An automated microfluidic platform for toxicity testing based on *Caenorhabditis elegans*

Jiaying Wu[a,b,c,†], Jing Xi[a,b,†,*], Zhouhai Zhu[a,†], Wei Zhao[d], Qiyuan Peng[a], Chunbo Liu[a], Ziqian Wan[e], Yiyi Cao[b], Xiang Chen[f*], Yang Luan[a,b,*]

[a] Joint Institute of Tobacco and Health, 41 Keyi Road, Kunming, Yunnan, China

[b] School of Public Health, Shanghai Jiao tong University School of Medicine, 227 South Chongqing Road, Shanghai, China

[c] Shanghai Municipal Center for Disease Control and Prevention, 1380 West Zhongshan Road, Shanghai, China

[d] Technology Center, China Tobacco Yunnan Industrial Co., Ltd. 181 Hongjin Road,Kunming 650231

[e] Biological science department, Xi'an jiaotong-Liverpool University, 111 Renai Road, Suzhou, Jiangsu, China

[f] National Key Laboratory of Science and Technology on Micro/Nano Fabrication, Department of Micro/Nano Electronics, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai, China

[†] Jiaying Wu, Jing Xi and Zhouhai Zhu contributed equally to this work.

*Correspondence to:

Jing Xi, School of Public Health, Shanghai Jiao tong University School of Medicine, 227 South Chongqing Road, 200025 Shanghai, China. E-mail: xijing@shsmu.edu.cn

Xiang Chen, National Key Laboratory of Science and Technology on Micro/Nano Fabrication, Department of Micro/Nano Electronics, Shanghai Jiao Tong University,

23 800 Dongchuan Road, Shanghai, China. xiangchen@sjtu.edu.cn

24 Yang Luan, School of Public Health, Shanghai Jiao tong University School of Medicine,

25 227 South Chongqing Road, 200025 Shanghai, China. E-mail: yluan@sjtu.edu.cn

26 **Abbreviations**[1]

27 **Abstract**

28 Humans are frequently exposed to a multitude of chemicals daily, necessitating

29 efficient methods for rapidly assessing toxicity and potential health risks.

30 Microfluidics has shown promise as an intelligent tool for rapid compound testing,

31 owing to its flexibility in integrating with automated devices. The article introduces

32 an automated microfluidic platform, based on *Caenorhabditis elegans (C. elegans)*,

33 designed for chemical toxicity testing. This platform consists of three modules –

34 worm culture, monitoring, and image analysis – which enable automated worm

35 culturing, drug delivery, periodic monitoring, and automated phenotypic analysis.

36 Researchers have designed a bridged microfluidic chip that permits worms to move

37 freely during experiments and established an economical monitoring module for

38 long-term tracking and periodic imaging. Furthermore, they have developed an

39 automated image analysis algorithm to automatically determine worm bending

40 frequency. The platform was subsequently utilized for long-term toxicological

41 assessments of the organophosphate pesticide and environmental pollutants. Results

42 indicated that the platform can effectively evaluate the general and developmental

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[1] ***Abbreviations:*** C. elegans, Caenorhabditis elegans; MCP, monocrotophos; BDE-47, 2,2',4,4'-Tetrabromodiphenyl ether; MEMS, Micro-Electro-Mechanical Systems; PDMS, Polydimethylsiloxane; NGM, nematode growth medium; 5-FUdR, 5-Fluoro-20-deoxyuridine; DMSO, dimethyl sulfoxide;; SE, standard error; PBDEs, polybrominated diphenyl ethers;

impacts of chemicals. The automated microfluidic worm analysis platform holds significant potential for applications in drug safety assessmentand drug screening research, contributing to human health and industry advancement.

Keywords: automated microfluidic platform, Caenorhabditis elegans, chemical toxicity testing

Introduction

With the development of technology, large numbers of newly registered chemicals discharge into the environment every day (https://www.cas.org/). Humans are consistently exposed to various chemicals, which demands efficient methods to rapidly evaluate the toxicity and potential health risks to humans. Microfluidics has shown promise as an intelligent tool for rapid testing of compounds due to its flexibility to integrate with automation devices. Current researches based on *in vitro* organ chips or microphysiological systems cannot simulate *in vivo* dynamic process in human bodies[1, 2]. *Caenorhabditis elegans (C. elegans)*, a classic model organism, is a powerful alternative *in vivo* assay system that has proven to be a useful tool in toxicology studies[3, 4]. Recently, platforms combining microfluidic chip with *C. elegans* are promising for toxicity testing of numerous chemicals[5, 6].

Numerous researchers have developed microfluidic platforms for short-term observation of worms in acute toxicity testing assays[7, 8]. These platforms exhibit significant benefits, including minimal reagent usage and the capacity for seamless integration with automated systems. The microchambers can be configured to prevent the overlap of worms along the Z-axis, thereby simplifying the process of

automated imaging and monitoring throughout the experiments. In chronic toxicity studies, periodic observation and phenotype analysis are needed to provide information on the cumulative effect of toxins, thus demanding long-term cultivation with periodic drug delivery and medium replenishment. Sahand *et al*. developed a platform for age synchronization and analyzing subtle aging phenotypes of worms, on which worms were fed manually outside and transferred daily into the chip for progeny filtration[9]. Researchers have reported the development of platforms capable of sustaining the culture of worms and conducting time-lapse imaging over extended periods within microfluidic chips[10-12]. More recently, Le *et al*. introduced a microfluidics-based automated system known as HeALTH, designed to facilitate the lifelong cultivation and behavioral monitoring of worms[13]. Traditionally, the observation and analysis of phenotypic characteristics have involved immobilizing worms within microfluidic chips[9, 10, 14]. However, there has been a growing trend towards the creation of chips with specialized designs that permit the free movement of worms, thereby enabling the observation of behavioral phenotypes in their entirety. To this end, researchers have also devised algorithms capable of automatically identifying, tracking, and analyzing the behavioral traits of worms[15-18]. Despite these advancements, the approaches necessitate substantial computing resources and are computationally intensive for standard computers. In recent times, artificial intelligence (AI) has been integrated into the image processing of worms[19], yet there are limited studies on the use of deep learning for the analysis of worm phenotypes, particularly in images with complex backgrounds such as those from

87 microfluidic chips. In summary, while the aforementioned platforms have made
88 strides in the automation of toxicity testing assays using worms, there remains
89 considerable room for further development.

90 In this study, we presented a microfluidic platform designed for the automated
91 assessment of chemical toxicity using *C. elegans*, which consists of worm cultivation,
92 drug delivery, regular monitoring and automated phenotype analysis. Initially, we
93 designed a bridge-like microfluidic chip for worm culturing, which allows worms to
94 move freely form larval stage to adult. Additionally, we constructed an economical
95 monitoring module capable of longitudinal tracking and periodic image capture of
96 the worms. . The bend frequency of worms can be automatically determined using
97 an image analysis algorithm previously developed in our study[20]. Subsequently, we
98 carried out a whole-life-covered worm culture (17 days) and toxicity evaluation of
99 monocrotophos (MCP) and 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) using this
100 microfluidic platform, to validate the capacity of the platform for long-term toxicity
101 testing assays on worms.

102 **Materials and methods**

103 **Microfluidic device fabrication**

104 The microfluidic chip mold was developed by MEMS (Micro-Electro-Mechanical
105 Systems) process, including metal sputtering and electroplating[21]. Liquid PDMS
106 mixture (Sylgard 184 Silicone Elastomer Base : Curing Agent = 10:1) was cast on a
107 silicon mold and cured overnight at 70 °C to fabricate a PDMS chip. The inlet and
108 outlet were punched with needles. Finally, the PDMS chip was bonded to a clean

109 glass by air plasma treatment and stored overnight at 135 °C. For more details, see
110 supplementary materials.

111 **Worm strains and maintenance**

112 The *C. elegans* strains used in this study were wild-type Bristol N2， which was
113 obtained from the Caenorhabditis Genetics Center (St. Paul, Minnesota, USA).
114 Nematodes were cultured in the nematode growth medium (NGM, 17 g/L Agar B, 3
115 g/L NaCl, 2.5 g/L peptone, 25 mM KPO_4 buffer pH 6.0, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5
116 mg/L cholesterol in ethanol) or in M9 buffer (17 mM K_2HPO_4, 42 mM Na_2HPO_4, 85
117 mM NaCl, 1 mM $MgSO_4$) with the *E. coli* strain OP50 as nutrient at 20 °C. To obtain a
118 synchronized nematode population, a bleach-sodium hydroxide mixture (0.5 M
119 NaOH and 1% (v/v) NaClO) was used with an established method[22].

120 **Chemicals**

121 5-Fluoro-20-deoxyuridine (5-FUdR) and dimethyl sulfoxide (DMSO) were
122 purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Monocrotophos (MCP) was
123 purchased from LGC Science (ShangHai Ltd) and dissolved in ultrapure water.
124 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) was obtained from Matrix Scientific
125 (Columbia, South Carolina, USA) and dissolved in DMSO.The stock solutions were
126 subsequently diluted with M9 buffer to obtain the working solutions. Ultrapure
127 water was produced using a Milli-Q system (Millipore, Bedford, MA, USA).

128 **Lifespan assays**

129 The lifespan assay was performed on age-synchronized adult worms (48 h after
130 hatching) in 96-well plates and PDMS microfluidic chips. Worms were cultured in

131 culturing medium (*E. coli* OP50:M9 buffer 1:200, v/v) and 5-FUdR (final concentration
132 of 20 mg/mL) was added to inhibit reproduction. In the PDMS microfluidic chips,
133 culturing medium was refreshed every day to provide the worms with sufficient
134 nutrients. The worms were stimulated with an acupuncture needle (96-well plates)
135 or by increasing the flow rate (microfluidic chips) every 24 h. If a worm did not
136 respond to any stimulation within 1 min, it was considered dead.

137 **Developmental toxicity assays**

138 For comparison of the developmental toxicity assays in PDMS microfluidic chips
139 and multi-well plates, N2 worms were treated with MCP (0, 250, 500 μM and 1 mM)
140 and BDE-47 (0, 3, 10 and 30 μg/ml) from the L1 stage (no feeding after hatching).
141 After 24 h, videos of worms in each chamber were recorded and analyzed with
142 automated image analysis module. The same assay was repeated with multi-well
143 plates, and the body length of worms were calculated manually.

144 **Neurotoxicity assays**

145 Synchronized N2 worms in L4 stage (feeding for 48 h after hatching) were treated
146 with chemicals for 24 h in this assay, and 20 mg/mL 5-FUdR was added to inhibit
147 reproduction. The concentrations of MCP were 0, 250, 500 μM and 1 mM, BDE-47
148 were 0, 3, 10 and 30 μg/ml. After treatment, worms were rinsed with M9 and
149 recorded by monitoring module. The bend frequencies of worms were calculated
150 with the image analysis module.

151 **Statistical analysis**

152 GraphPad Prism version 8.0 (GraphPad Software, La Jolla, California, USA) and IBM

153 SPSS Statistics version 25 (SPSS, Chicago, IL, USA) were used for statistical analysis. All
154 data are expressed as mean ± standard error (SE). The log-rank test was used to
155 compare the survival curves of worms cultured on PDMS microfluidic chips and
156 micro-well plates. One-way analysis of variance (one-way ANOVA) followed by the
157 *Dunnett t*-test were used to determine the differences between the treatment
158 groups and control group. A value of $P < 0.05$ was considered statistically significant.

159 **Results**

160 **Overview of microfluidic platform for worm toxicity testing**

161 Since current microfluidic platforms for worm toxicity testing usually operate at a
162 low level of automation, we developed an integrated microfluidic platform, which
163 consists of three modules (Fig. 1A). The worm cultivation module includes a
164 microfluidic chip for worm culturing and observation, equipped with pressure-based
165 delivery system. Based on this module, diets and test substances can be
166 automatically added at regular intervals, and worms can be cultured in microfluidic
167 chip until death. During chronic toxicity testing assays, the phenotypes of worms in
168 bright filed can be monitored and collected regularly with the monitoring module,
169 which includes a motion stage and a Pi camera. After capturing images or videos of
170 worms, the body length and bend frequency can be calculated automatically with the
171 image analysis module. Fig.1B shows the physical photograph of the entire platform,
172 which is controlled through the control center.

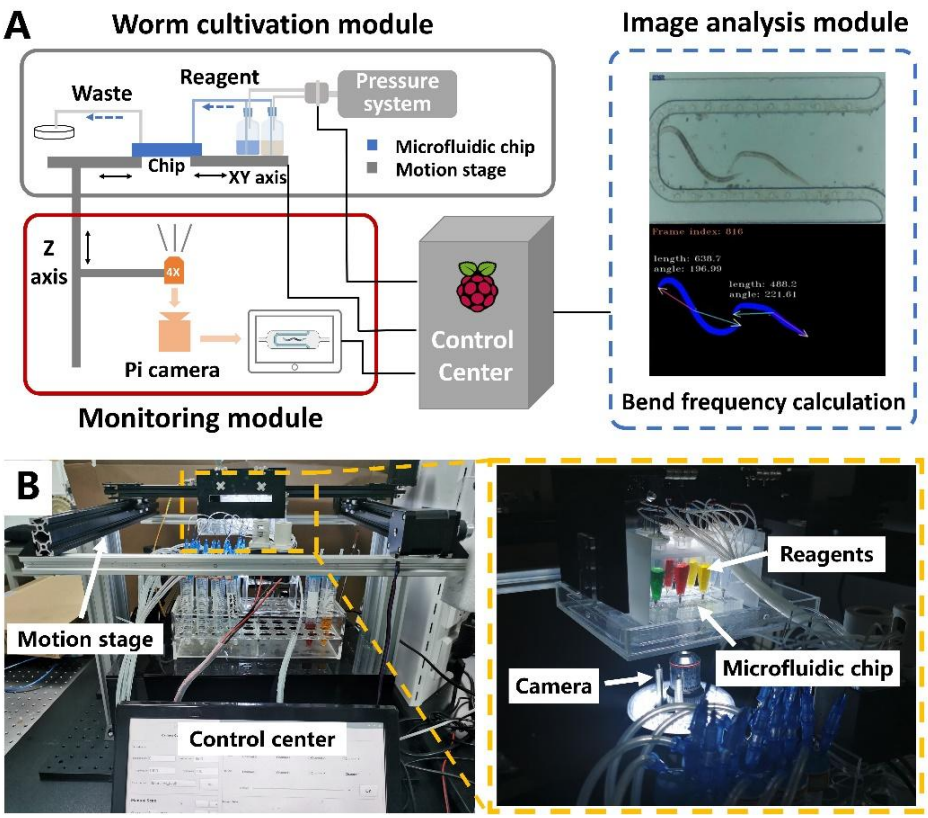


Fig.1. Schematic of the microfluidic platform. (A) Diagram of the designed platform consisting of the "worm cultivation module" “monitoring module” and "image analysis module". **(B)** Physical photograph of the whole platform. Inside the yellow dotted box (graph on the right) is magnified photograph of "worm culturing module".

The bridge-like microfluidic chip for worm cultivation

We designed a bridge-like microfluidic chip that allows worms to move freely during the experiment (Fig. 2A). Five parallel chambers were mounted on one chip, and each chamber had a bridge-like structure to allow medium replacement and prevent the worms from escaping. To meet the requirements of worm experiments from the larval stages to adult, the gap should be designed to arrest the worms in L1 stage. Since L1 worms are approximately 10-15 μm wide and sufficiently flexible to squeeze through narrow gaps, an ideal gap should not only arrest the worms but also allow the medium to flow smoothly under relatively low pressure. We tested the working conditions with gap sizes of 2, 5 and 10 μm and confirmed that a 5 μm gap

188 can meet the above requirements (see supplementary materials). To prevent the
189 "bridge deck" from collapsing, pillars were set as support structures at intervals of
190 100 μm. The designed height of the chambers is 40 μm (approximately equal to the
191 diameter of adult worms) to avoid overlapping more than two worms along the
192 *Z*-axis, which is beneficial for observation and automated analysis. Five inlets and
193 outlets are merged on each chip (Fig. 2C).

194 The operation procedure of the microfluidic chip is demonstrated as Fig. 2B. Firstly,
195 synchronized worm suspension in the reservoir was loaded into microfluidic chip.
196 During the experiment, culturing medium can be replenished and consistently loaded
197 into the chip at a low flow rate, to provide sufficient nutrients for worms.

198 To validate the biocompatibility of this microfluidic platform, we cultured worms in
199 multi-well plates and PDMS microfluidic chips until death. Briefly, 24 and 17 worms
200 were included in the multi-well plate group and PDMS microfluidic chips. As depicted
201 in Fig. 2D, the growth status of worms cultured on microfluidic platform are
202 consistent with those in traditional multi-well plates. The average lifespan (± SD) of
203 the worms in multi-well plates and microfluidic chips were 12 (± 4) days and 11 (± 3)
204 days, respectively, which revealed no significant difference between the survival
205 curves of two groups ($P > 0.05$) (Fig. 2D). The above results indicated that the
206 platform is suitable for worm growth and automated long-term cultivation, which
207 can relive labor and time compared with traditional assays on multi-well plates.

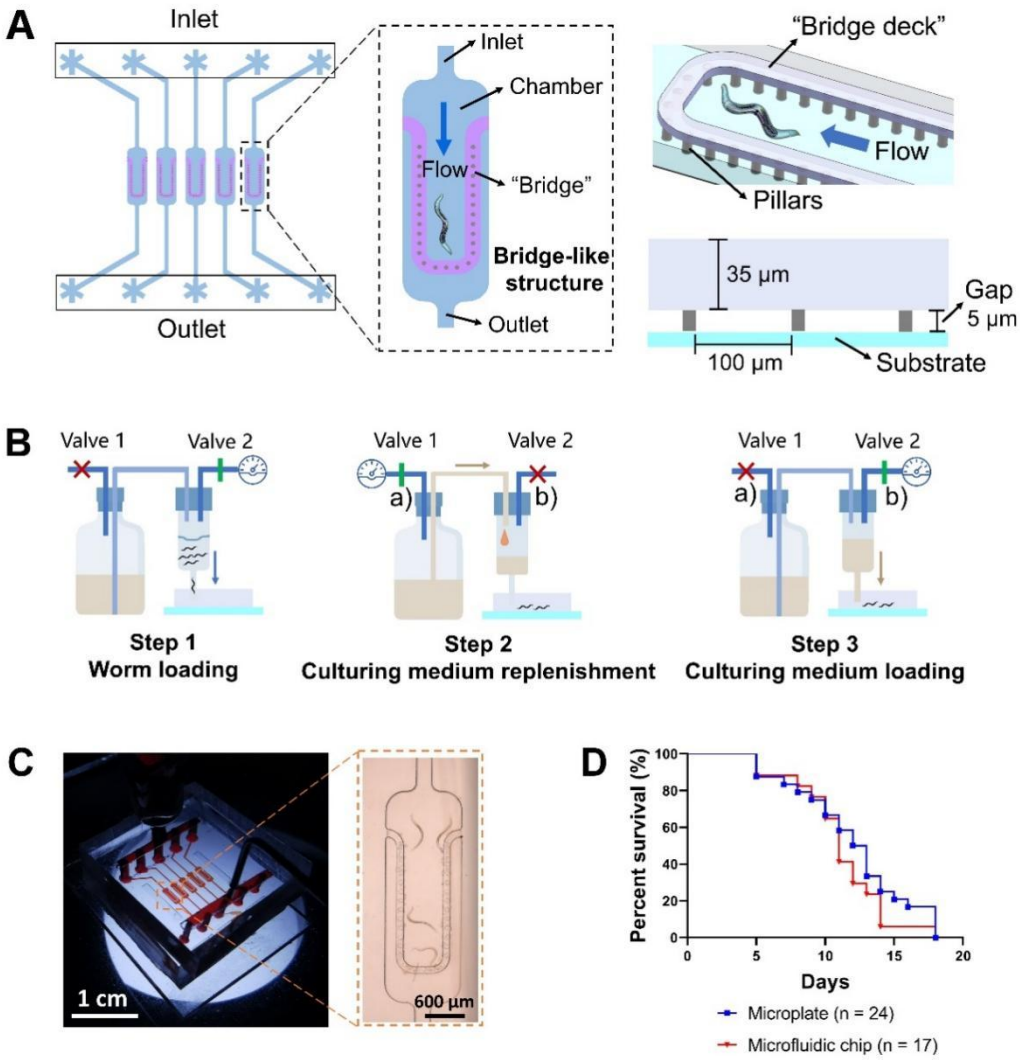


Fig.2. Design and operation of the microfluidic chip. (A) Five structures are paralleled in one chip. The inlet and outlet are 150 μm wide and the chamber is 40 μm in height. The "bridge deck" has a thickness of 35 μm and a 5 μm gap underneath to prevent the worms from escaping while allowing the culturing medium to flow. Pillars are set as support structures at intervals of 100 μm. Five inlets and outlets are merged and soaked in liquid to avoid water evaporation. **(B) Procedure of worm loading and nutrient replenishment.** Step 1: valves 1 is closed (red bar) and valve 2 is open (green bar), then worm suspension is loaded into the chip. Step 2: when reagents are exhausted, valves 1 is open and valve 2 is closed. Step 3: nutrient is loaded into the chip. **(C) The physical photograph of the microfluidic chip. (D) Comparison of worm lifespan on multi-well plate and PDMS chip.** The survival curves of the two groups have no significant difference ($p > 0.05$).

Low-cost monitoring module for longitudinal observation and image acquisition

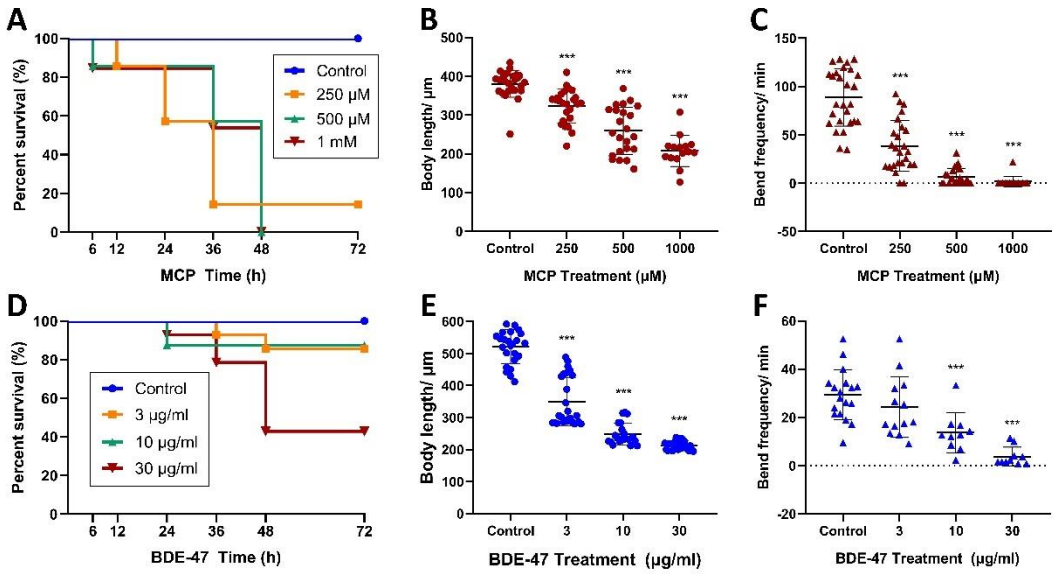
The monitoring module is a low-cost optical imaging system that can ensure stability for the long-term culturing of worms. We built a low-cost three-axis displacement platform using aluminum profiles. Cost of the monitoring module was

223 less than US$ 200. The displacement platform was controlled by an Arduino circuit
224 connected to a Raspberry Pi CPU. The GRBL code library was pre-written in Arduino.
225 The stepper motor and its driver were connected. The Raspberry Pi could send the G
226 code to Arduino to control the motion of the stage. The motion accuracy of this
227 module can easily reach 10 μm. We used a 4× lens and a low-cost Raspberry Pi CCD
228 for imaging.
229
230 **Automated image analysis module for phenotype analysis of worms**
231 With the large amount of image data generated in toxicity testing assays,
232 automated data processing can significantly improve the efficiency of the experiment.
233 Therefore, we used the automated image analysis algorithm developed in our
234 previous study[20] to analyze the body length and bend frequency of worms. We used
235 deep learning to extract worm contours from videos that contain chamber structure
236 and impurities in the culture environment. The following process is described as
237 before.
238
239 **Toxicity evaluation of MCP and BDE-47 on the platform**
240 Based on this microfluidic platform, we chose MCP and BDE-47 to demonstrate
241 the feasibility of the platform for automated toxicity testing of exogenous chemicals.
242 MCP is an organophosphorus pesticide widely used in agricultural production, the
243 residues of which may be detected frequently in food. As a cholinesterase inhibitor
244 acting on an acetylcholine receptor, MCP is highly neurotoxic[23]. As a kind of
245 commercial flame retardant, BDE-47 is the most abundant and prevalent

246 polybrominated diphenyl ethers (PBDEs) congeners in the environment, which has
247 been known to introduce neurodevelopmental toxicity[24].

248 We treated N2 worms with MCP and BDE-47 for 72 h and evaluated the body
249 length and bend frequency of worms at 24 h. Fig. 3A showed that treatment of MCP
250 and BDE-47 induced significant general toxicity in worms on this platform. The body
251 length was used as the toxic endpoints to evaluate the development toxicity. As
252 shown in Fig. 3B&E, MCP and BDE-47 treatment caused dose-dependent decreases
253 ($p < 0.001$), implying evident developmental toxicity to nematodes. Fig. 3C&F showed
254 that the bend frequency of worms decreased significantly ($p < 0.001$) with the
255 increase in dose after 24 h treatment.

256 The above results demonstrated that this platform can be applied to automated
257 toxicity testing of numerous chemicals that may cause neurotoxicity and
258 development toxicity. In the future, more phenotypes such as brood size and cell
259 apoptosis, can be measured to expand the scope of toxicity evaluation.



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261 Fig. 3. Toxicity evaluation of MCP and BDE-47 on the platform. (A-C) The survival curve, body length and bend

frequency of worms treated with MCP. (D-F) The survival curve, body length and bend frequency of worms treated with BDE-47.

Discussion

With the development of science and technology, microfluidic technology is more and more widely used in the biomedical field. Microfluidic technology has the characteristics of high throughput, low consumption and precise control and is widely used in cell culture, drug screening, disease diagnosis and other fields. As a model organism, *C. elegans* has the characteristics of short life cycle, simple genome and relatively simple nervous system and are ideal models for studying neurotoxicology and neural effects of drugs. In this study, we introduced an automated microfluidic platform based on *C. elegans*, which can realize automation of worm toxicity testing assays including worm culturing, monitoring and phenotype analysis.

Toxicity assays require periodic rinsing and recording of the worms' phenotypes to evaluate the toxicity of compounds with extended exposure times. In experiments lasting more than 24 hours, the diet-containing culturing medium should also be replenished regularly. Therefore, an automated liquid delivery and switching system is needed. NemaLife has introduced a product that can realize programmable regimens of feeding and reagent addition for toxicity testing (https://www.nemalifeinc.com/infinity-system), but in the latest report, they still use hand-held syringes to manually perform fluid manipulation[25]. Some researchers that developed an open-source liquid handling device to realize reagent delivery from large reservoirs to microfluidic chips were faced with the problems of complicated

motion platforms and extra on-chip backpressure designs[26]. Other researchers applied rotary valves[27] or robotic devices[28] to liquid manipulation, but these fluid switch and delivery systems are limited to complex structures with high-cost, thus limiting the automation of chemical screening. In our study, we designed an automated medium replenishment device to relive the tedious operation of manual liquid change, the cost of which is low enough for laboratories.

Combined with microfluidic technology and nematode model, the neural effects of chemicals or drugs can be studied efficiently, quickly and accurately. Through the platform, the exposure environment of different concentrations of chemicals or drugs to nematodes can be simulated, and the behavioral changes of nematodes, such as movement trajectory, movement speed, reaction time can be observed and recorded. Through the analysis of these data, the neural effects of chemicals or drugs can be evaluated and the scientific basis for the safety assessment can be provided.

In general, the automated microfluidics nematode analysis platform is expected to play a crucial role in the safety assessment of tobacco products, drug screening and neurotoxicology research, contributing greatly to people's health and the sustainable development of the industry.

Supplementary Information

Microfluidic device fabrication

Determination of the gap height of the microfluidic chips

Declarations

Availability of data and material

The data used and analyzed in the present study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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