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

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Abbreviations[1]

[1] ***Abbreviations:*** C. elegans, Caenorhabditis elegans; MCP, monocrotophos; BDE-47, 2,2',4,4'-Tetrabromodiphenyl ether; ACh, acetyl choline; DA, dopamine; GABA, gamma aminobutyric

Abstract

Humans are frequently exposed to a multitude of chemicals daily, necessitating efficient methods for rapidly assessing toxicity and potential health risks. Microfluidics has shown promise as an intelligent tool for rapid compound testing, owing to its flexibility in integrating with automated devices. The article introduces an automated microfluidic platform, based on *Caenorhabditis elegans (C. elegans)*, designed for chemical toxicity testing. This platform consists of three modules – worm culture, monitoring, and image analysis – which enable automated worm culturing, drug delivery, periodic monitoring, and automated phenotypic analysis. Researchers have designed a bridged microfluidic chip that permits worms to move freely during experiments and established an economical monitoring module for long-term tracking and periodic imaging. Furthermore, they have developed an automated image analysis algorithm to automatically determine worm bending frequency. The platform was subsequently utilized for long-term toxicological assessments of the organophosphate pesticide and environmental pollutants, as well as to investigate the effects of tobacco components on various neuronal pathways within the worm. Results indicated that the platform can effectively evaluate the neurotoxic and developmental impacts of chemicals, offering scientific evidence for safety evaluations of tobacco products. The automated microfluidic worm analysis platform holds significant potential for applications in tobacco product safety

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acid; 5-HT, serotonin; MEMS, Micro-Electro-Mechanical Systems; PDMS, Polydimethylsiloxane; NGM, nematode growth medium; 5-FUdR, 5-Fluoro-20-deoxyuridine; DMSO, dimethyl sulfoxide; GFP, Green fluorescent protein; SE, standard error; PBDEs, polybrominated diphenyl ethers;

assessment, drug screening, and neurotoxicology research, contributing to human health and industry advancement.

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

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

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

86 strides in the automation of toxicity testing assays using worms, there remains
87 considerable room for further development.

88 In this study, we presented a microfluidic platform designed for the automated
89 assessment of chemical toxicity using *C. elegans*, which consists of worm cultivation,
90 drug delivery, regular monitoring and automated phenotype analysis. Initially, we
91 designed a bridge-like microfluidic chip for worm culturing, which allows worms to
92 move freely form larval stage to adult. Additionally, we constructed an economical
93 monitoring module capable of longitudinal tracking and periodic image capture of
94 the worms. . The bend frequency of worms can be automatically determined using
95 an image analysis algorithm previously developed in our study[20]. Subsequently, we
96 carried out a whole-life-covered worm culture (17 days) and toxicity evaluation of
97 monocrotophos (MCP) and 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) using this
98 microfluidic platform, to validate the capacity of the platform for long-term toxicity
99 testing assays on worms. Finally, we applied this microfluidic platform to examine the
100 neurotoxic effects of various tobacco constituents. Since *C. elegans* share similar
101 nervous system and neurotransmitters [eg. acetyl choline (ACh), dopamine (DA),
102 gamma aminobutyric acid (GABA), serotonin (5-HT)] with mammals, it is suitable for
103 investigating the effects of chemicals on nervous system. Consequently, we also
104 investigated the influence of tobacco constituents on different types of worm
105 neurons, which may offer insights relevant to tobacco regulation.

106 **Materials and methods**

107 **Microfluidic device fabrication**

108 The microfluidic chip mold was developed by MEMS (Micro-Electro-Mechanical
109 Systems) process, including metal sputtering and electroplating[21]. Liquid PDMS
110 mixture (Sylgard 184 Silicone Elastomer Base : Curing Agent = 10:1) was cast on a
111 silicon mold and cured overnight at 70 °C to fabricate a PDMS chip. The inlet and
112 outlet were punched with needles. Finally, the PDMS chip was bonded to a clean
113 glass by air plasma treatment and stored overnight at 135 °C. For more details, see
114 supplementary materials.

115 **Worm strains and maintenance**

116 The *C. elegans* strains used in this study were wild-type Bristol N2, NW1229
117 [*F25B3.3*::GFP *(evIs111)*], EG1285 [*unc-47*p::GFP *(oxIs12)*], LX929 [*unc-17*::GFP
118 *(vsIs48)*] and GR1366 [*tph-1*::GFP *(mgIs42)*], which were obtained from the
119 Caenorhabditis Genetics Center (St. Paul, Minnesota, USA). Nematodes were
120 cultured in the nematode growth medium (NGM, 17 g/L Agar B, 3 g/L NaCl, 2.5 g/L
121 peptone, 25 mM KPO_4 buffer pH 6.0, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mg/L cholesterol
122 in ethanol) or in M9 buffer (17 mM K_2HPO_4, 42 mM Na_2HPO_4, 85 mM NaCl, 1 mM
123 $MgSO_4$) with the *E. coli* strain OP50 as nutrient at 20 °C. To obtain a synchronized
124 nematode population, a bleach-sodium hydroxide mixture (0.5 M NaOH and 1% (v/v)
125 NaClO) was used with an established method[22].

126 **Chemicals**

127 5-Fluoro-20-deoxyuridine (5-FUdR) and dimethyl sulfoxide (DMSO) were

128 purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Monocrotophos (MCP) was
129 purchased from LGC Science (ShangHai Ltd) and dissolved in ultrapure water.
130 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) was obtained from Matrix Scientific
131 (Columbia, South Carolina, USA) and dissolved in DMSO. L511A, X6145A, Fla-1, Cig-1
132 and Cig-7 stocks were provided by Joint Institute of Tobaccl and Health. The stock
133 solutions were subsequently diluted with M9 buffer to obtain the working solutions.
134 Ultrapure water was produced using a Milli-Q system (Millipore, Bedford, MA, USA).

135 **Lifespan assays**

136 The lifespan assay was performed on age-synchronized adult worms (48 h after
137 hatching) in 96-well plates and PDMS microfluidic chips. Worms were cultured in
138 culturing medium (*E. coli* OP50:M9 buffer 1:200, v/v) and 5-FUdR (final concentration
139 of 20 mg/mL) was added to inhibit reproduction. In the PDMS microfluidic chips,
140 culturing medium was refreshed every day to provide the worms with sufficient
141 nutrients. The worms were stimulated with an acupuncture needle (96-well plates)
142 or by increasing the flow rate (microfluidic chips) every 24 h. If a worm did not
143 respond to any stimulation within 1 min, it was considered dead.

144 **Developmental toxicity assays**

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

150 plates, and the body length of worms were calculated manually.

151 **Neurotoxicity assays**

152 Synchronized N2 worms in L4 stage (feeding for 48 h after hatching) were treated
153 with chemicals for 24 h in this assay, and 20 mg/mL 5-FUdR was added to inhibit
154 reproduction. The concentrations of MCP were 0, 250, 500 μM and 1 mM, BDE-47
155 were 0, 3, 10 and 30 μg/ml. The concentrations of L551A, X6145A, Fla-1, Cig-1 and
156 Cig-7 were 0, 1/100000, 1/10000, 1/1000 and 1/200 of the stock solutions. After
157 treatment, worms were rinsed with M9 and recorded by monitoring module. The
158 bend frequencies of worms were calculated with the image analysis module.

159 **Neuronal degeneration assays**

160 Green fluorescent protein (GFP) transgenic strains NW1229, BZ555, LX929 and
161 GR1366 were used to relatively visualize pan-neurons, dopaminergic neurons,
162 cholinergic neurons and serotonergic neurons of worms. Synchronized worms were
163 treated with L551A, X6145A, Fla-1, Cig-1 and Cig-7 (0, 1/100000, 1/10000, 1/1000
164 and 1/200 of the stock solutions) from L1 to L4 stage (48 h). After treatment, worms
165 were immobilized with 20mM levamisole and scanned by Confocal Quantitative
166 Image Cytometer CQ1 (Yokogawa China Co,. Ltd.) at a 10× objective. The fluorescent
167 intensity of worms was quantified with ImageJ software.

168 **Statistical analysis**

169 GraphPad Prism version 8.0 (GraphPad Software, La Jolla, California, USA) and IBM
170 SPSS Statistics version 25 (SPSS, Chicago, IL, USA) were used for statistical analysis. All
171 data are expressed as mean ± standard error (SE). The log-rank test was used to

172 compare the survival curves of worms cultured on PDMS microfluidic chips and
173 micro-well plates. One-way analysis of variance (one-way ANOVA) followed by the
174 *Dunnett t*-test were used to determine the differences between the treatment
175 groups and control group. A value of $P < 0.05$ was considered statistically significant.

176 **Results**

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

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

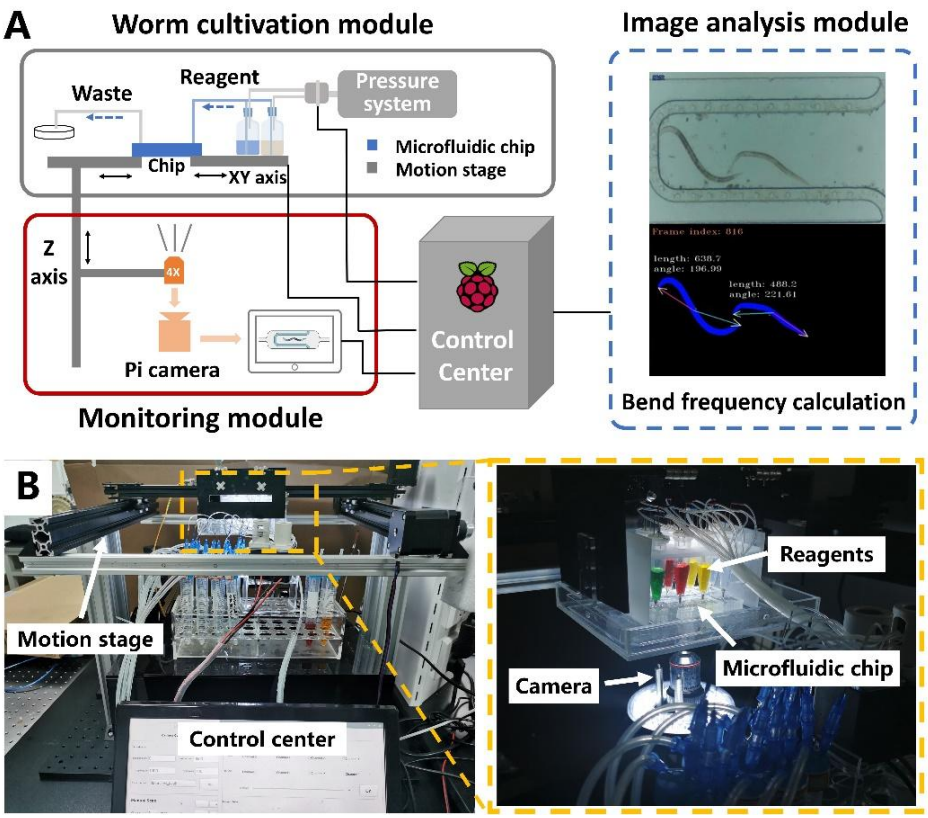


Fig.1. Schematic of the microfluidic platform. (A) Diagram of the designed platform consisting of the "worm cultivation module" "monitoring module" and "image analysis module". **(B)** Physical photograph of the whole platform. Inside the yellow dotted box (graph on the right) is magnified photograph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

205 can meet the above requirements (see supplementary materials). To prevent the
206 “bridge deck” from collapsing, pillars were set as support structures at intervals of
207 100 μm. The designed height of the chambers is 40 μm (approximately equal to the
208 diameter of adult worms) to avoid overlapping more than two worms along the
209 *Z*-axis, which is beneficial for observation and automated analysis. Five inlets and
210 outlets are merged on each chip (Fig. 2C).

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

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

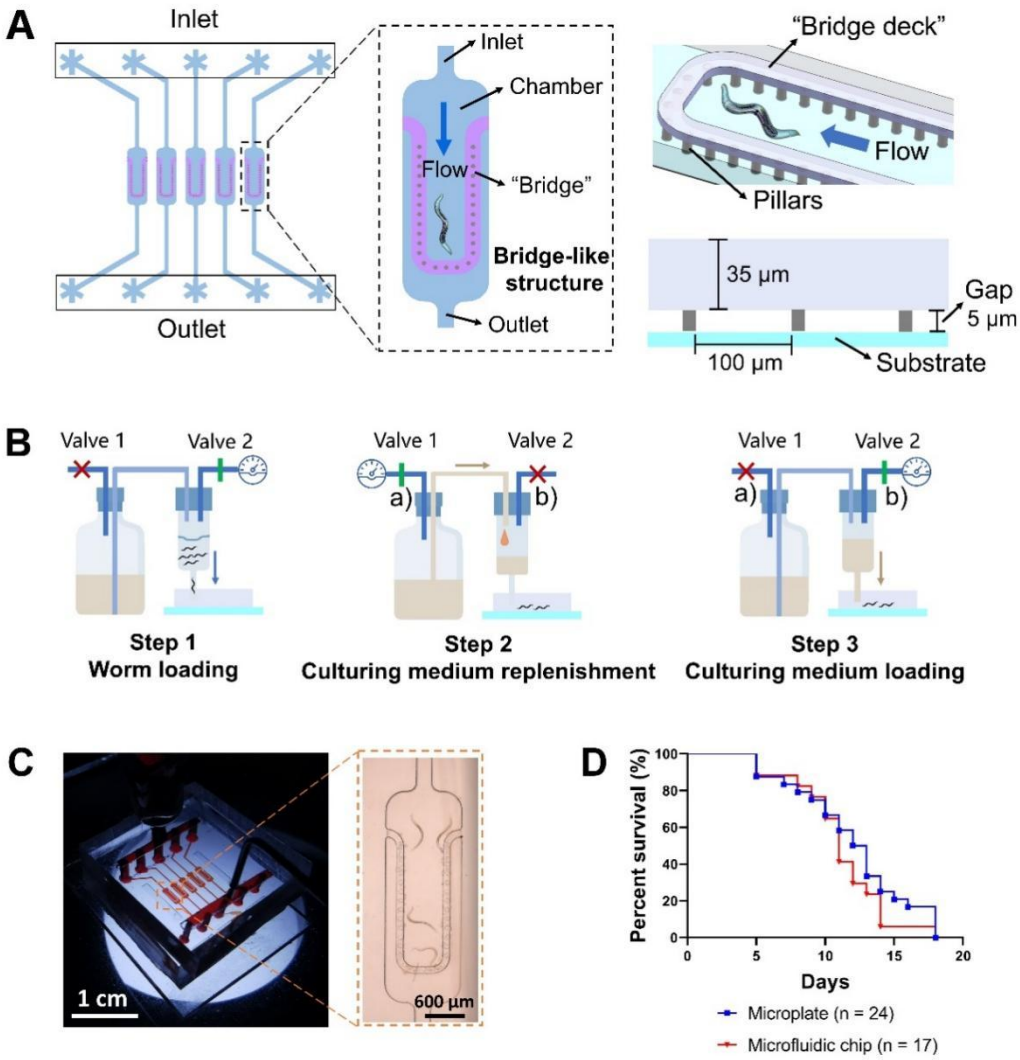


Fig.2. Design and operation of the microfluidic chip. (A) Five structures are paralleled in one chip. The inlet and outlet are 150 μm wide and the chamber is 40 μm in height. The "bridge deck" has a thickness of 35 μm and a 5 μm gap underneath to prevent the worms from escaping while allowing the culturing 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

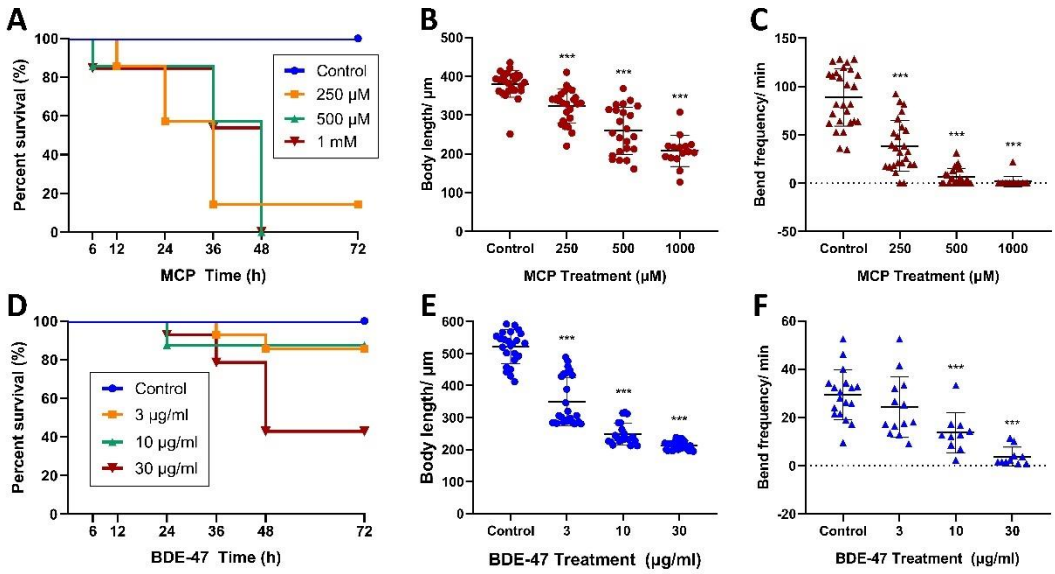
The monitoring module is a low-cost optical imaging system that can ensure stability for the long-term culturing of worms. We built a low-cost three-axis displacement platform using aluminum profiles. Cost of the monitoring module was

240 less than US$ 200. The displacement platform was controlled by an Arduino circuit
241 connected to a Raspberry Pi CPU. The GRBL code library was pre-written in Arduino.
242 The stepper motor and its driver were connected. The Raspberry Pi could send the G
243 code to Arduino to control the motion of the stage. The motion accuracy of this
244 module can easily reach 10 μm. We used a 4× lens and a low-cost Raspberry Pi CCD
245 for imaging.
246
247 **Automated image analysis module for phenotype analysis of worms**
248 With the large amount of image data generated in toxicity testing assays,
249 automated data processing can significantly improve the efficiency of the experiment.
250 Therefore, we used the automated image analysis algorithm developed in our
251 previous study[20] to analyze the body length and bend frequency of worms. We used
252 deep learning to extract worm contours from videos that contain chamber structure
253 and impurities in the culture environment. The following process is described as
254 before.
255
256 **Toxicity evaluation of MCP and BDE-47 on the platform**
257 Based on this microfluidic platform, we chose MCP and BDE-47 to demonstrate
258 the feasibility of the platform for automated toxicity testing of exogenous chemicals.
259 MCP is an organophosphorus pesticide widely used in agricultural production, the
260 residues of which may be detected frequently in food. As a cholinesterase inhibitor
261 acting on an acetylcholine receptor, MCP is highly neurotoxic[23]. As a kind of
262 commercial flame retardant, BDE-47 is the most abundant and prevalent

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

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

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



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

279 frequency of worms treated with MCP. (D-F) The survival curve, body length and bend frequency of worms
280 treated with BDE-47.
281

282 **Neurotoxicity testing of tobacco constituents on the platform**

283 **Bend frequency**

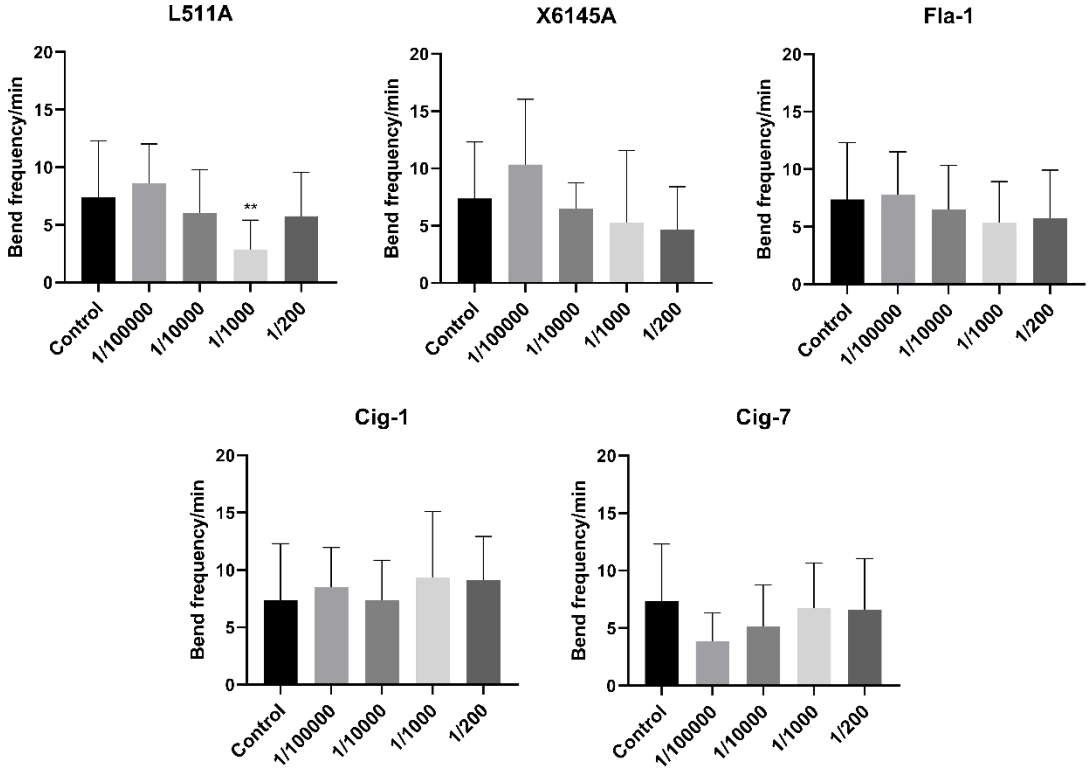
284 We treated worms with two commercially available flavor mixtures (codenamed
285 L511A and X6145A), two cigarette brands of smoke Cambridge filter collections
286 (codenamed Cig-1 and Cig-7), and a single tobacco component (solanone,
287 codenamed Fla-1) to investigate their effects on bend frequency of worms. The
288 experimental design covered four dose groups of 1/100000, 1/10000, 1/1000 and
289 1/200 of the stock solution, and 0.5% DMSO was set up as the solvent control.

290 As shown in Fig. 4, after exposing wild-type nematode N2 to L511A in each dose
291 group, its bend frequency per minute was basically consistent with that of the
292 control group. In the 1/100000 dose group, the bend frequency of nematodes
293 increased slightly, but there was no significant difference compared with the solvent
294 control. However, the bend frequency of the higher dose group decreased, especially
295 the 1/1000 dose group decreased by about 50 % and there was a statistical
296 difference compared with the solvent control. For X6145A and Fla-1, the bend
297 frequency of worms also showed a trend of slightly increasing in low-dose group and
298 decreasing in high-dose group. This suggests that these two flavoring substances and
299 tobacco component may produce neuroexcitatory effects at low doses in nematodes,
300 but may exhibit neurotoxicity at high doses.

301 On the other hand, after exposure to Cig-1, the bend frequency of worms
302 increased with the increase of dose, but there was no statistical difference compared

303 with the solvent control. There was a dose-dependent increase in the frequency of
304 worm bend in each dose group of Cig-7, but there was no significant difference with
305 the control group.

306 Combining the above results, we can draw the following conclusions: cigarette
307 Cig-1 may have nerve excitatory effect on nematode and flavor mixtures L511A and
308 X6145A may produce neuroexcitatory effects at low doses, but cause neurotoxicity to
309 nematodes at high doses.



310

311 Fig. 4. Bend frequency of worms treated with five tobacco constituents.

312

313 **Neuronal degeneration**

314 **Pan-neuron**

315 Nematode strain NW1229, which expressed GFP in pan-neuron, was exposed to 5
316 tobacco samples (1/100000, 1/10000, 1/1000, 1/200) for 48 h from L1 stage, and

317 then the fluorescence intensity of nematode was analyzed.

318 As shown in Fig. 5A, the overall fluorescence intensity of all GFP-labeled neurons
319 in worms decreased with the increase of L511A exposure concentration, suggesting
320 that exposure to flavor mixture L511A caused pan-neuronal damage to worms.
321 Quantitative statistical analysis of relative fluorescence intensity showed that after
322 exposure to L511A for 48 h, compared with the control group, the relative
323 fluorescence intensity of the 1/200 dose group was reduced by about 33 %, with
324 significant statistical difference. In addition, fluorescence images showed that the
325 body length of nematodes after exposure to L511A was significantly shorter than
326 that of the control group, suggesting that the flavoring substance L511A may have
327 growth and developmental toxicity.

328 However, as shown in Fig. 5B-E, pan-neuron fluorescence intensity of flavorings
329 X6145A, tobacco single component Fla-1, cigarette samples Cig-1 and Cig-7 showed
330 no significant changes in each dose group, indicating that pan-neuron damage was
331 not caused by X6145A, Fla-1, Cig-1 and Cig-7.

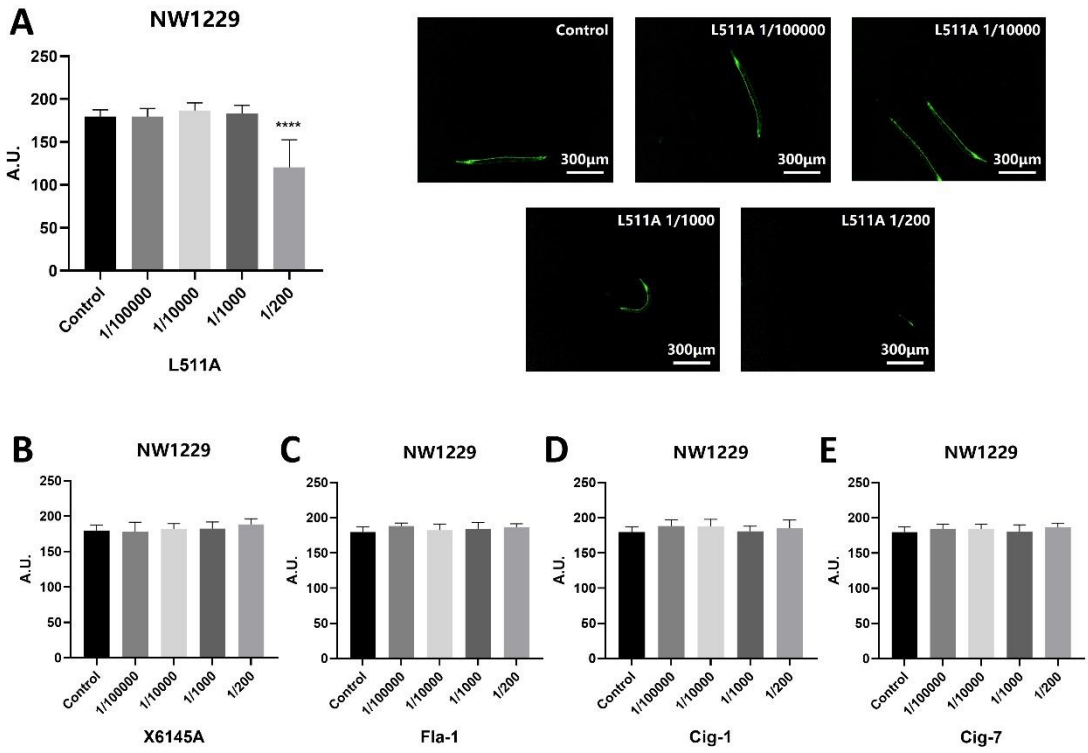


Fig. 5. Fluorescence intensity of NW1229 worms treated with five tobacco constituents for 48 h.

Dopaminergic neuron

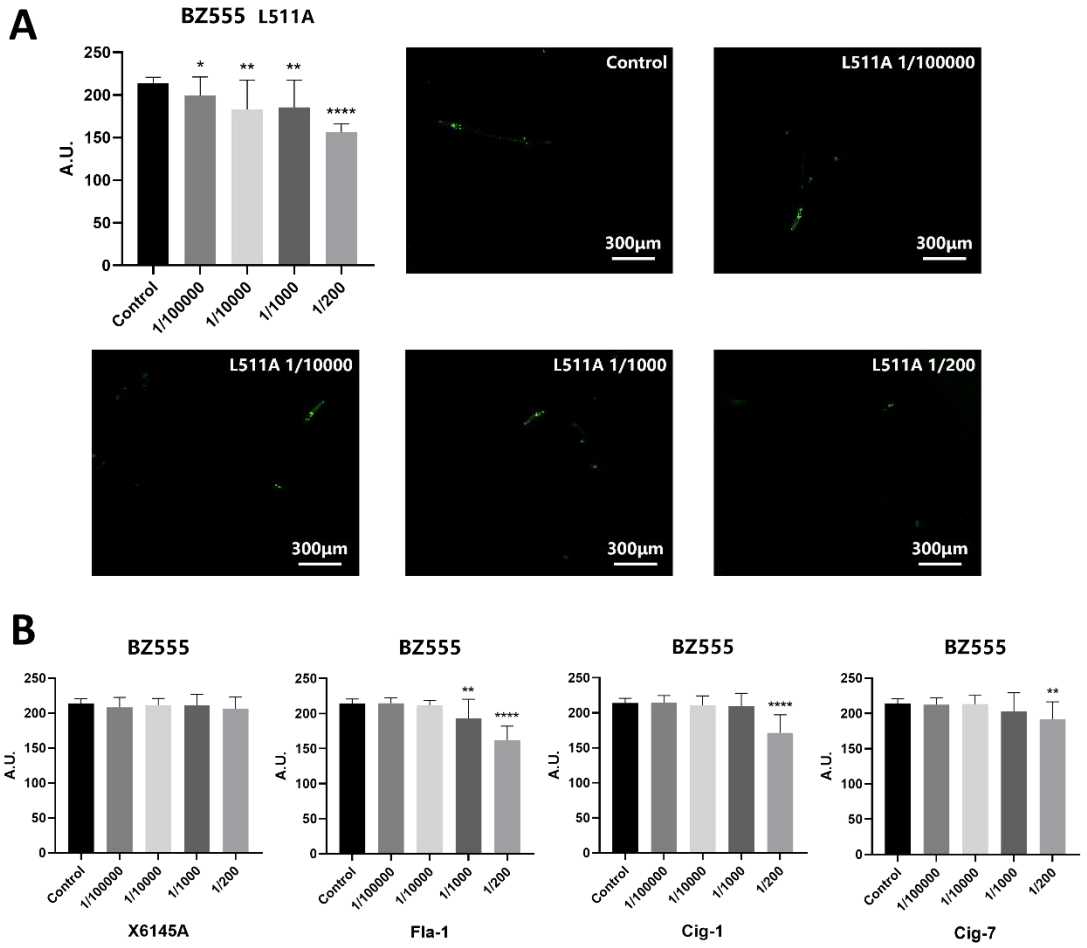
The visualization nematode dopaminergic neuron strain BZ555 *dat-1*p::GFP was exposed to 5 tobacco samples (1/100000, 1/10000, 1/1000, 1/200) for 48 h from L1 stage, and the fluorescence intensity of nematode was analyzed.

As shown in Fig. 6A, the fluorescence intensity in worms decreased with the increase of L511A exposure concentration, suggesting that exposure to L511A caused damage to dopaminergic neurons in C. elegans. Quantitative statistical analysis showed that after exposure to L511A for 48 h, compared with the control group, the total body relative fluorescence intensity of each dose group decreased by 7 % (1/100000), 14.5 % (1/10000), 13.6 % (1/1000) and 27.1 % (1/200), respectively, with statistical differences. In addition, fluorescence images also showed that the body length of nematodes exposed to L511A in the high-dose group was shorter than that

347 in the control group, suggesting that L511A may have growth and developmental
348 toxicity.

349 As shown in Fig. 6B, 1/200 dose groups of Fla-1, Cig-1 and Cig-7 all caused a
350 significant decrease in the fluorescence intensity of nematode worms, which was
351 24.3 %, 20.1 % and 10.7 %, respectively. X6145A had no effect on the fluorescence
352 intensity of worms.

353 These results suggested that L511A, Fla-1 and cigarette samples Cig-1 and Cig-7
354 could cause dopaminergic neuron damage in worms.



355

356 Fig. 6. Fluorescence intensity of BZ555 worms treated with five tobacco constituents for 48 h.

357

358 **Cholinergic neuron**

359 Visualized nematode cholinergic neuron strain LX929 *unc-17*::GFP was exposed to
360 5 tobacco samples (1/100000, 1/10000, 1/1000, 1/200) for 48 h from L1 stage, and
361 then the fluorescence intensity of nematode was analyzed.

362 As shown in Fig. 7A, there was no significant change in the fluorescence intensity
363 of cholinergic neurons in each dose group after exposure to L511A for 48 hours. It
364 suggested that L511A did not cause significant damage to cholinergic neurons in
365 nematodes. Based on fluorescence images, it was found that the body length of
366 nematodes exposed to L511A in the high-dose group (1/200) was shorter than that in
367 the control group, suggesting that L511A may have growth and developmental
368 toxicity.

369 Fig. 7B showed that the fluorescence intensity of nematodes in each dose group of
370 X6145A, Fla-1, Cig-1 and Cig-7 had no significant changes, suggesting that these 5
371 samples did not cause damage to cholinergic neurons of nematodes.

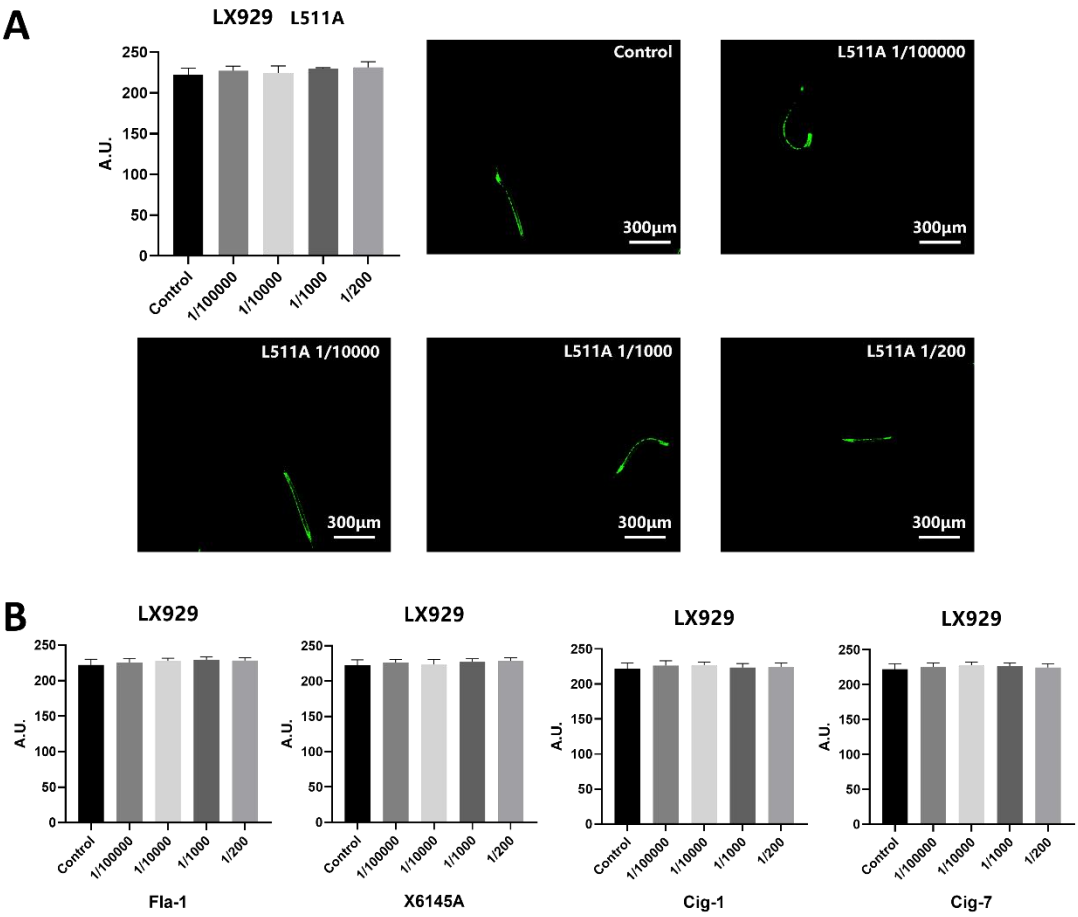


Fig. 7. Fluorescence intensity of LX929 worms treated with five tobacco samples for 48 h

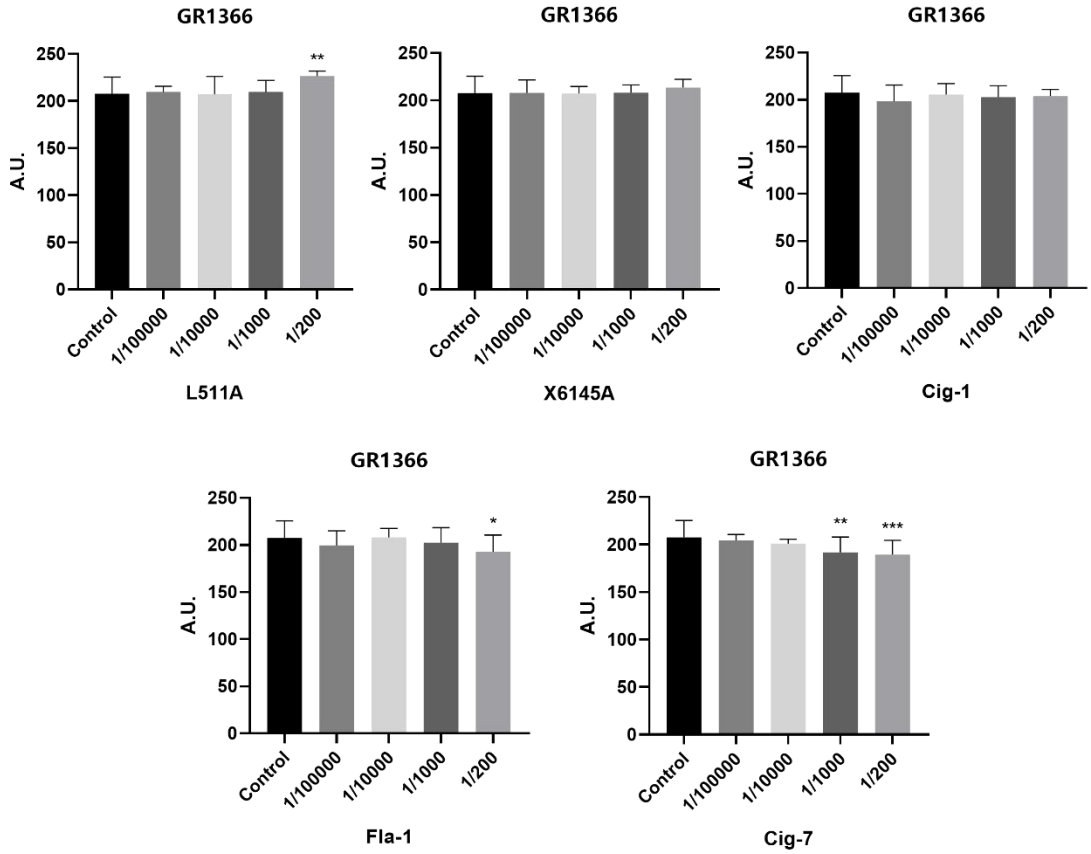
Serotonergic neuron

The visualization nematode serotonergic neuron strain GR1366 *tph-1*::GFP was exposed to 5 tobacco samples (1/100000, 1/10000, 1/1000, 1/200) for 48 h from L1 stage, and then the fluorescence intensity of nematode was analyzed.

As shown in Fig. 8, the fluorescence intensity of nematodes in the 1/200 dose group of Fla-1 decreased by 6.8 % compared with the control group, and the fluorescence intensity in the 1/1000 and 1/200 dose groups of Cig-7 decreased by 7.7 % and 8.7 %, respectively, with statistical differences.

It is suggested that tobacco single component Fla-1 and cigarette samples Cig-7

384 can damage serotonergic neurons of nematodes.



385

386 Fig. 8. Fluorescence intensity of GR1366 worms treated with five tobacco samples for 48 h

387

388 **Discussion**

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

397 of worm toxicity testing assays including worm culturing, monitoring and phenotype
398 analysis.

399 Toxicity assays require periodic rinsing and recording of the worms' phenotypes to
400 evaluate the toxicity of compounds with extended exposure times. In experiments
401 lasting more than 24 hours, the diet-containing culturing medium should also be
402 replenished regularly. Therefore, an automated liquid delivery and switching system
403 is needed. NemaLife has introduced a product that can realize programmable
404 regimens of feeding and reagent addition for toxicity testing
405 (https://www.nemalifeinc.com/infinity-system), but in the latest report, they still use
406 hand-held syringes to manually perform fluid manipulation[25]. Some researchers that
407 developed an open-source liquid handling device to realize reagent delivery from
408 large reservoirs to microfluidic chips were faced with the problems of complicated
409 motion platforms and extra on-chip backpressure designs[26]. Other researchers
410 applied rotary valves[27] or robotic devices[28] to liquid manipulation, but these fluid
411 switch and delivery systems are limited to complex structures with high-cost, thus
412 limiting the automation of chemical screening. In our study, we designed an
413 automated medium replenishment device to relive the tedious operation of manual
414 liquid change, the cost of which is low enough for laboratories.

415 Combined with microfluidic technology and nematode model, the neural effects of
416 tobacco products can be studied efficiently, quickly and accurately. Through the
417 platform, the exposure environment of different concentrations of tobacco products
418 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 tobacco products can be evaluated and the scientific basis for the safety assessment of tobacco products can be provided.

As people pay more and more attention to health problems, the safety requirements of tobacco products are getting higher and higher. The application of automatic microfluidic nematode analysis platform in the study of neural effects of tobacco products is still in the initial stage. With the continuous improvement of technology and in-depth research, the platform is expected to be more widely used and industrialized prospects in the future. On the one hand, it can be applied to the actual production to evaluate the safety of tobacco products quickly and accurately. At the same time, relevant testing equipment and kits can be developed to provide technical support for the production and regulation of tobacco products. On the other hand, it can be applied to other types of neurotoxicological studies, such as the neurological effects of drugs and the neurotoxicity of environmental pollutants. Drug screening and neurotoxicology research services based on the platform also can be developed to provide technical support and consulting services for pharmaceutical enterprises and research institutions.

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.

441 **Supplementary Information**

442 Microfluidic device fabrication

443 Determination of the gap height of the microfluidic chips

444 **Declarations**

445 **Availability of data and material**

446 The data used and analyzed in the present study are available from the

447 corresponding author on reasonable request.

448 **Competing interests**

449 The authors declare that they have no competing interests.

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