technology allows **large-scale studies** for the parallel characterization of drugs and chemicals in *C. elegans*. The robotic platform provides **fully-automated culture, treatment, imaging and analysis** of the worms over long-term experiments. The high-content information extracted using our image processing and data interpretation algorithms enables detailed multi-phenotypic screening at the whole-organism level.



Features

- **Patented** microfluidic design, relying on passive hydrodynamics
- **16 fluidic lines**, enabling tests of 16 independent conditions
- **Plug & play** chip-to-device connectivity and fluidic operations

Features

- **Active culture**, treatment and study of 64+ independent conditions
- **Programmable acquisitions** of BF and fluo images and videos
- **Active temperature control** in the 10-40°C range

Features

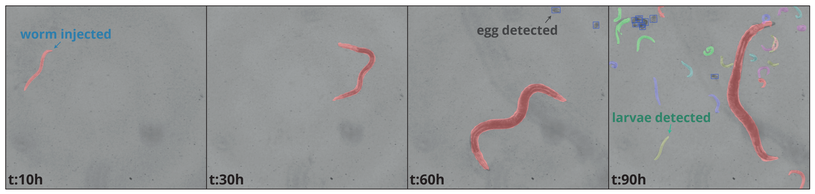
- **User-friendly software** to design, run and monitor experiments
- **Time-resolved/high-content data** extraction based on AI
- **Integrated statistical analysis** /data interpretation algorithms

Worms are **automatically injected into the microfluidic** platform and confined within dedicated chambers of the microfluidic chips. They are then continuously fed with *E. coli* solution and can be exposed to the test compounds according to the defined treatment plan. The pictures of each microfluidic chamber **are acquired via time-lapse microscopy** at desired frequency.

Data processing pipeline

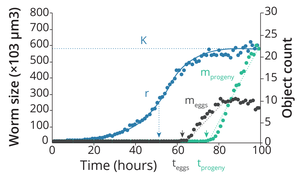
The time-lapse brightfield pictures obtained during the experimental procedure are post-processed by a **machine learning (ML) software (A)**. Through this ML software we monitor the **growth rate** of the worm population within the microfluidic chip over several days, as well as the **fertility** (eggs appearance and number) and **progeny production (B)**, with a high degree of reproducibility accross replicates **(C)**.

A. ML-based objects' detection



The object recognition allows the ML algortihms to extract the following parameters: maximal length (K_{length}), time required to reach ½ max length(r_{length}), the time point when the first egg is detected (t_{eggs}), the time point when the first larvae is detected ($t_{progeny}$), the speed of egg (m_{eggs}) and progeny accumulation ($m_{progeny}$).

B. Automated data analysis



C. Objects detected

● Worm (size) ● Eggs (number) ● Progeny (number)

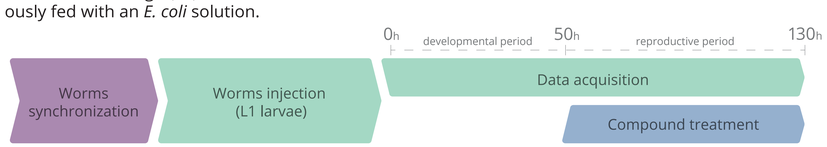
Reproducibility

Parameter	Value	Variation	Experiments
Maximum size (K)	570'388 (µm³)	±6.5%	15 repeats
Growth dynamic (r)	2.24 (days)	±5.6%	15 repeats
Sexual maturity (t_{eggs})	56.44 (hours)	±5.4%	15 repeats

Reproductive toxicity assay

Purpose: the assay is designed to monitore the potential adverse effects (AE) of molecules on the reproductive capacity of *C. elegans*. It can be used as a prediction for **fertilization problems/embryotoxic effects** of the test compounds.

Method description: a synchronized population of *C. elegans* is injected into the microfluidic platform at the first larval stage (L1). Worms are confined within dedicated microfluidic chambers and are continuously fed with an *E. coli* solution.



Worms are then chronically exposed to the test compounds starting **from the last larval stage prior to sexual maturity (L4)** for 80 hours (day 3 of adulthood). The protocol was specifically designed to avoid a treatment with the compounds during the developmental phase: the goal is **to evaluate the potential adverse effects on *C. elegans* reproduction only**.

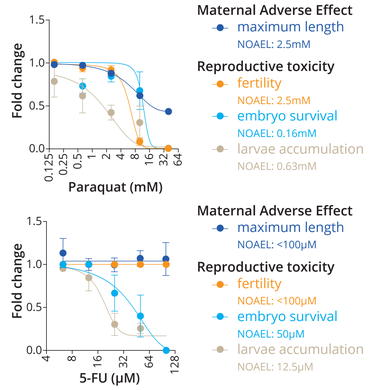
The images of each microfluidic chamber are recorded every hour. **Time-resolved phenotypic readouts** are then extracted from the collected images.

Data interpreation

Maternal effects	Adverse effects if p<0.05 and if:	Conclusion:
Maxium length (K length)	< 1.0 - CTLneg sd (threshold ≈0.90)	Smaller adult worms
Growth dynamic (r length)	<1.0 - 2x CTLneg sd (threshold≈0.85) or >1.0 + 2x CTLneg sd (threshold≈1.15)	Developmental arrest Slower development
Reproductive toxicity		
Fertility (% eggs detected)	<100%	Lower fertility
Sexual maturity (t eggs)	> 1.0 + CTLneg sd (threshold≈1.05)	Delay in sexual maturity
Egg accumulation (m eggs)	<1.0 - 0.5x CTLneg sd (threshold≈0.71)	Lower egg laying
Embryo survival (% eggs hatched)	<100%	Higher embryotoxicity
Larvae emergence (t progeny)	> 1.0 + CTLneg sd (threshold≈1.05)	Longer egg maturation
Larvae accumulation (m progeny)	<1.0 - 0.5x CTLneg sd (threshold≈0.70)	Lower egg viability

Results

A



B

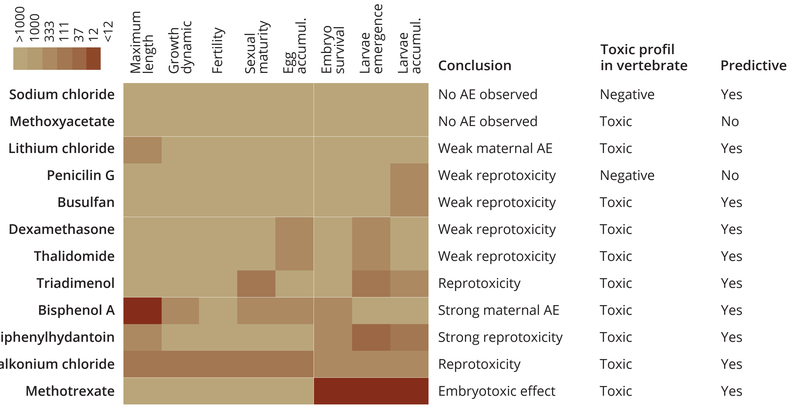
	End points	NOAEL (mM)
Maternal effects	Maximum length	2.5
	Growth dynamic	2.5
Repro toxicity	Fertility	0.63
	Sexual maturity	0.16
	Egg accumulation	0.16
	Embryo survival	0.16
	Larvae emergence	0.16
	Larvae accumulation	0.63

>40 40 10 2.5 0.63 0.16 <0.16

	End points	NOAEL (µM)
Maternal effects	Maximum length	>100
	Growth dynamic	>100
Repro toxicity	Fertility	>100
	Sexual maturity	>100
	Egg accumulation	50
	Embryo survival	50
	Larvae emergence	50
	Larvae accumulation	12.5

>100 100 50 25 12.5 6.25 <6.25

Worms were treated on the platform with the herbicide **Paraquat** (**A-B**, top row) and the cytotoxic chemotherapy medication **5-fluorouracil** (**A-B**, bottom row). This test set was employed to validate our Reprotox protocol with molecules known to induce different toxic effects in *C. elegans*. Curves depicted on graphics (**A**) show the respective dose-effect on development (max. length) and reproductive capacity (fertility, embryo survival and larvae accumulation). No-Observed-Adverse-Effect Level (NOAEL) were computed for both Paraquat and 5-FU and represented for all the end points (**B**).
Conclusion: Paraquat induced **major maternal AE** at high doses (NOAEL: 2.5mM) and significant reprotoxic effect at lower doses (NOAEL: 0.16mM). As reported, 5-FU at the doses tested has no maternal AE but a **strong reprotoxic impact** at mid doses (NOAEL: 50µM).



Twelve benchmark chemicals known to induce or not reprotoxic effects were tested in blind with the reproductive toxicity assay on the microfluidic platform. Five concentrations for each chemical (1mM, 333µM, 111µM, 37µM and 12µM) were tested and compared to the negative control (DMSO 1%). NOAEL was then determined and their respective toxicity profiles described.
Conclusion: After unblinding, 10 out of 12 were predicted correctly (according to the ECHA database), providing **a good predictivity of 83%**.

Conclusion and Outlook

We presented a novel microfluidic platform designed for fully automated analyses of *C. elegans* nematodes. This study highlighted the strong advantages of our innovative approach, including (1) a good reproducibility and accurate results thanks to standardized protocols, (2) an automated and dynamic dosing of chemicals with low liquid consumption (700µl in total for a full run) and (3) multi-phenotypic readouts in real time.
Our approach allowed identifying 10 toxic/reprotoxic chemicals among the 12 tested. The unblinding showed a good success rate with 1 false negative (Methoxyacetaet) and 1 false positive (Penicilin G), providing an overall predictivity of 83%.
In conclusion, we believe this platform represents the first "all-in-one" *C. elegans* microfluidic laboratory allowing rapid identification of toxic compounds in the early stages of the drug/chemical discovery pipeline.

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