All-in-one microfluidic-based robotic platform for automated Tox screenings in *C. elegans*

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Abstract

Background and Objectives: The current alternative methods to animal testing are typically based on cellular models. Nematode *Caenorhabditis elegans* constitute a valuable alternative in predictive toxicology studies, that can complement *in vitro* models to better predict the outcomes in mammals. This microscopic worm gained popularity due to its small size, short life cycle, ease of cultivation and propagation and a powerful genetic toolkit. However, the current methods for *C. elegans* experimentation often lack automation and standardization, representing the main limitation for their wider use in screening.

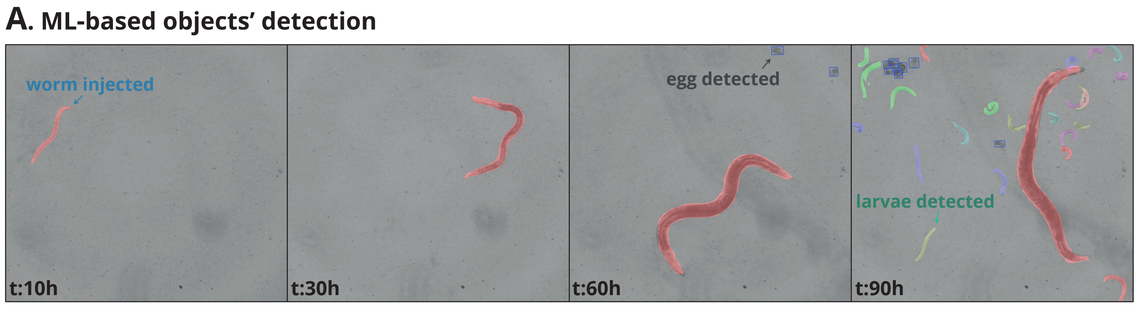
Material and Methods: We developed a microfluidic-based robotic platform that automates the entire process of culture, treatment, imaging and high-content phenotypic analysis of *C. elegans*, and executes different types of toxicology assays. The imaging potential of the platform is further extended by a possibility to acquire fluorescent pictures, allowing to benefit from the existing large collection of reporter strains.

Results: We assessed the effects on reproduction and development of twenty-one benchmark chemicals, amongst which were bisphenol A, thalidomide, hydroxyurea, paraquat, busulfan, 5-fluorouracil. Synchronized populations of worms were chronically exposed to 5 doses of test compounds starting from the last larval stage (L4). 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, progeny accumulation rate, progeny size and survival rate. Out of the tested compounds, methotrexate showed the most pronounced adverse effects on embryonic viability, while bisphenol A strongly impacted the development of the mothers.

Discussion and 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. Our technology allows not only to perform endpoint measurements, but to monitor the dynamics of biological responses.

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

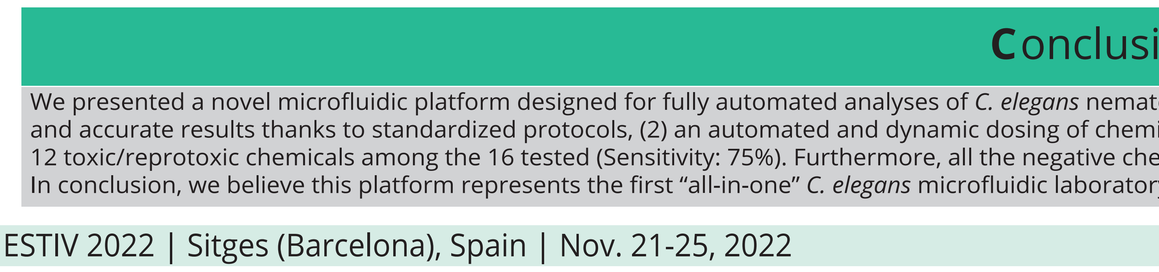
- **User-friendly software** to design, run and monitor experiments
- **Time-resolved/high-content data** extraction based on AI
- **Integrated statistical analysis** /data interpretation algorithms

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 ($\mathbf{K}_{length}$), time required to reach ½ max length($\mathbf{r}_{length}$), the time point when the first egg is detected ($\mathbf{t}_{eggs}$), the time point when the first larvae is detected ($\mathbf{t}_{progeny}$), the speed of egg ($\mathbf{m}_{eggs}$) and progeny accumulation ($\mathbf{m}_{progeny}$).

B. Automated data analysis

C. Objects detected

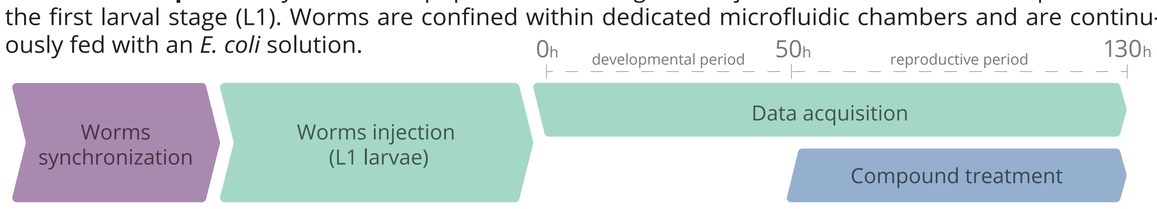
● Worm (size) ● Eggs (number) ● Progeny (number)

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

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.

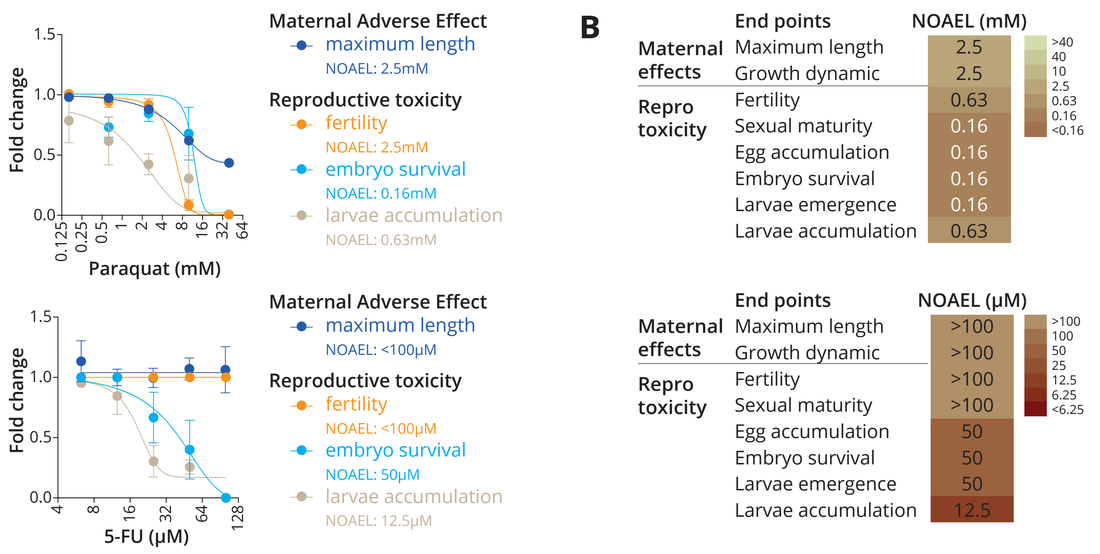


Worms are then chronically exposed to the test compounds starting **from the last larval stage prior to sexual maturity (L4)** for 80 hours (day 3 of adulthood). The protocol was specifically designed to avoid a treatment with the compounds during the developmental phase: the goal is **to evaluate the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

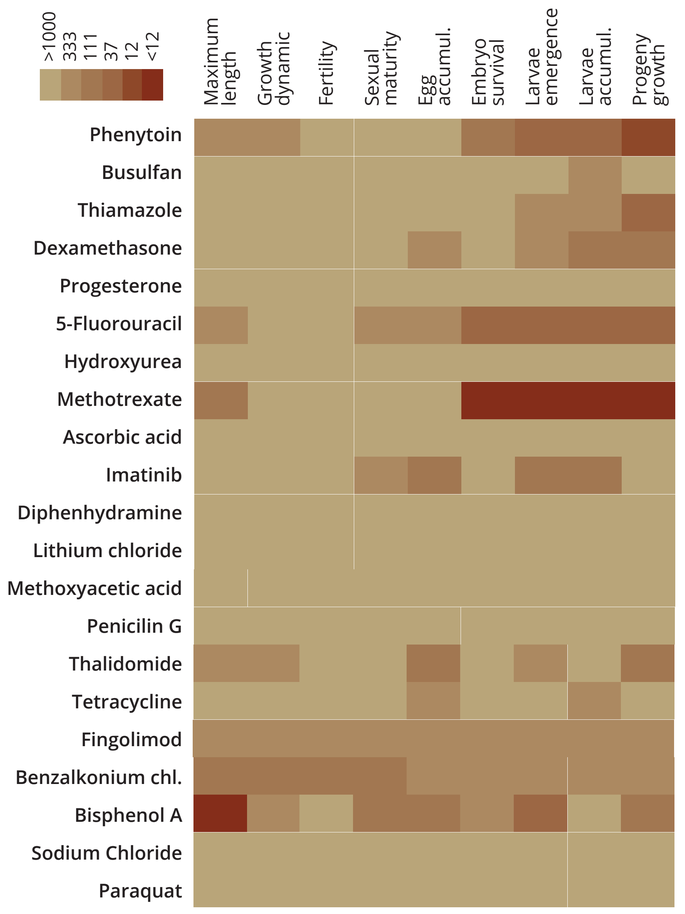
Data interpreation

Maternal effects	Adverse effects if $p<0.05$ and if:	Conclusion:
Maxium size (K value)	< 1.0 - CTLneg sd (threshold ≈0.90)	Smaller adult worms
Growth dynamic (r value)	<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
Progeny growth (min size/dev)	<1.0 - CTLneg sd (threshold≈0.90)	Smaller progeny

Results



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).



	Conclusion	Toxic profil in vertebrate	Predictive
Phenytoin	Reprotoxicity	Toxic	Yes
Busulfan	Reprotoxicity	Toxic	Yes
Thiamazole	Reprotoxicity	Toxic	Yes
Dexamethasone	Reprotoxicity	Toxic	Yes
Progesterone	No AE Observed	Negative	Yes
5-Fluorouracil	Reprotoxicity	Toxic	Yes
Hydroxyurea	No AE Observed	Toxic	No
Methotrexate	Embryotoxic effect	Toxic	Yes
Ascorbic acid	No AE Observed	Negative	Yes
Imatinib	Reprotoxicity	Toxic	Yes
Diphenhydramine	No AE Observed	Negative	Yes
Lithium chloride	No AE Observed	Toxic	No
Methoxyacetic acid	No AE Observed	Toxic	No
Penicilin G	No AE Observed	Negative	Yes
Thalidomide	Maternal AE	Toxic	Yes
Tetracycline	Reprotoxicity	Toxic	Yes
Fingolimod	Maternal AE	Toxic	Yes
Benzalkonium chl.	Maternal AE	Toxic	Yes
Bisphenol A	Maternal AE	Toxic	Yes
Sodium Chloride	No AE Observed	Negative	Yes
Paraquat	No AE Observed	Toxic	No

Twenty-one benchmark chemicals known to induce or not reprotoxic effects were tested in blind on the microfluidic platform. Five concentrations for each chemical (1mM, 333µM, 111µM, 37µM and 12µM) were tested and compared to the neg. control (DMSO 1%). Three technical repeats were executed, each chemicals were tested twice. NOAEL was then determined and their respective toxicity profiles described.
Conclusion: After unblinding, 17 out of 21 were predicted correctly (according to the ECHA database), providing **a balanced accuracy of 87.5% (Sensitivity: 75% / Specificity: 100%)**.

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 and (3) multi-phenotypic readouts in real time. Our approach allowed identifying 12 toxic/reprotoxic chemicals among the 16 tested (Sensitivity: 75%). Furthermore, all the negative chemicals were correctly identified (5 negatives among the 5 tested), providing an overall balanced accuracy of 87.5%. 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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