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"Worms-on-chip" technology as a new alternative for toxicology studies

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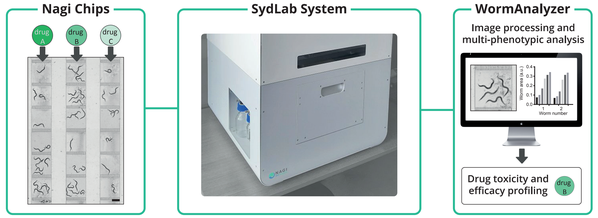
Purpose: Currently existing biological testing strategies still involve an extensive animal experimentation in vertebrate models, which is expensive and is associated with a number of important ethical concerns and regulatory constraints. The alternative methods to animal testing are typically based on cellular models. The main limitation of these *in vitro* techniques is that they cannot predict complex responses at the level of an organism, usually involving a multi-organ crosstalk. *Caenorhabditis elegans* (*C. elegans*) has been known for more than 60 years as a powerful model organism in fundamental research, allowing exploration of different facets of aging, development, neurosciences and genomics. This nematode gained popularity amongst the scientific community due to its small size, short life cycle, ease of cultivation and propagation and a powerful genetic toolkit. Despite their relative simplicity, nematodes present a wide range of behaviors and possess well-defined tissues. Nowadays *C. elegans* starts to get recognition as a valuable alternative for rodent and cell models in predictive toxicology studies. However, experimentation in *C. elegans* is still mainly based on manual labor and requires a specific skill-set from the person manipulating the worms. These testing strategies relying on manual handling techniques and direct observation by the operator hence lack reproducibility and standardization, overall largely limiting the potential of the worms for high-throughput and high-content screenings required for toxicology studies.

Methods: Nagi Bioscience SA developed a microfluidic-based platform for automated long-term worm culture and phenotyping. By integrating a microfluidic chip within a system for automated multiplexed liquid delivery and large-scale image acquisition we are capable to perform high-content phenotypic analysis of *C. elegans*. The imaging potential is further extended by a possibility to acquire fluorescent pictures. Finally, an integrated incubator guarantees a fine-tuned temperature control for the whole duration of the experiment. Overall, our platform offers the possibility of data acquisition on up to 64 conditions in parallel.

Results: Using this platform, we successfully executed different types of toxicological studies in *C. elegans*, including the assessments of reproductive toxicity, developmental toxicity, acute toxicity and embryotoxicity. Moreover, we applied these new test methods to the characterization and safety assessment of different classes of substances, comprising small molecules, nanoparticles, anthelmintics or food additives in worms. Finally, the possibility of performing fluorescent imaging permitted to profit from the large existing collection of *C. elegans* reporter strains: in this manner a user can collect additional information on the underlying molecular mechanisms by monitoring the actors of different stress pathways.

Microfluidic platform overview

Our microfluidic technology allows **large-scale studies** for the parallel characterization of multiple drugs and chemicals in different *C. elegans* populations. 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 dedicated image processing and data interpretation algorithms enables detailed multi-phenotypic screening at the whole-organism level.

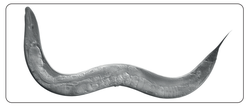


Features
- **Patented** microfluidic design, relying on passive hydrodynamics
- **16 fluidic lines**, enabling tests of 16 independent conditions
- **Plug & play** chip-to-device connectivity and fluidic operations

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 treatment plan defined by the user. The pictures of each microfluidic chamber **are collected via time-lapse microscopy** at desired frequency.

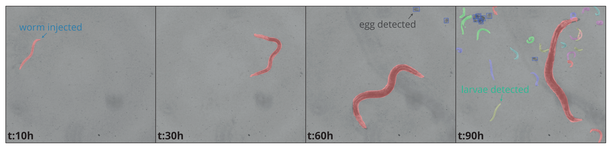


C. elegans offers an **excellent ethical alternative to vertebrate animal testing**, providing fast and high-throughput results through a whole-organism, validated by 60+ years of scientific research. Toxicity data obtained in worms proved themselves **to be predictive** of outcomes in mammals and the LC_{50} ranking in *C. elegans* matches the LD_{50} ranking in mouse and rat.

Data processing pipeline

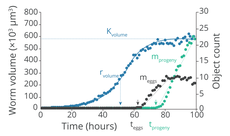
The time-lapse brightfield pictures obtained during the experimental procedure are post-processed by an extensively trained **machine learning (ML) software**. Through this ML software we monitor the **growth rate** of the worm population within the microfluidic chip over several days, as well as the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

ML-based objects' detection



The efficient object recognition allows the ML algorithms to extract the following parameters: maximal volume (K_{volume}), time required to reach ½ max volume (r_{volume}), 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 ($m_{progeny}$).

Automated data analysis

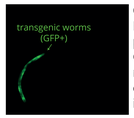


Objects detected

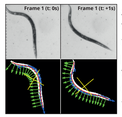
● Worm (size) ● Eggs (number) ● Progeny (number)

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



Our platform implements **both BF and fluorescent microscopy**. With the possibility to track GFP signal in a time resolved manner, we can take advantage of the large collection of existing *C. elegans* reporter strains.

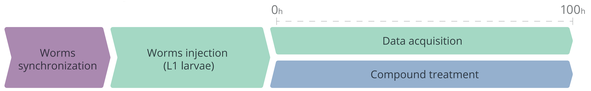


An option to **record videos** instead of pictures is also available. By processing the acquired videos we are capable to extract **multiple motility parameters**.

Developmental toxicity assay

Purpose: the assay is designed to explore the potential adverse effects of molecules on the development of *C. elegans*. It can be used as a prediction for **teratogenic potency** of the test compounds.

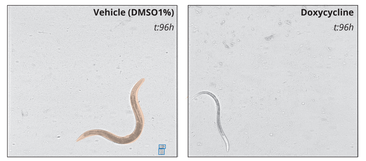
Method description: a synchronized population of *C. elegans* is injected into the microfluidic platform at the **first larval stage (L1)**. Worms are confined within dedicated microfluidic chambers and are continuously fed with an *E. coli* solution.



Worms are **chronically exposed** to the test compounds right after their injection (L1 stage) for 100 hours (corresponding to the full larval development + 2 day of adulthood). The compounds to be tested are mixed with the *E. coli* solution. Freeze-dried OP50 *E. coli* are used as a food source for the whole duration of the experiment, **preventing the metabolization** of the tested molecules by the bacteria.

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

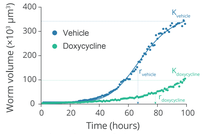
Readouts:
- worm lethality
- worm size
- growth dynamics
- sexual maturity
- worms' shape



C. elegans development over 96 hours post-hatching for wild-type worms treated with the antibiotic doxycycline starting from the L1 larval stage (right) and untreated worms (left), as observed via brightfield imaging by the SydLab system. Overlay masks for the detection of worms and eggs are automatically generated by SydLab's AI-based computer vision algorithm.

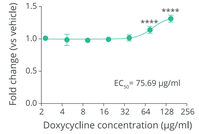
Results analyzed:

Worm's development



Temporal evolution of the average worm size for wild-type worms treated with the antibiotic doxycycline starting from the L1 larval stage vs untreated worms (vehicle). The treatment significantly impacts on worms' development, size and growth rate.

Growth dynamics (dose-response)



Average time to reach half of the maximal size (r) for wild-type worms treated with the antibiotic doxycycline at different doses (data normalized to the value measured for the vehicle). Doxycycline treatments delay worms' development in a dose-dependent manner.

Data interpretation

Lethality	no
Small	**YES**
Arrested dev.	no
Slow dev.	**YES**
Shape	no

Conclusion

Doxycycline treatment significantly impacts the worms' development, with a slower growth dynamic and smaller worms.

Mode of action

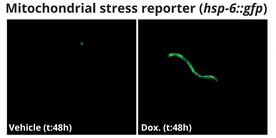
Purpose: the assay is designed to establish which commonly known **stress defense pathways** are activated by the test compounds in *C. elegans.*

Method description: for this assay we take advantage of the existing *C. elegans*-reporter strains for a variety of key molecular stress pathways, as well **as the capacity of our device to perform fluorescent imaging**.

Readouts:
- dev/repro readouts
- oxidative stress
- ER stress
- mitochondrial stress
- cytosolic stress

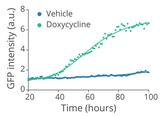
... (non exhaustive list)

Mitochondrial stress reporter (*hsp-6::gfp*)



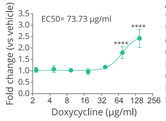
Dynamics of mitochondrial stress activation in *hsp-6::gfp C. elegans* treated with the antibiotic doxycycline starting from the L1 larval stage (right) vs untreated worms (left), as observed *via* fluorescence imaging by the SydLab system.

Pathway activation (dynamics)



Temporal evolution of the average fluo intensity measured for *hsp-6::gfp* worms treated with the antibiotic doxycycline vs untreated worms (vehicle). Increased expression of the *hsp-6::gfp* reporter reveals the activation of the mitochondrial stress induced by the drug treatment.

Pathway activation (intensity - dose-response)

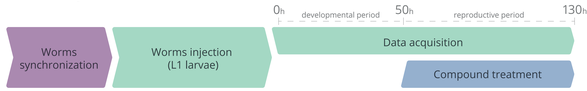


Average peak fluo intensity for *hsp-6::gfp* worms treated with the antibiotic doxycycline at different doses. Doxycycline treatment activate the mitochondrial stress response in a dose-dependent manner.

Reproductive toxicity assay

Purpose: the assay is designed to explore the potential adverse effects 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.

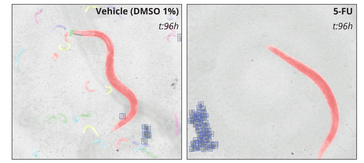


Worms are chronically exposed to the test compounds starting **from the last larval stage prior to sexual maturity (L4)** for 80 hours (day 3 of adulthood). The protocol was specifically designed to avoid a treatment with the compounds during the developmental phase: the goal was **to evaluate the potential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

Readouts:
- worm lethality
- worm size
- growth dynamics
- sexual maturity
- worms' shape
- fertility
- embryonic viability
- progeny accumulation

Maternal effect readouts

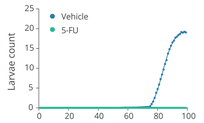
Reprotox readouts



C. elegans reproduction over 96 hours post-hatching for wild-type worms treated with the anticancer drug 5-FU starting from the L4 larval stage (right) and untreated worms (left) as observed via brightfield imaging by the SydLab system. Overlay masks for the detection of worms and eggs are automatically generated by SydLab's AI-based computer vision algorithm.

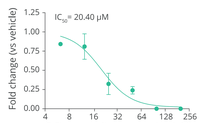
Results analyzed:

Progeny production dynamics



Temporal evolution of the average number of larvae produced by wild-type *C. elegans* treated with the anticancer drug 5-FU starting from the L4 larval stage vs untreated worms (vehicle). The drug treatment significantly impacts the reproductive process.

Reproduction rate (dose-response)



Progeny production rate over the first 24 hours of reproduction for wild-type worms treated with 5-FU at different doses (data normalized to the value measured for the vehicle). 5-FU treatments induce an embryotoxic effect in a dose-dependent manner.

Data interpretation

Maternal effect	no
Sterility	no
Delayed sex maturity	no
Embryotoxicity	**YES**
Low embryo surival	no
Delayed ef maturation	no
Low progeny production	no

Conclusion

5-FU treatment specifically affects the worms' reproduction, with a strong embryotoxic phentoype.

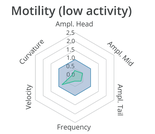
Motility assay

Purpose: the assay is designed to evaluate the effects of test compounds on *C. elegans* **behavior and motility**.

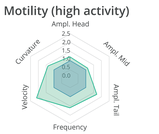
Method description: for this assay, short video sequences are recorded every 1 to 6 hours for each microfluidic chamber. Time-resolved readouts are then extracted from the acquired videos for a **diversified characterization of the worms' motility and behavior**.

Readouts:
- bending frequency
- velocity
- amplitude of the head
- amplitude of the mid body
- amplitude of the tail
- curvature

Motility (low activity)



Motility (high activity)



Representative multi-phenotypic fingerprint plot comparing a control worm and a more (left) or less (right) active worm at day 1 of adulthood, which includes readouts of body bends frequency, velocity, curvature and amplitude of the movement at the head, tail and middle of the body. This high-content motion analysis reveals very subtle changes in *C. elegans* movements, beyond average motility measurements.

Conclusion & Outlook

We presented a novel microfluidic platform designed for fully automated analyses of *C. elegans* nematodes. Our technology offers unprecedented levels of control and automation in long-term *C. elegans* experiments. Dedicated ML software allows monitoring of a variety of worm phenotypes, including body size, fertility, reproduction and motility that are relevant for early toxicity identification. 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.