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

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Abbreviations[1]

Abstract

Humans are frequently exposed to a multitude of chemicals daily, necessitating efficient methods for rapidly assessing toxicity and potential health risks.

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

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

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

41 **Introduction**

42 With the development of technology, large numbers of newly registered chemicals
43 discharge into the environment every day (https://www.cas.org/). Humans are
44 consistently exposed to various chemicals, which demands efficient methods to
45 rapidly evaluate the toxicity and potential health risks to humans. Microfluidics has
46 shown promise as an intelligent tool for rapid testing of compounds due to its
47 flexibility to integrate with automation devices. Current researches based on *in vitro*
48 organ chips or microphysiological systems cannot simulate *in vivo* dynamic process in
49 human bodies[1, 2]. *Caenorhabditis elegans (C. elegans)*, a classic model organism, is a
50 powerful alternative *in vivo* assay system that has proven to be a useful tool in
51 toxicology studies[3, 4]. Recently, platforms combining microfluidic chip with *C.*
52 *elegans* are promising for toxicity testing of numerous chemicals[5, 6].
53 Numerous researchers have developed microfluidic platforms for short-term
54 observation of worms in acute toxicity testing assays[7, 8]. These platforms exhibit
55 significant benefits, including minimal reagent usage and the capacity for seamless
56 integration with automated systems. The microchambers can be configured to
57 prevent the overlap of worms along the Z-axis, thereby simplifying the process of
58 automated imaging and monitoring throughout the experiments. In chronic toxicity
59 studies, periodic observation and phenotype analysis are needed to provide
60 information on the cumulative effect of toxins, thus demanding long-term cultivation
61 with periodic drug delivery and medium replenishment. Sahand *et al*. developed a
62 platform for age synchronization and analyzing subtle aging phenotypes of worms,

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

83 In this study, we presented a microfluidic platform designed for the automated
84 assessment of chemical toxicity using *C. elegans*, which consists of worm cultivation,

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

101 **Materials and methods**

102 **Microfluidic device fabrication**

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

107 outlet were punched with needles. Finally, the PDMS chip was bonded to a clean
108 glass by air plasma treatment and stored overnight at 135 °C. For more details, see
109 supplementary materials.

110 **Worm strains and maintenance**

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

121 **Chemicals**

122 5-Fluoro-20-deoxyuridine (5-FUdR) and dimethyl sulfoxide (DMSO) were
123 purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Monocrotophos (MCP) was
124 purchased from LGC Science (ShangHai Ltd) and dissolved in ultrapure water.
125 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) was obtained from Matrix Scientific
126 (Columbia, South Carolina, USA) and dissolved in DMSO. L511A, X6145A, Fla-1, Cig-1
127 and Cig-7 stocks were provided by Joint Institute of Tobaccl and Health. The stock
128 solutions were subsequently diluted with M9 buffer to obtain the working solutions.

129 Ultrapure water was produced using a Milli-Q system (Millipore, Bedford, MA, USA).

130 **Lifespan assays**

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

139 **Developmental toxicity assays**

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

146 **Neurotoxicity assays**

147 Synchronized N2 worms in L4 stage (feeding for 48 h after hatching) were treated
148 with chemicals for 24 h in this assay, and 20 mg/mL 5-FUdR was added to inhibit
149 reproduction. The concentrations of MCP were 0, 250, 500 μM and 1 mM, BDE-47
150 were 0, 3, 10 and 30 μg/ml. The concentrations of L551A, X6145A, Fla-1, Cig-1 and

151 Cig-7 were 0, 1/100000, 1/10000, 1/1000 and 1/200 of the stock solutions. After
152 treatment, worms were rinsed with M9 and recorded by monitoring module. The
153 bend frequencies of worms were calculated with the image analysis module.

154 **Neuronal degeneration assays**

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

163 **Statistical analysis**

164 GraphPad Prism version 8.0 (GraphPad Software, La Jolla, California, USA) and IBM
165 SPSS Statistics version 25 (SPSS, Chicago, IL, USA) were used for statistical analysis. All
166 data are expressed as mean ± standard error (SE). The log-rank test was used to
167 compare the survival curves of worms cultured on PDMS microfluidic chips and
168 micro-well plates. One-way analysis of variance (one-way ANOVA) followed by the
169 *Dunnett t*-test were used to determine the differences between the treatment
170 groups and control group. A value of $P < 0.05$ was considered statistically significant.

171 **Results**

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

173 Since current microfluidic platforms for worm toxicity testing usually operate at a

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

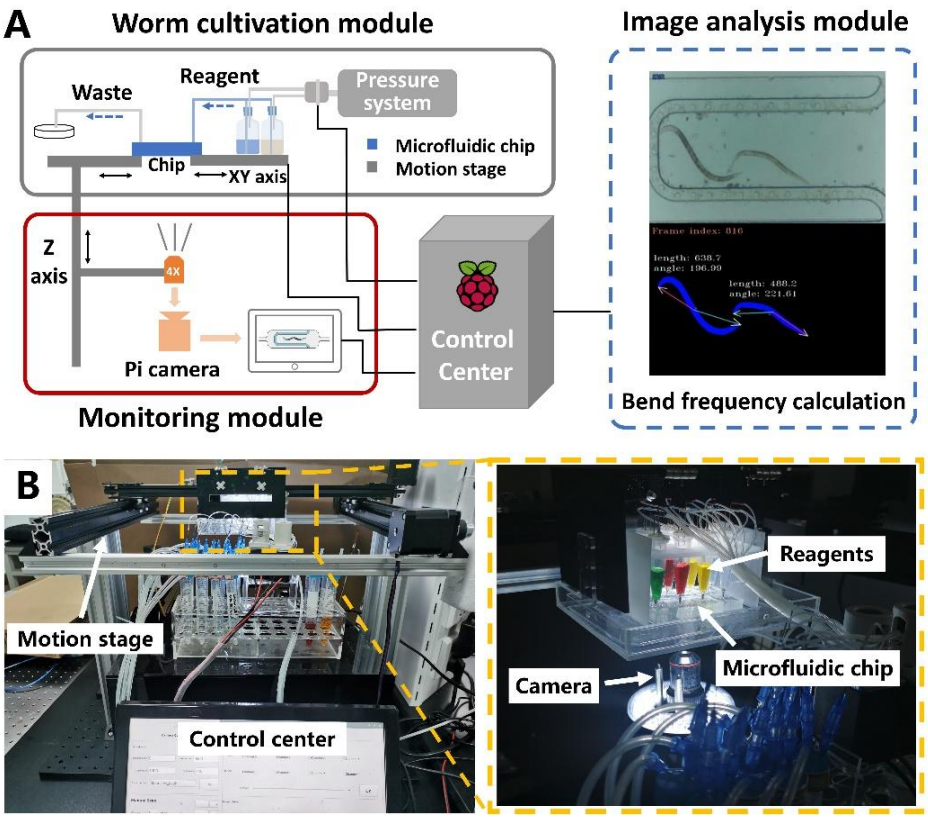


Fig.1. Schematic of the microfluidic platform. (A) Diagram of the designed platform consisting of the "worm cultivation module" "monitoring module" and "image analysis module". **(B)** Physical photograph of the whole

188 platform. Inside the yellow dotted box (graph on the right) is magnified photograph of "worm culturing module".

189

190 **The bridge-like microfluidic chip for worm cultivation**

191 We designed a bridge-like microfluidic chip that allows worms to move freely
192 during the experiment (Fig. 2A). Five parallel chambers were mounted on one chip,
193 and each chamber had a bridge-like structure to allow medium replacement and
194 prevent the worms from escaping. To meet the requirements of worm experiments
195 from the larval stages to adult, the gap should be designed to arrest the worms in L1
196 stage. Since L1 worms are approximately 10-15 μm wide and sufficiently flexible to
197 squeeze through narrow gaps, an ideal gap should not only arrest the worms but also
198 allow the medium to flow smoothly under relatively low pressure. We tested the
199 working conditions with gap sizes of 2, 5 and 10 μm and confirmed that a 5 μm gap
200 can meet the above requirements (see supplementary materials). To prevent the
201 "bridge deck" from collapsing, pillars were set as support structures at intervals of
202 100 μm. The designed height of the chambers is 40 μm (approximately equal to the
203 diameter of adult worms) to avoid overlapping more than two worms along the
204 *Z*-axis, which is beneficial for observation and automated analysis. Five inlets and
205 outlets are merged on each chip (Fig. 2C).

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

210 To validate the biocompatibility of this microfluidic platform, we cultured worms in

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

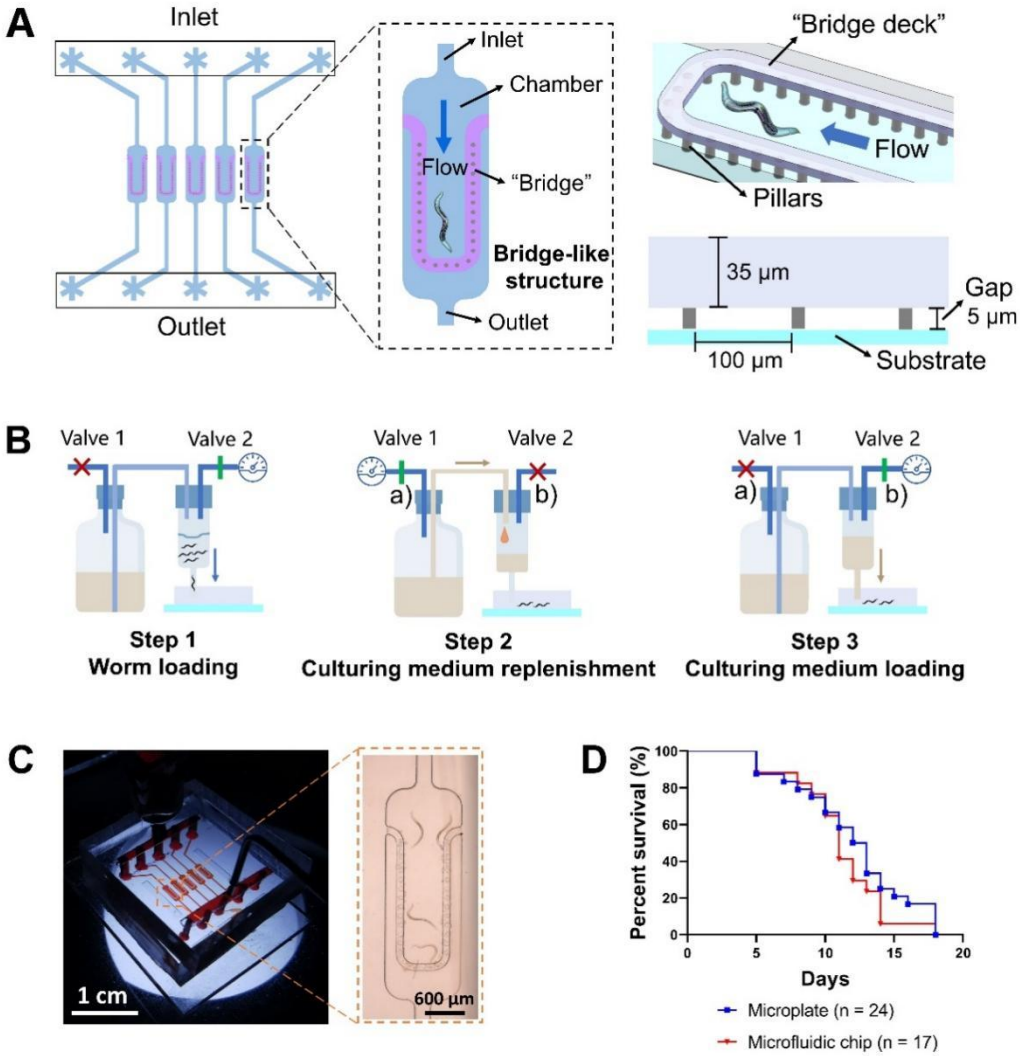


Fig.2. Design and operation of the microfluidic chip. (A) Five structures are paralleled in one chip. The inlet and outlet are 150 μm wide and the chamber is 40 μm in height. The "bridge deck" has a thickness of 35 μm and a 5 μm gap underneath to prevent the worms from escaping while allowing the culturing 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 less than US$ 200. The displacement platform was controlled by an Arduino circuit connected to a Raspberry Pi CPU. The GRBL code library was pre-written in Arduino. The stepper motor and its driver were connected. The Raspberry Pi could send the G code to Arduino to control the motion of the stage. The motion accuracy of this module can easily reach 10 μm. We used a 4× lens and a low-cost Raspberry Pi CCD for imaging.

Automated image analysis module for phenotype analysis of worms

With the large amount of image data generated in toxicity testing assays, automated data processing can significantly improve the efficiency of the experiment. Therefore, we used the automated image analysis algorithm developed in our previous study[20] to analyze the body length and bend frequency of worms. We used deep learning to extract worm contours from videos that contain chamber structure

248 and impurities in the culture environment. The following process is described as
249 before.
250
251 **Toxicity evaluation of MCP and BDE-47 on the platform**
252 Based on this microfluidic platform, we chose MCP and BDE-47 to demonstrate
253 the feasibility of the platform for automated toxicity testing of exogenous chemicals.
254 MCP is an organophosphorus pesticide widely used in agricultural production, the
255 residues of which may be detected frequently in food. As a cholinesterase inhibitor
256 acting on an acetylcholine receptor, MCP is highly neurotoxic[23]. As a kind of
257 commercial flame retardant, BDE-47 is the most abundant and prevalent
258 polybrominated diphenyl ethers (PBDEs) congeners in the environment, which has
259 been known to introduce neurodevelopmental toxicity[24].
260 We treated N2 worms with MCP and BDE-47 for 72 h and evaluated the body
261 length and bend frequency of worms at 24 h. Fig. 3A showed that treatment of MCP
262 and BDE-47 induced significant general toxicity in worms on this platform. The body
263 length was used as the toxic endpoints to evaluate the development toxicity. As
264 shown in Fig. 3B&E, MCP and BDE-47 treatment caused dose-dependent decreases
265 ($p < 0.001$), implying evident developmental toxicity to nematodes. Fig. 3C&F showed
266 that the bend frequency of worms decreased significantly ($p < 0.001$) with the
267 increase in dose after 24 h treatment.
268 The above results demonstrated that this platform can be applied to automated
269 toxicity testing of numerous chemicals that may cause neurotoxicity and
270 development toxicity. In the future, more phenotypes such as brood size and cell

apoptosis, can be measured to expand the scope of toxicity evaluation.

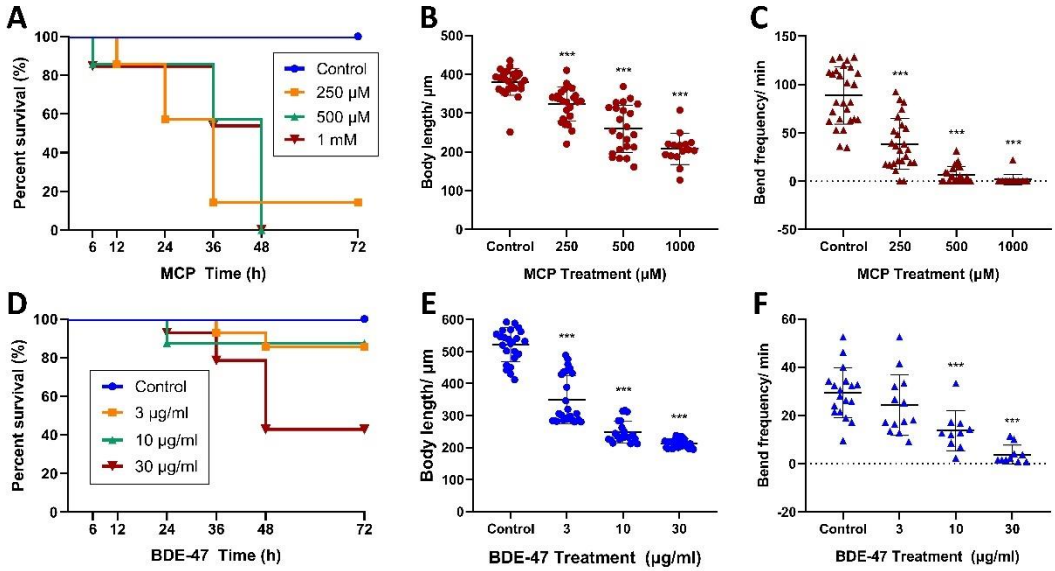


Fig. 3. Toxicity evaluation of MCP and BDE-47 on the platform. (A-C) The survival curve, body length and bend frequency of worms treated with MCP. (D-F) The survival curve, body length and bend frequency of worms treated with BDE-47.

Neurotoxicity testing of tobacco constituents on the platform

Bend frequency

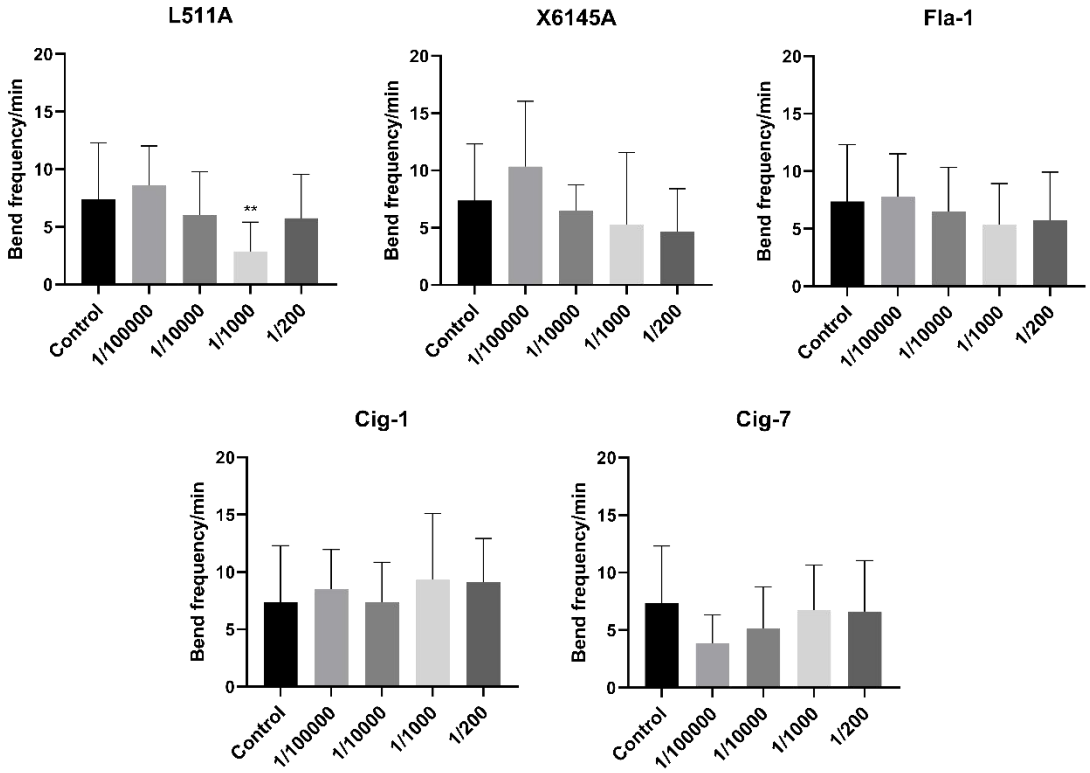
We treated worms with two commercially available flavor mixtures (codenamed L511A and X6145A), two cigarette brands of smoke Cambridge filter collections (codenamed Cig-1 and Cig-7), and a single tobacco component (solanone, codenamed Fla-1) to investigate their effects on bend frequency of worms. The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

As shown in Fig. 4, after exposing wild-type nematode N2 to L511A in each dose group, its bend frequency per minute was basically consistent with that of the control group. In the 1/100000 dose group, the bend frequency of nematodes increased slightly, but there was no significant difference compared with the solvent

289 control. However, the bend frequency of the higher dose group decreased, especially
290 the 1/1000 dose group decreased by about 50 % and there was a statistical
291 difference compared with the solvent control. For X6145A and Fla-1, the bend
292 frequency of worms also showed a trend of slightly increasing in low-dose group and
293 decreasing in high-dose group. This suggests that these two flavoring substances and
294 tobacco component may produce neuroexcitatory effects at low doses in nematodes,
295 but may exhibit neurotoxicity at high doses.

296 On the other hand, after exposure to Cig-1, the bend frequency of worms
297 increased with the increase of dose, but there was no statistical difference compared
298 with the solvent control. There was a dose-dependent increase in the frequency of
299 worm bend in each dose group of Cig-7, but there was no significant difference with
300 the control group.

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



305

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

307

308 **Neuronal degeneration**

309 **Pan-neuron**

310 Nematode strain NW1229, which expressed GFP in pan-neuron, was exposed to 5

311 tobacco samples (1/100000, 1/10000, 1/1000, 1/200) for 48 h from L1 stage, and

312 then the fluorescence intensity of nematode was analyzed.

313 As shown in Fig. 5A, the overall fluorescence intensity of all GFP-labeled neurons

314 in worms decreased with the increase of L511A exposure concentration, suggesting

315 that exposure to flavor mixture L511A caused pan-neuronal damage to worms.

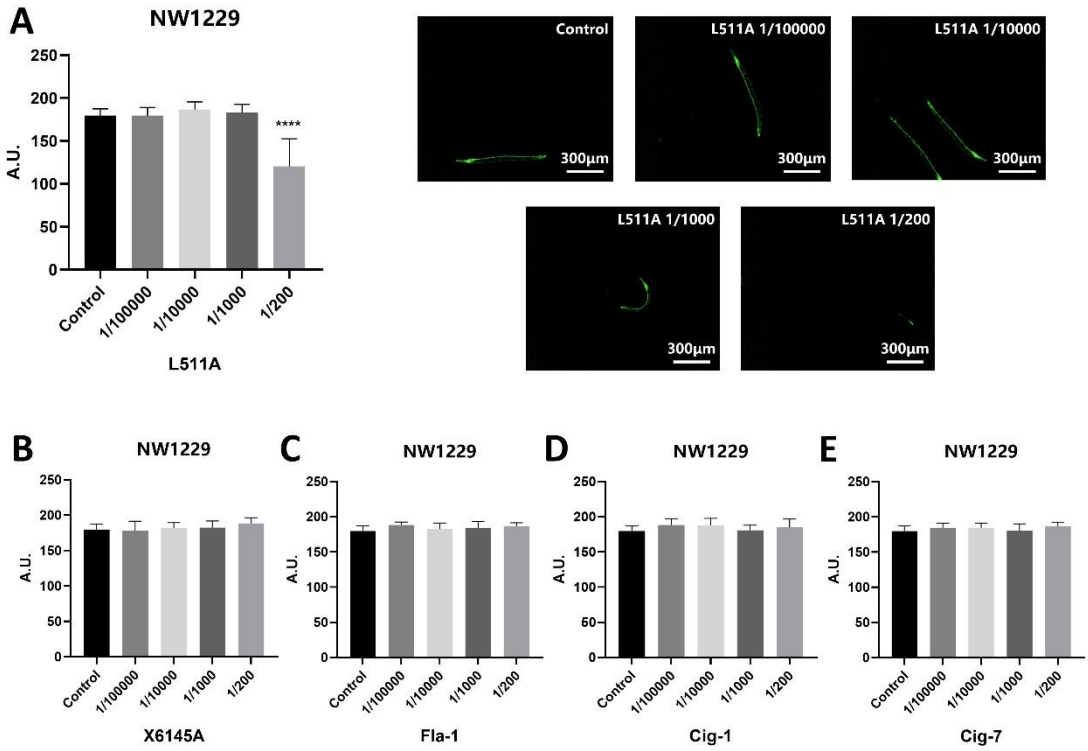
316 Quantitative statistical analysis of relative fluorescence intensity showed that after

317 exposure to L511A for 48 h, compared with the control group, the relative

318 fluorescence intensity of the 1/200 dose group was reduced by about 33 %, with

319 significant statistical difference. In addition, fluorescence images showed that the
320 body length of nematodes after exposure to L511A was significantly shorter than
321 that of the control group, suggesting that the flavoring substance L511A may have
322 growth and developmental toxicity.

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



327
328 Fig. 5. Fluorescence intensity of NW1229 worms treated with five tobacco constituents for 48 h.
329

330 **Dopaminergic neuron**

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

334 As shown in Fig. 6A, the fluorescence intensity in worms decreased with the
335 increase of L511A exposure concentration, suggesting that exposure to L511A caused
336 damage to dopaminergic neurons in C. elegans. Quantitative statistical analysis
337 showed that after exposure to L511A for 48 h, compared with the control group, the
338 total body relative fluorescence intensity of each dose group decreased by 7 %
339 (1/100000), 14.5 % (1/10000), 13.6 % (1/1000) and 27.1 % (1/200), respectively, with
340 statistical differences. In addition, fluorescence images also showed that the body
341 length of nematodes exposed to L511A in the high-dose group was shorter than that
342 in the control group, suggesting that L511A may have growth and developmental
343 toxicity.

344 As shown in Fig. 6B, 1/200 dose groups of Fla-1, Cig-1 and Cig-7 all caused a
345 significant decrease in the fluorescence intensity of nematode worms, which was
346 24.3 %, 20.1 % and 10.7 %, respectively. X6145A had no effect on the fluorescence
347 intensity of worms.

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

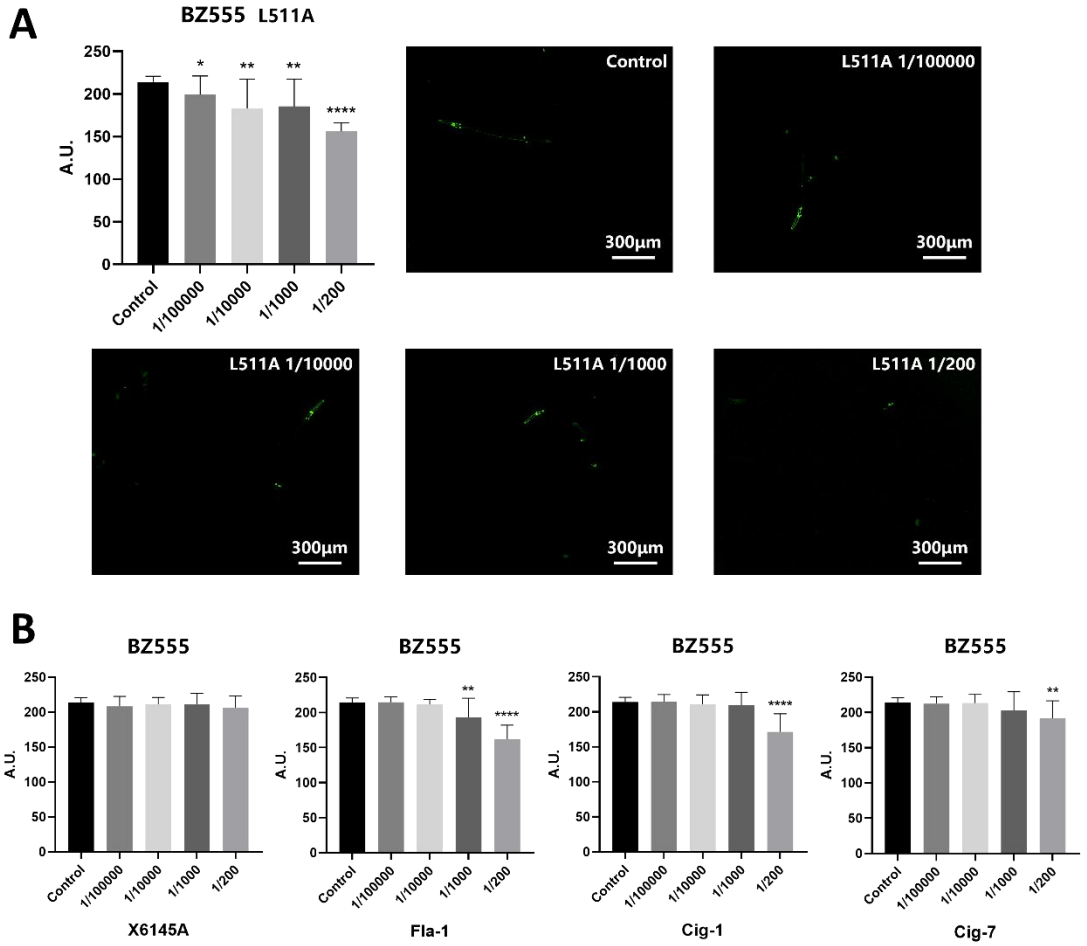


Fig. 6. Fluorescence intensity of BZ555 worms treated with five tobacco constituents for 48 h.

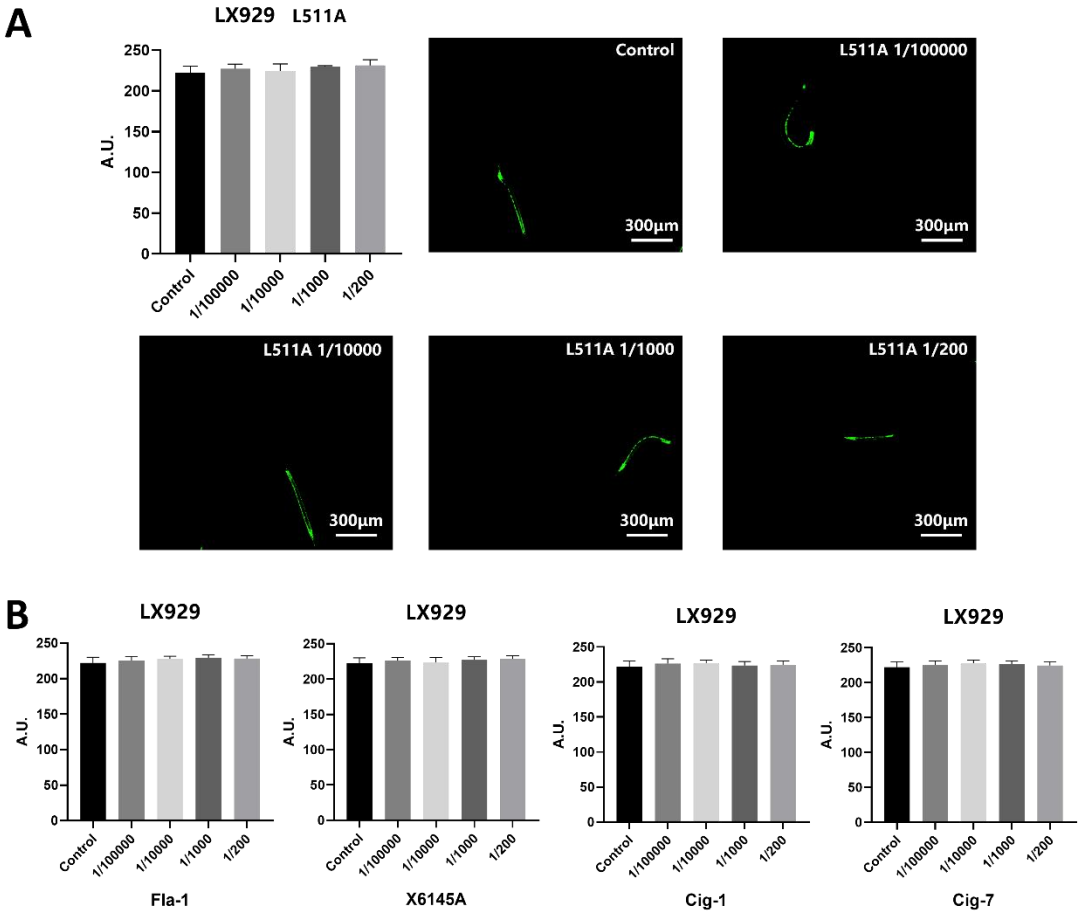
Cholinergic neuron

Visualized nematode cholinergic neuron strain LX929 *unc-17*::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. 7A, there was no significant change in the fluorescence intensity of cholinergic neurons in each dose group after exposure to L511A for 48 hours. It suggested that L511A did not cause significant damage to cholinergic neurons in nematodes. Based on fluorescence images, it was found that the body length of nematodes exposed to L511A in the high-dose group (1/200) was shorter than that in

362 the control group, suggesting that L511A may have growth and developmental
363 toxicity.

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



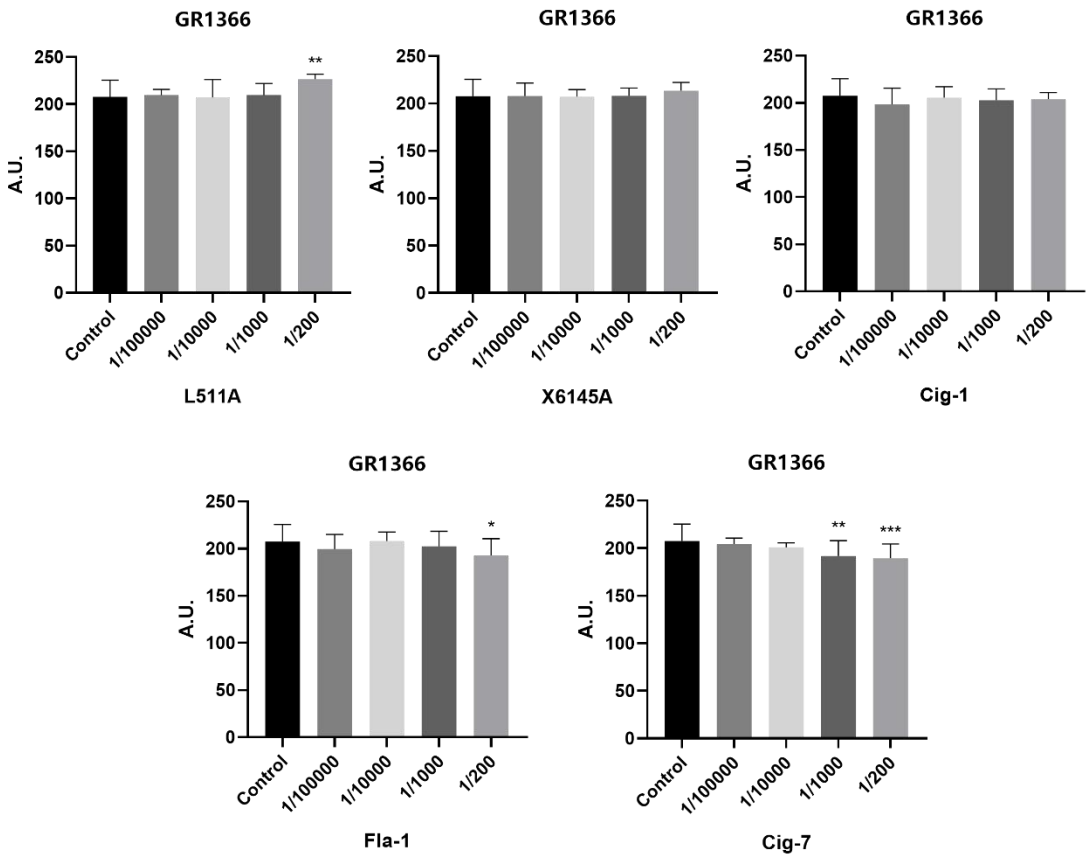
367
368 Fig. 7. Fluorescence intensity of LX929 worms treated with five tobacco samples for 48 h
369

370 **Serotonergic neuron**

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

374 As shown in Fig. 8, the fluorescence intensity of nematodes in the 1/200 dose
375 group of Fla-1 decreased by 6.8 % compared with the control group, and the
376 fluorescence intensity in the 1/1000 and 1/200 dose groups of Cig-7 decreased by
377 7.7 % and 8.7 %, respectively, with statistical differences.

378 It is suggested that tobacco single component Fla-1 and cigarette samples Cig-7
379 can damage serotonergic neurons of nematodes.



380

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

382

383 **Discussion**

384 With the development of science and technology, microfluidic technology is more
385 and more widely used in the biomedical field. Microfluidic technology has the
386 characteristics of high throughput, low consumption and precise control and is

387 widely used in cell culture, drug screening, disease diagnosis and other fields. As a
388 model organism, *C. elegans* has the characteristics of short life cycle, simple genome
389 and relatively simple nervous system and are ideal models for studying
390 neurotoxicology and neural effects of drugs. In this study, we introduced an
391 automated microfluidic platform based on *C. elegans*, which can realize automation
392 of worm toxicity testing assays including worm culturing, monitoring and phenotype
393 analysis.

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

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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