White paper

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Neurotoxicity in *C. elegans* : Neuron-specific degeneration assessment using Newormics' high-content *vivo*Chip™ platform

Introduction

Current neurotoxicity assessment of environmental toxicants, pharmaceutical compounds, and industrial products is mandated by international regulatory bodies and largely relies on testing in mammalian species (rats, rabbits, dogs, and monkeys). However, high screening costs, long experimental times, and desire and increasingly changing legislative requirements to reduce dependence on animal testing have led many large industries to search for alternative models.

Currently available neuronal *in vitro* models can only provide limited insight on neurotoxicity, as they lack biologically relevant cellular network structures.

Caenorhabditis elegans can fill this unmet niche by enabling *in vivo* neurotoxicological assessment at high throughput rates in a complex, intact nervous system. Additionally, detailed information on the specific neuron types and behavioral outputs can be obtained. The ability to demonstrate neuron-specific mode-of-action in a complex nervous system makes *C. elegans* a powerful alternative model that can provide complementary information to *in vitro* models, helping to reduce the necessity for toxicity testing in large animal models.

Advantages of *C. elegans* as a HTS model for neurotoxicity and development:

- Short life-cycle
- Transparent body
- Precise gene editing
- Homologous genome
- Easy to grow in solid/liquid media
- Well-characterized nervous system
- All major neurotransmitter types
- Ethically compatible *in vivo* model

Figure 1: *C. elegans* is an attractive *in vivo* model for high-throughput toxicology studies.

Advantages of *C. elegans* for neurotoxicity assessment

The nematode, *C. elegans,* possesses several advantages as a whole animal, *in vivo* model system (**Figure 1**). *C. elegans* hermaphrodites have a very well-defined nervous system, comprising one-third of all its somatic cells (302/959). The 302 neurons form ~7,000 chemical synapses, similar in structure and function to synapses found in mammals. The major neurotransmitter classes found in mammals have been identified in *C. elegans* including GABA, dopamine, acetylcholine, glutamate, and serotonin, which are among the most studied. The short life-span, transparent body, simplified architecture, tractable genetics, easy of culturing, and conserved toxicological pathways make *C. elegans* an attractive model for high-throughput toxicology studies. *C. elegans* has been widely used for environmental toxicity studies with behavioral outputs, and developmental and reproductive endpoints. The advent of fluorescent markers for specific cells has also facilitated individual cell monitoring in *C. elegans* and enabled health assessments of specific neurons in live animals at different developmental stages.

Newormics' unique offering to enable neurotoxicity testing

Despite advancements in gene editing tools, well-characterized neuronal anatomy, and homologous genetic pathways, the lack of high-throughput techniques to assess individual neurons has been a major barrier to the use of *C. elegans* for neurotoxicity assessment.

Newormics, an Austin-based biotechnology company, has developed the first *in vivo* high-throughput, high-content screening platform for use with *C. elegans*. Utilizing a proprietary microfluidic-based screening device, *vivo*Chip™, we can immobilize *C. elegans* and examine individual neurons at high-resolutions in a cost- and time-effective manner for neurotoxicity assessments.

Leveraging unique *vivo*Chip™ technology, Newormics is offering a fee-for-service neuron-specific toxicity assessment of individual or combinations of compounds, using our proprietary *C. elegans* neurotoxicity assays. The end-points are multi-parametric phenotypes of structural neurodegeneration and neuron-specific, dose-dependent mode of action, as visualized using fluorescence reporters.

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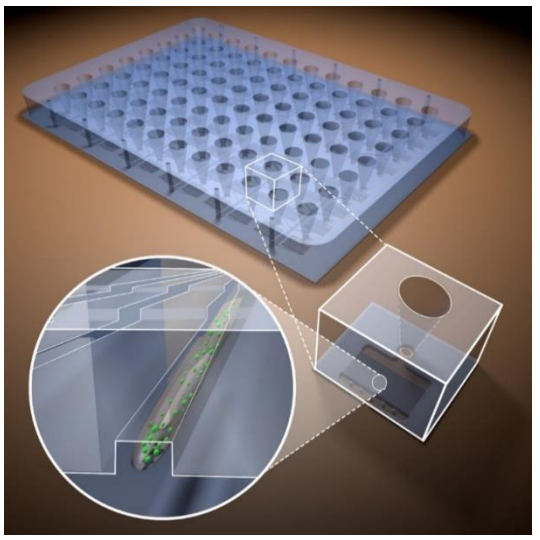

Figure 2: Newormics' *vivo*Chip™ platform to immobilize ~4,000 *C. elegans* in 3 min and rapidly image their neurons for high-content phenotyping for neurotoxicity assessments.

*vivo*Chip™ screening platform

High-content analysis of *C. elegans* requires their complete immobilization, which can be a labor-intensive and time-consuming process, as commonly performed manually. Newormics developed the only available high-throughput platform (*vivo*Chip™) that can immobilize 4,000 *C. elegans* simultaneously in 3 minutes from 96 different populations (**Figure 2**). Its proprietary microfluidic channel geometry provides easy access to immobilized *C. elegans* for rapid acquisition of high-resolution images of single neurons and their processes in all 4,000 individuals. The microplate format enables full integration with currently available liquid handling robotic platforms.

For neurotoxicity assessment, we image animals at high-resolutions to capture morphological phenotypes along the entire body using an automated microscopy system. We then analyze the images using our custom-developed graphical user interface (GUI) based software (*vivo*Score™). The GUI enables the user to define phenotypes of interest, streamlines image processing of large numbers of *C. elegans* from the corresponding large data sets, and provides automated statistical analyses.

Newormics' *in vivo* neurotoxicity assay using *C. elegans*

Neurons are characterized and identified by the neurotransmitters they use to communicate signals across synapses. The four types of neurotransmitters that are most studied in both humans and *C. elegans* are dopamine, GABA, acetylcholine, and serotonin. In our first assay, all eight dopaminergic neurons (**Figure 3**) were examined using a GFP-labeled model (dat-1::gfp) under a 20× objective. Among those neurons, the four cephalic sensilla (CEP) neurons are well-suited for neurotoxicity assessment associated with chemical exposure because their proximity to the mouth. This makes them the first neurons exposed to toxicants. We therefore assessed these four CEP neurons from 15 z-stack fluorescence images of immobilized animals after they were exposed to various concentrations of chemicals.

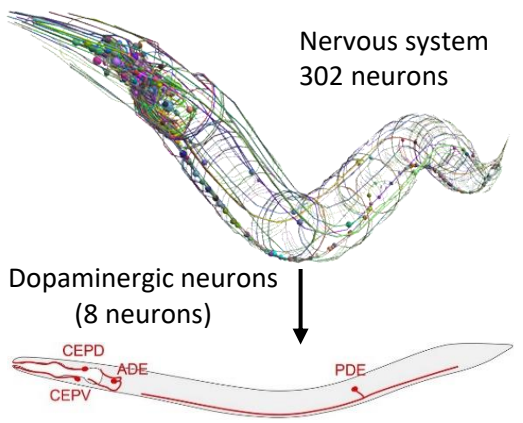

Figure 3: Schematic of *C. elegans* nervous system and eight dopaminergic neurons that fall into four different sets of bilaterally symmetric pairs of neurons: the dorsal cephalic sensillum (CEPD), ventral CEP (CEPV), anterior deirid sensillum (ADE), and posterior deirid sensillum (PDE).

***Large-scale, synchronized C. elegans culturing*.** We maintain *C. elegans* strains on nematode growth culture media at 20 °C following standard protocols (**Figure 4**). We age-synchronize the animals by bleaching gravid worms with NaOH/sodium hypochlorite solution, which dissociates the cuticle of the adults and enables collection of eggs. The eggs are incubated in minimal culture media at 20 °C for 24 hours and allowed to hatch. Though eggs hatch at different times, they all arrest developmentally at larval 1 (L1) stage due to a lack of essential nutrients for growth, thus providing a synchronized population.

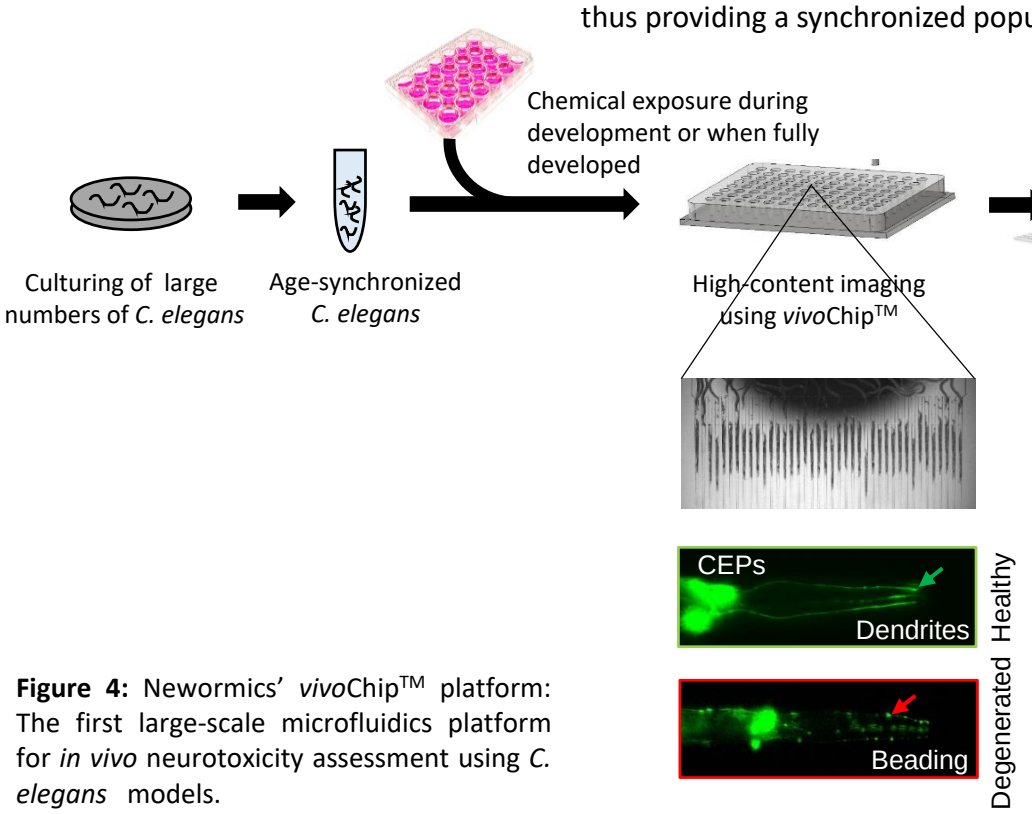

Figure 4: Newormics' *vivo*Chip™ platform: The first large-scale microfluidics platform for *in vivo* neurotoxicity assessment using *C. elegans* models.

Neurotoxicity and DNT endpoints:

- Multi-parametric phenotypes of cell body & neuronal process degeneration
- Neuron-specificity of mode-of-action:
  - Dopaminergic
  - GABAergic
  - Cholinergic
  - Serotonergic neurons

Developmental endpoints:

- Developmental defects (body length and diameter, germline and vulva development, number of eggs and viability)

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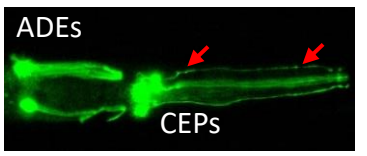
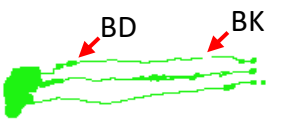
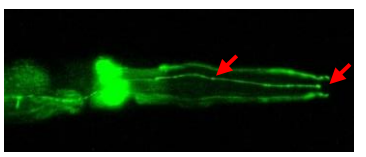
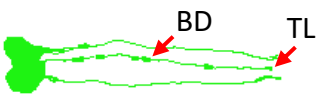
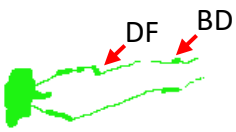
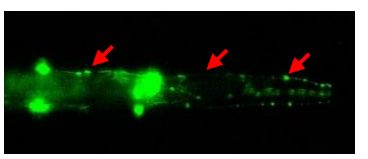
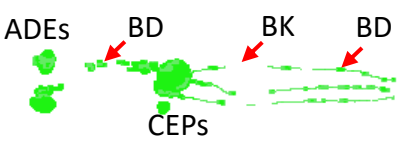
Chemical treatment: Approximately 80 isogenic and age-synchronized L1 *C. elegans* are placed into each well of a 24-well plate and fed with HB101 bacteria. We introduce the chemicals of interest either at L1 or larval 4 (L4) stage, to test neurotoxicity during development or after their development is complete, respectively. For toxicity assessments, animals are kept at 20°C for 72 hours, which represents chronic exposure, given their short lifespan.

General developmental phenotypes: To assess general developmental features of L1-stage treated animals, we image individual worms in the wells with 4× and 10× objectives. The first qualitative endpoint that we assess is whether the animals form a normal Christmas-tree shaped vulva at 48 hours, as a marker of developmental delay. At 72 hours, additional qualitative endpoints of general health features are assessed such as remaining food density, worm diameter, and the live/dead population ratio. Only populations of live animals are then loaded into the *vivo*Chip™ where we can quantitatively assess developmental endpoints from bright field images with 4× objective. Specifically, we measure body length and diameter, count seam cells as a marker of developmental stage, and count the number of eggs in the body as a measure of fertility.

Neurodegeneration phenotypes: The exposure of dopaminergic neurons to toxicants primarily results in dendritic degeneration that shows four main phenotypes: beading, breaking, deformation, and tip loss (**Table 1**). Resolving these dendritic phenotypes requires imaging of the neurons with sub-micron resolutions and collecting multiple z-stack fluorescence images of *C. elegans* (20× objective, providing 360 nm pixel resolution). For our positive control in developmental neurotoxicity (DNT) assays, where we expose the animals to a toxicants starting at L1 stage, we use one of the most widely studied neurotoxic chemicals, 1-Methyl-4-phenylpyridinium iodide (MPP+). MPP+ induces cellular damage through mitochondrial disfunction in mammals as well as in *C. elegans*. Using the *vivo*Score™ GUI, we count how many neurons (out of 4 labeled with GFP) show degeneration of all 4 phenotypes in individual *C. elegans*, up to 40 animals for each population, exposed to different concentrations of MPP+.

Table 1: Examples of neuronal degeneration phenotypes characterized in dat-1::gfp animals where dopaminergic neurons are labeled with GFP. High-resolution fluorescence images of young adult animals are captured using the *vivo*Chip™ platform. The identified neuronal structures are the anterior deirides (ADE) and cephalic sensilla (CEP) neurons. We counted the following four neurodegeneration phenotypes in all four dendritic processes of CEP neurons: beading (BD), breaking (BK), tip loss (TL), and deformation (DF).

*vivo*Chip™ Image Capture	*vivo*Score™ Image Analysis
	
	
	
	

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Results

We analyzed the four CEP dopaminergic neurons in dat-1::gfpexpressing animals treated with five different concentrations of MPP+ and DMSO, as the negative control. **Figure 5** shows the average percentage of occurrences of each degeneration phenotype (bars) found for each treatment condition. There is an increase in all degeneration phenotypes at doses of MPP+ >0.5mM. The average percentage of all four degeneration phenotypes (black dots) show statistically significant dose dependent increase in neurotoxicity.

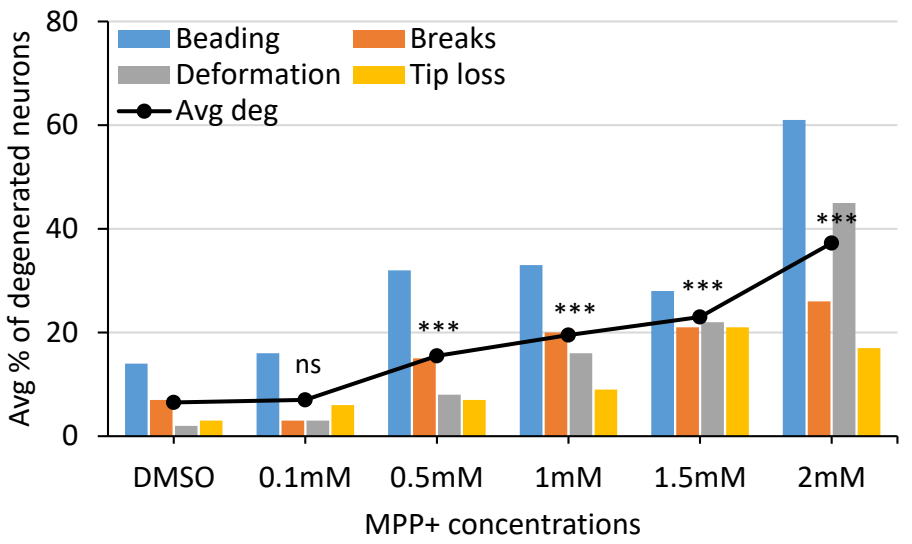

Figure 5: Dose-dependent degeneration phenotypes in the dat-1::gfp animals, as observed from the high-resolution images of young adult animals. Animals were treated at the early developmental stage of L1 with 5 different doses of MPP+ and imaged at young adult stage. The multiple z-stack images are analyzed to score for different degeneration phenotypes in the CEP neurons. The bars represent the average percentage of neurons with a specific neurodegeneration phenotype and the black dots represent the average percentage of all four degeneration phenotypes found per treatment condition. Fisher exact test with respect to negative control (DMSO-treatment), $p < 0.001$ (***).

Summary

C. elegans is recognized as a powerful alternative *in vivo* model. The Newormics' *vivo*Chip™ enables the use of *C. elegans* for high-content, multi-parametric analysis of neurodegeneration phenotypes using high-resolution fluorescence images of neurons. To demonstrate our unique capabilities, we studied dendritic neurodegeneration of dopaminergic neurons in *C. elegans* induced by a well-accepted neurotoxicant, MPP+, showing dose-dependent increase in neurotoxicity.

This neuron-specific assay provides the ability to assess developmental neurotoxicity in an intact, live, whole animal model. We are currently offering this assay on a fee-for-service basis.

Our near-term goal is to establish neurotoxicity assessment reference standards from a small library of chemical compounds that would be of interest to the pharmaceutical, agrichemical, manufacturing, and petroleum industries for methods validation purposes.

We are looking for interested partners who would like to share with us their chemicals of interest to test with our dopaminergic neurodegeneration assay. This information can be used for validation of the assay. Additionally, to explore potential future collaborations or service contracts for ongoing monitoring of industrial exposures to existing chemicals, safety testing of novel compounds, mixtures.

We plan to provide a complete standardized set of the four types of neuron-specific *C. elegans* assays for neurotoxicity assessment services.

References

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