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TITLE:

Studying Neurobehavioral Effects of Environmental Pollutants on Zebrafish Larvae

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KEYWORDS:

neurobehavioral effects, environmental pollutants, zebrafish larvae, neurotoxicity, light stimuli, behavior test

SUMMARY:

A detailed experimental protocol is presented in this paper for the evaluation of neurobehavioral toxicity of environmental pollutants using a zebrafish larvae model, including the exposure process and tests for neurobehavioral indicators.

ABSTRACT:

Recent years more and more environmental pollutants have been proved neurotoxic, especially at the early development stages of organisms. Zebrafish larvae are a preeminent model for the neurobehavioral study of environmental pollutants. Here, a detailed experimental protocol is provided for the evaluation of the neurotoxicity of environmental pollutants using zebrafish larvae, including the collection of the embryos, the exposure process, neurobehavioral indicators, the test process, and data analysis. Also, the culture environment, exposure process, and experimental conditions are discussed to ensure the success of the assay. The protocol has been used in the development of psychopathic drugs, research on environmental neurotoxic pollutants, and can be optimized to make corresponding studies or be helpful for mechanistic studies. The protocol demonstrates a clear operation process for studying neurobehavioral

effects on zebrafish larvae and can reveal the effects of various neurotoxic substances or pollutants.

INTRODUCTION:

In recent years more and more environmental pollutants have been proved neurotoxic[1-4]. However, the assessment of neurotoxicity in vivo after exposure to environmental pollutants is not as easy as that of endocrine disruption or developmental toxicity. In addition, early exposure to pollutants, especially at environmentally relevant doses, has attracted increasing attention in toxicity studies[5-8].

Zebrafish is being established as an animal model fit for neurotoxicity studies during early development after exposure to environmental pollutants. Zebrafish are vertebrates that develop faster than other species after fertilization. The larvae do not need to be fed because the nutrients in the chorion are enough for sustain them for 7 days postfertilization (dpf)[9]. Larvae come out from the chorion at ~2 dpf and develop behaviors such as swimming and turning that can be observed, tracked, quantified, and analyzed automatically using behavior instruments[10-13] starting at 3–4 dpf[14-18]. In addition, high-throughput tests can also be realized by behavior instruments. Thus, zebrafish larvae are an outstanding model for the neurobehavioral study of environmental pollutants[19]. Here, a protocol is offered using high-throughput monitoring to study the neurobehavioral toxicity of environmental pollutants on zebrafish larvae under light stimuli.

Our lab has studied the neurobehavioral toxicity of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47)[20,21], 6'-Hydroxy/Methoxy-2,2',4,4'-tetrabromodiphenyl ether (6-OH/MeO-BDE-47)[22], deca-brominated diphenyl ether (BDE-209), lead, and commercial chlorinated paraffins[23] using the presented protocol. Many labs also use the protocol to study the neurobehavioral effects of other pollutants on larvae or adult fish[24-27]. This neurobehavioral protocol was used to help provide mechanistic support showing that low-dose exposure to bisphenol A and replacement bisphenol S induced premature hypothalamic neurogenesis in embryonic zebrafish[27]. In addition, some researchers optimized the protocol to perform corresponding studies. A recent study eliminated the toxicity of amyloid beta (Aβ) in an easy, high-throughput zebrafish model using casein-coated gold nanoparticles (βCas AuNPs). It showed that βCas AuNPs in systemic circulation translocated across the blood-brain barrier of zebrafish larvae and sequestered intracerebral Aβ42, eliciting toxicity in a nonspecific, chaperone-like manner, which was supported by behavioral pathology[28].

Locomotion, path angle, and social activity are three neurobehavioral indicators used to study the neurotoxicity effects of zebrafish larvae after exposure to pollutants in the presented protocol. Locomotion is measured by the swimming distance of larvae and can be damaged after exposure to pollutants. Path angle and social activity are more closely related with the function of the brain and the central nervous system[29]. The path angle refers to the angle of the path of animal motion relative to the swimming direction[30]. Eight angle classes from ~-180°–~+180° are set in the system. To simplify the comparison, six classes in the final outcome are defined as routine turns (-10° ~ 0°, 0° ~ +10°), average turns (-10° ~ -90°, +10° ~ +90°), and responsive turns (-180° ~ -90°, +90° ~ +180°) according to our previous studies[21,22]. Two-fish social activity is

fundamental of group shoaling behavior; here a distance of <0.5 cm between two larvae valid is defined as social contact.

The protocol presented here demonstrates a clear process for studying neurobehavioral effects on zebrafish larvae and provides a way to reveal the neurotoxicity effects of various substances or pollutants. The protocol will benefit researchers interested in studying the neurotoxicity of environmental pollutants.

PROTOCOL:

The protocol is in accordance with guidelines approved by the Animal Ethics Committee of Tongji University.

1. Zebrafish embryo collection

1.1. Put two pairs of healthy adult Tubingen zebrafish into the spawning box on the night before exposure, keeping the sex ratio at 1:1.

1.2. Remove the adult fish back to the system 30–60 min after daylight the next morning.

1.3. Remove the embryos out of the spawning box.

1.4. Rinse the embryos with system water.

1.5. Transfer the embryos into a glass Petri dish (9 cm diameter) with enough system water.

1.6. Observe the embryos under the microscope and select healthy embryos for later exposure.

NOTE: Healthy embryos are usually transparent with light golden color under the microscope. The unhealthy embryos are usually pale and clumped together as observed under the microscope.

2. Preparation before exposure

2.1. Prepare the Hanks' solution according to the guidelines of the zebrafish book[31].

NOTE: The Hanks' solution includes 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 4.2 mM $NaHCO_3$.

2.2. Dilute the Hanks' solution to 10% Hanks' solution using sterile water.

2.3. Add 1 mL of DMSO into 999 mL of 10% Hanks' solution to make a control solution of 10% Hanks' solution including 0.1% DMSO.

NOTE: The next steps use BDE-47 as an example of an exposure solution.

2.4. Dissolve 5 mg of the neat BDE-47 in 1 mL of 100% DMSO to make a standard exposure solution of 5 mg/mL.

2.5. Vortex the 5 mg/mL solution for 1 min to completely dissolve the BDE-47 in the DMSO.

2.6. Transfer 10 µL of the 5 mg/mL solution to a 12 mL brown glass bottle.

2.7. Add sterilized water to a final volume of 10 mL to make the concentration of BDE-47 exposure solution 5 mg/L and DMSO ratio at 0.1%, then vortex for 1 min.

2.8. Transfer 10 µL and 100 µL of the 5 mg/L solution into two 100 mL volumetric flasks respectively.

2.9. Add 10% Hanks' solution including 0.1% DMSO (prepared in step 2.3) to 100 mL to make the final concentrations of the BDE-47 exposure solutions 5 µg/L and 50 µg/L, respectively.

2.10. Transfer the solutions into 100 mL brown glass bottles and store them at 4 °C.

3. Exposure of embryos

3.1. Transfer ~50 embryos into each of the three glass Petri dishes (6 cm diameter) 3–5 hours post fertilization (hpf).

3.2. Use a 1 mL pipette tip to blot the system water around the embryos.

3.3. Use a pipette to transfer the control and two BDE-47 exposure solutions (control, 5 µg/L, 50 µg/L) into the three glass Petri dishes, respectively.

3.4. Shake the glass Petri dishes gently one by one to make the embryos disperse in the bottom of the plate.

3.5. Put the glass Petri dishes into the light incubator under 28.5 °C.

3.6. Renew half of the exposure solutions every 24 h until 5 dpf.

3.7. Check the dead embryos of every group on 1 dpf and 2 dpf and calculate the death rate.

3.8. Check the incubated embryos of every group on 2 dpf and 3 dpf and calculate the hatchability.

3.9. Check the deformity of the larvae every day after they come out from the chorion and calculate the deformity rate of every group.

NOTE: The deformity indicators include pericardial cyst, spinal curvature, tail curvature, among others factors[32].

4. Preparation for the behavior test

4.1. Prepare a 48 well microplate for the locomotion and path angle test and three 6 well microplates for the social activity test on the morning of 5 dpf.

4.2. Transfer 800 µL of exposure solution into every well of the 48 well microplate.

NOTE: Use 16 wells for every group (i.e., the control solution, 5 µg/L, and 50 µg/L group).

4.3. Use a 1 mL pipette tip to transfer 200 µL of exposure solution with one larva from the glass Petri dish into one well of the 48 well microplate.

4.4. Transfer 4 mL of exposure solution into every well of the 6 well microplate.

NOTE: Use one 6 well microplate for every group.

4.5. Use a 1 mL pipette tip to transfer 200 µL of exposure solution with two larvae into each well of the 6 well microplate.

NOTE: Every group has six repeating groups.

4.6. Make sure the temperature of the test room is 28 °C 2 h before the test.

NOTE: Behavioral tests are usually performed in the afternoon.

5. Behavioral test

5.1. Locomotion and path angle test

5.1.1. Click the launcher icon on the computer desk to open the software (see **Table of Materials**) that controls the high-throughput monitoring enclosure to start the program.

5.1.2. Choose the "**Tracking, Rotations, Path Angles**" module to enter the operating interface.

5.1.3. Transfer the 48 well microplate prepared in step 4.3 to the recording platform and pull down the cover.

5.1.4. Click the "**File**" "**Generate Protocol**" button in turn in the software to begin generating a new protocol.

5.1.5. Input "**48**" in the "**Location count**" position and click the "**OK**" button.

5.1.6. Click the "**Parameters**" "**Protocol Parameters"** "**Time**" button in turn in the software. Set the experiment duration for 1 h and 10 min and set the integration period to 60 s[33].

5.1.7. Draw the detected areas.

5.1.7.1. Select the elliptical shape and draw the first circle around the first top left well.

5.1.7.2. Select the circle, click the "**Copy**" "**Top-Right Mark**" "**Paste**" **"Select"** button in turn, and use the mouse to drag the copied circle to the top right well.

5.1.7.3. Select the circle, click the "**Copy**" "**Bottom Mark**" "**Paste**" **"Select"** button in turn, and use the mouse to drag the copied circle to the bottom right well.

5.1.7.4. Click the "**Build**" "**Clear marks**" button in turn.

NOTE: The system will automatically draw every other well of the plate. The newly created areas should perfectly fit each well between the actual fish and its reflection on the side of the well.

5.1.8. Click the "**Draw Scale**" button, draw a calibration line on the screen (a diagonal path or parallel to the side of the microplate), enter its length and set the "**Unit**". Then click the "A**pply to group**" button.

5.1.9. Set the detection threshold at 16–18 to allow the detection of the animals.

5.1.10. Set the animal color at "**Black**" in the software.

5.1.11. Input inactive/small and small/large speed at 0.5 cm/s and 2.5 cm/s respectively.

5.1.12. Set the path angle classes. Input "-90, -30, -10, 0, 10, 30, and 90" to make path angle classes from -180°–+180°.

5.1.13. Set the light conditions

5.1.13.1. Click the "**Parameters**" "**Light driving**" "**Uses one of the 3 triggering methods below**" "**Enhanced stimuli**" button in turn to set the light conditions.

5.1.13.2. Choose the "**Edge**" button, then set a dark period of 10 min, followed by three cycles of alternating 10 min light and dark periods.

5.1.14. Save the protocol and turn down the light of the test room.

5.1.15. Acclimate the larvae in the system for 10 min and click the "**Experiment**" "**Execute**" button in turn, then choose the folder where the experiment files are saved and enter the result

name.

5.1.16. Click the "**Background**" "**Start**" button in turn to start the test.

5.1.17. Click the "**Experiment**" "**Stop**" button in turn to stop the experiment when the test ends.

NOTE: The system shows the data tested when the system stops. The data include the tracked distance at three speed classes and path angle numbers at eight angle classes of every minute. For the locomotion test in the presented example, the total distance in every light period (10 min) is calculated and the difference between the control group and the treatment groups compared.

5.1.18. Transfer the 48 well microplate back to the light incubator for other experiments.

5.2. Social activity test

5.2.1. Click on the launcher icon on the computer desk to open the software that controls the high-throughput monitoring enclosure to start the program.

5.2.2. Choose the "**Social Interactions**" module to enter the operating interface.

5.2.3. Transfer the prepared 6 well microplate (control group) in step 4.4 to the recording platform and pull down the cover.

5.2.4. Click the "**File**" "**Generate Protocol**" button in turn in the software to begin generating a new protocol.

5.2.5. Input "**6**" in the "**Location count**" position and click the "**OK**" button.

5.2.6. Click the "**Parameters**" "**Protocol Parameters**" "**Time**" button in turn in the software. Set the experiment duration for 1 h and 10 min and set the integration period to 60 s.

5.2.7. Draw the detected areas.

5.2.7.1. Select the elliptical shape and draw the first circle around the first top left well.

5.2.7.2. Select the circle, click the "**Copy**", "**Top-Right Mark**" "**Paste**" **"Select"** button in turn, and use the mouse to drag the copied circle to the top-right well.

5.2.7.3. Select the circle, click the "**Copy**" "**Bottom Mark**" "**Paste**" **"Select"** button in turn, and use the mouse to drag the copied circle to the bottom right well.

5.2.7.4. Click the "**Build**" "**Clear marks**" button in turn.

NOTE: The newly created areas should fit each well perfectly and between the actual larvae and

its reflection on the side of the well.

5.2.8. Click the "**Draw Scale**" button, draw a calibration line in the screen (a diagonal path or parallel to the side of the microplate), enter its length and set the "**Unit**". Then click the "**Apply to group**" button.

5.2.9. Set the detection threshold at 16–18 to allow the detection of the animals.

5.2.10. Click the "**Black animal**" button in the software.

5.2.11. Choose the "**Distance Threshold**" button and input "**5**" in the software.

5.2.12. Set the light conditions.

5.2.12.1. Click the "**Parameters"** "**Light driving**" "**Uses one of the 3 triggering methods below**" "**Enhanced stimuli**" button in turn to set the light conditions.

5.2.12.2. Choose the "**Edge**" button, then set a dark period of 10 min, followed by three cycles of alternating 10 min light and dark periods.

5.2.13. Save the protocol and turn down the light of the room.

5.2.14. Acclimate the larvae in the system for 10 min and click the "**Experiment**" "**Execute**" button in turn, then choose the folder where the experiment files are saved and enter the result name.

5.2.15. Click the "**background**" "**start**" button in turn to start the test.

5.2.16. Click the "**Experiment**" "**Stop**" button in turn to stop the experiment in the software when the test ends.

NOTE: The system shows the data tested when the system stops.

5.2.17. Transfer the 6 well microplate (control group) back to the light incubator for other experiments.

5.2.18. Transfer the 6 well microplates (5 μg/L and 50 μg/L groups) in turn to the recording platform and repeat the steps from 5.2.4 to 5.2.17 by ordinal.

6. Data analysis

6.1. Open the spreadsheet file in the locomotion and path angle results.

6.2. Select the three distance columns (**inadist**, **smldist**, **lardist**) and add them up.

NOTE：The data of **inadist**, **smldist**, and **lardist** mean different distances recorded by the system in different speed classes (inactive/small/large), respectively.

6.3. For every 10 minute light period sum up the distance of every well, calculate the average distance of 16 wells, and compare the data of the three groups under light stimuli.

6.4. For every 10 minute light period sum up the angle number of every well in every light duration from **cl01** to **cl08** in turn, and compare the data of the three groups under light stimuli.

NOTE： The data of columns from **cl01** to **cl08** mean different path angle numbers recorded by the system in different path angles, respectively.

6.5. Open the spreadsheet file in the social activity results.

6.6. Select the **contct** and **contdur** columns, and for every 10 min light period sum up the social times and their duration for every well.

6.7. Calculate the average social times and duration of one group in every light duration and compare the data of the three groups under light stimuli.

REPRESENTATIVE RESULTS:

Here, we describe a protocol for studying the neurobehavioral effects of environmental pollutants using zebrafish larvae under light stimuli. The locomotion, path angle, and social activity tests are defined in the introduction. The setup of the microplates in the locomotion and path angle tests and the images of the software are shown below. In addition, our own research results are presented as examples. Two studies present the locomotion and path angle effects after exposure to BDE-47 and 6-OH/MeO-BDE-47. The third study presents the effects of four commercial chlorinated paraffins on social behavior.

The setup of the 48 well microplate and the movement locus of the larvae in the locomotion and path angle test.

Three groups, including one control group and two treatment groups, were used in the protocol. Because every group can have 16 animals, the system can be used to perform high-throughput tests of locomotion and path angle in one microplate. **Figure 1** shows one larva treated with the control solution, 5 μg/L solution, and 50 μg/L solution in each well of the first, middle, and last two rows, respectively.

Figure 1 also shows all movement loci of the larvae in the locomotion and path angle tests. The system tracked the locomotion of the larvae and calculated the swimming distance at different speed classes. The system calculated the path angle numbers of larvae at different path angle classes. Researchers can analyze the data recorded by the system in their own ways.

[Figure 1 here.]

The 6 well microplate in the social activity test.

Figure 2 shows a 6 well microplate in the social activity testing process. Every well had two larvae, and the system recorded the distance between the two larvae during the whole testing process. The system recorded the social activity numbers and duration in the set testing time (1 min in this protocol).

[Figure 2 here.]

BDE-47 exposure affected locomotion in zebrafish larvae at 5 dpf.

As shown in **Figure 3**, the highest concentration group of BDE-47 produced pronounced hypoactivity during the dark period. However, there were no observed changes due to BDE-47 exposure during the light periods.

[Figure 3 here.]

6-OH/MeO-BDE-47 exposure affected the path angles of zebrafish larvae at 5 dpf.

As shown in **Figure 4**, the high concentration group of 6-OH-BDE-47 performed fewer routine turns and average turns at 5 dpf. However, more responsive turns were induced by 6-MeO-BDE-47 exposure groups.

[Figure 4 here.]

CPs exposure affected social activity of zebrafish larvae at 5 dpf.

As shown in **Figure 5**, the social behaviors of zebrafish larvae were influenced by three CP products. The social activity was stimulated by CP-70 and the short-chain CP-52b. The long chain CP-52a shortened the duration per contact of the larvae.

[Figure 5 here.]

FIGURE AND TABLE LEGENDS:

Figure 1: The setup of the 48 well microplate and the movement loci of the larvae in the locomotion and path angle test. A1–A8, B1–B8 = the control group; C1–C8, D1–D8 = the 5 μg/L group; E1–E8, F1–F8 = the 50 μg/L group. The black color tracking line means inactivity or small movements; the green color tracking line means normal movements; and the red color tracking line means large movements.

Figure 2: The 6 well microplate in the social activity test. Every well had two larvae. The yellow line means the distance between two animals is <0.5 cm; the red line means the distance between two animals is >0.5 cm.

Figure 3: Effects of BDE-47 exposure on locomotion of larval zebrafish at 5 dpf. Locomotion (distance moved measured in cm) was recorded in alternating periods of darkness and light for a total duration of 70 min. Solid and open bars at the bottom indicate dark and light periods,

respectively. Data are presented as mean ± SEM (*p < 0.05 compared with the control group). This figure has been modified from Zhao et al.[17] with permission.

Figure 4: Effects of 6-OH/MeO-BDE-47 on the path angle of larval zebrafish during the dark period. Data are presented as the mean ± SEM (*p < 0.05 compared with control). This figure has been modified from Zhang et al.[18] with permission.

Figure 5: Effects of CPs on the average social duration per contact in different light/dark periods. (**A**) CP-42, (**B**) CP-52a, (**C**) CP-52b, (**D**) CP-70. The data are presented as the mean ± SEM (*p < 0.05 compared with the control). This figure has been modified from Yang et al.[19] with permission.

DISCUSSION:

This work provides a detailed experimental protocol to evaluate the neurotoxicity of environmental pollutants using zebrafish larvae. Zebrafish go through the process from embryos to larvae during the exposure period, which means that good care of the embryos and larvae is essential. Anything that affects the development of the embryos and larvae can influence the final result. Here the culture environment, exposure process, and experimental conditions are discussed to ensure the success of the whole assay.

For the culture environment, zebrafish embryos and larvae live under a stable temperature of ~28 °C. In this work, a light incubator that can set the light conditions automatically and keep the temperature stable is used to house the embryos and larvae. The embryos do not come out from the chorion at 1 dpf and 2 dpf, so care should be taken to avoid damaging the unhatched embryos when renewing the exposure solution. Also, the ratio of DMSO in the solution should be under 0.1%[34,35], and the fresh exposure solution should be at 28 °C before it is used for renewal.

The process of selecting embryos before exposure is also a key factor for the success of the experiment. Choosing healthy embryos developing concurrently for every group guarantees the accuracy of toxicity assessment. Zebrafish can live without food during the first 7 days after fertilization, so it is best to not feed the embryos or larvae during the whole exposure period because food could influence the final result. Also, it is best to prepare the exposure solution fresh when needed.

During the behavior test, it is essential to offer the larvae enough time to adapt to the environment of the high-throughput monitoring enclosure. Before the test, every step of the tested protocol should be checked carefully, including the light condition, testing time, etc. The testing room should be kept completely quiet and dark in order to not disturb the animals.

The protocol presented offers a fundamental frame to study the neurobehavioral toxicity of environmental pollutants. There are also other types of behaviors used when studying neurobehavioral effects, such as color-preference tests[36], bottom dwelling tests[37], light/dark preference tests[38,39], etc. However, these tests mainly use adult zebrafish, which are not fit for high-throughput tests. In addition, Weichert et al. videotaped to the behavior of spontaneous

tail movements which could be quantified just after 24 h exposure[40]. The evaluation of neurobehavioral toxicity also includes mechanism studies on the function of the brain and the central nervous system. The fundamental neurobehavioral indicators are introduced here and can form the basis for more complex indicators using other behavior instruments. Ultimately, the development of new neurobehavioral indicators accompanied with this study mechanism can be used in future studies.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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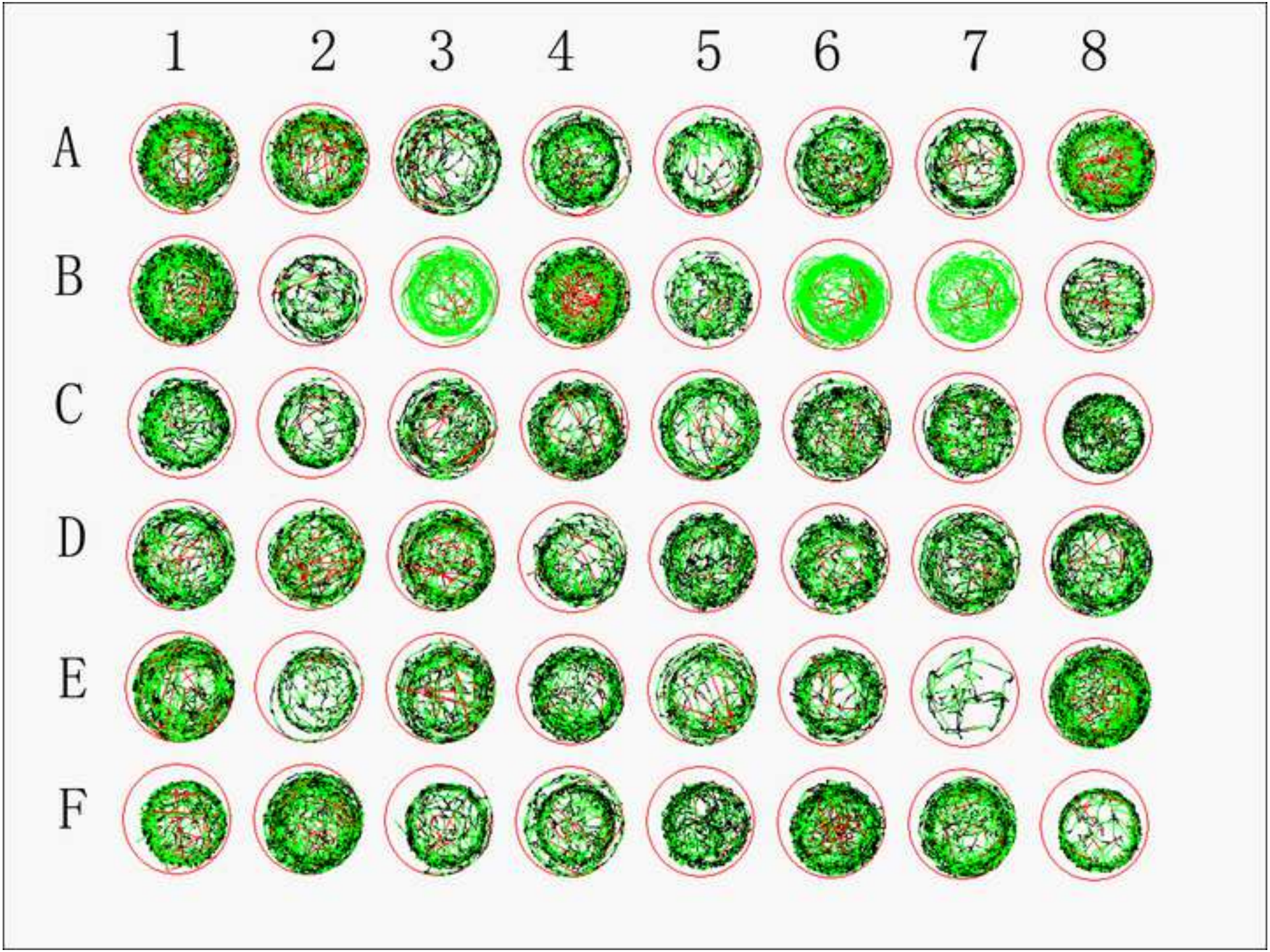

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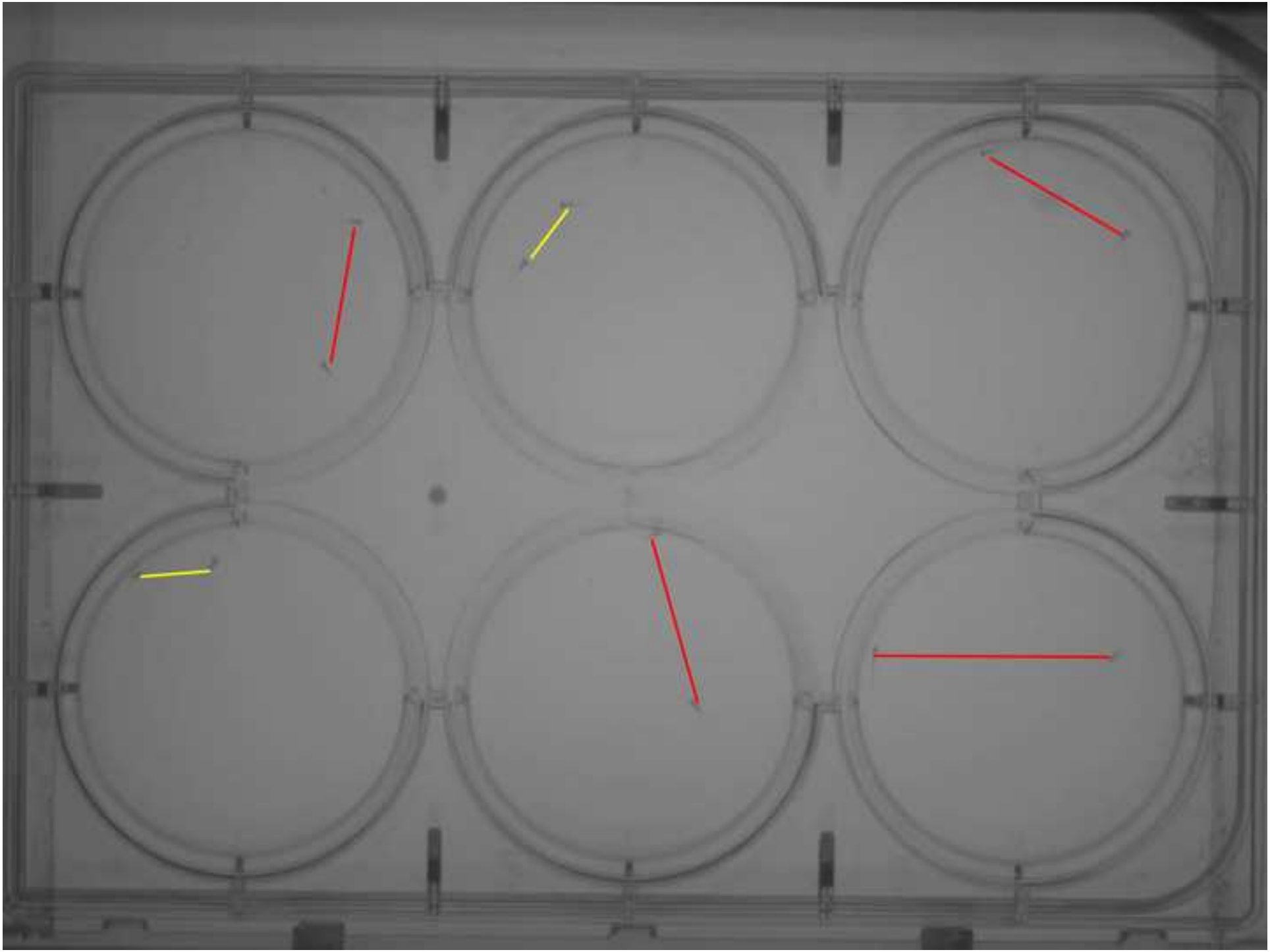

Figure

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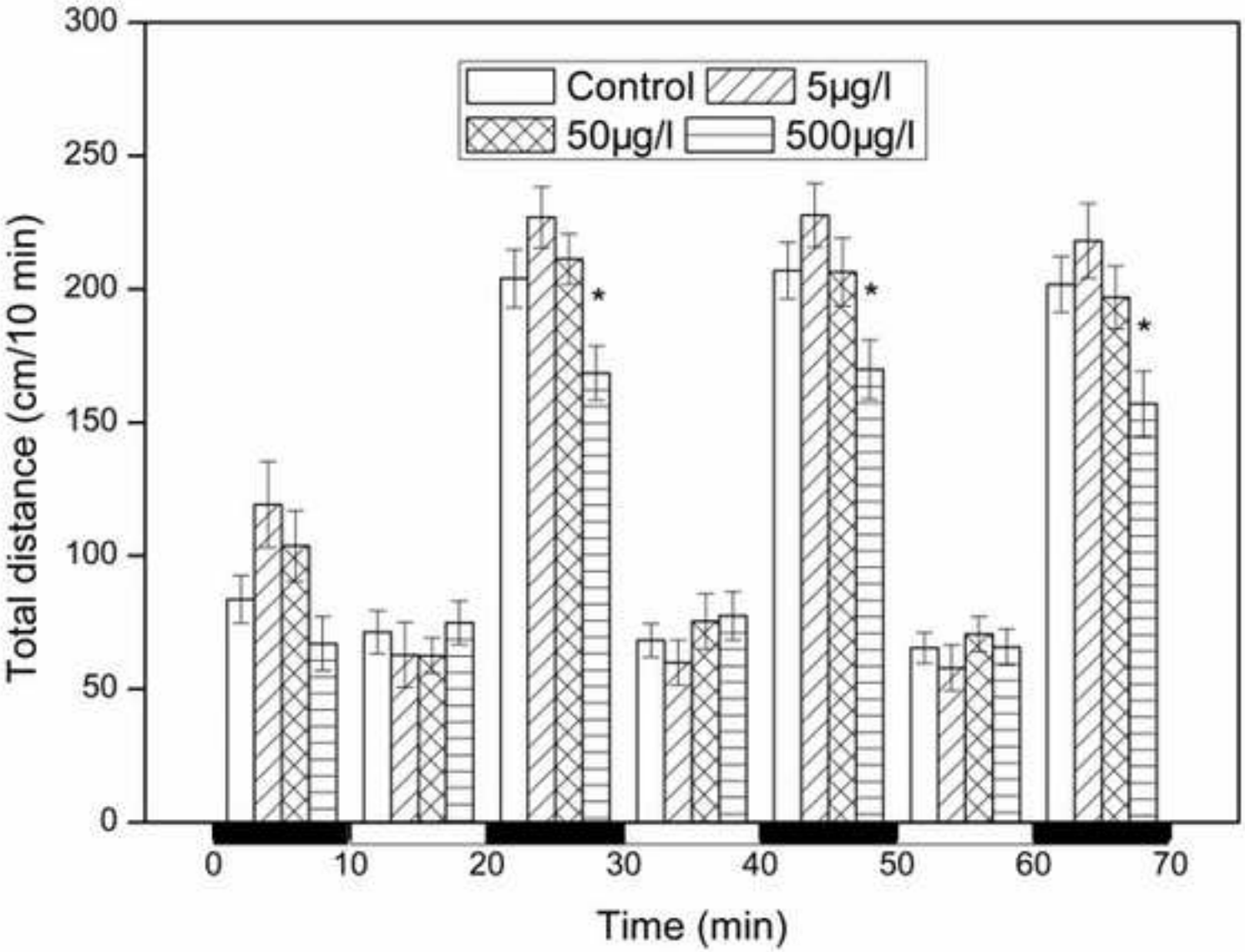

Figure

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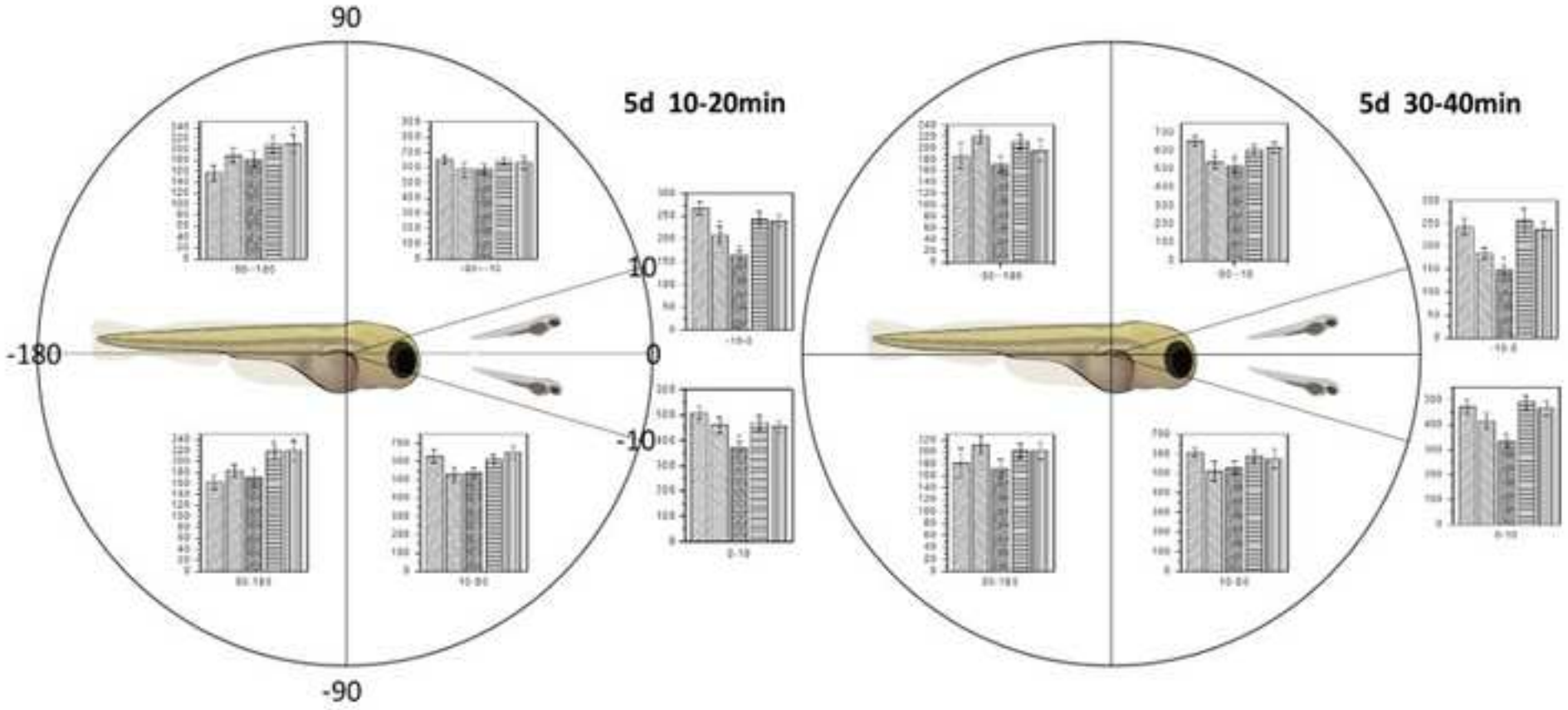

Figure

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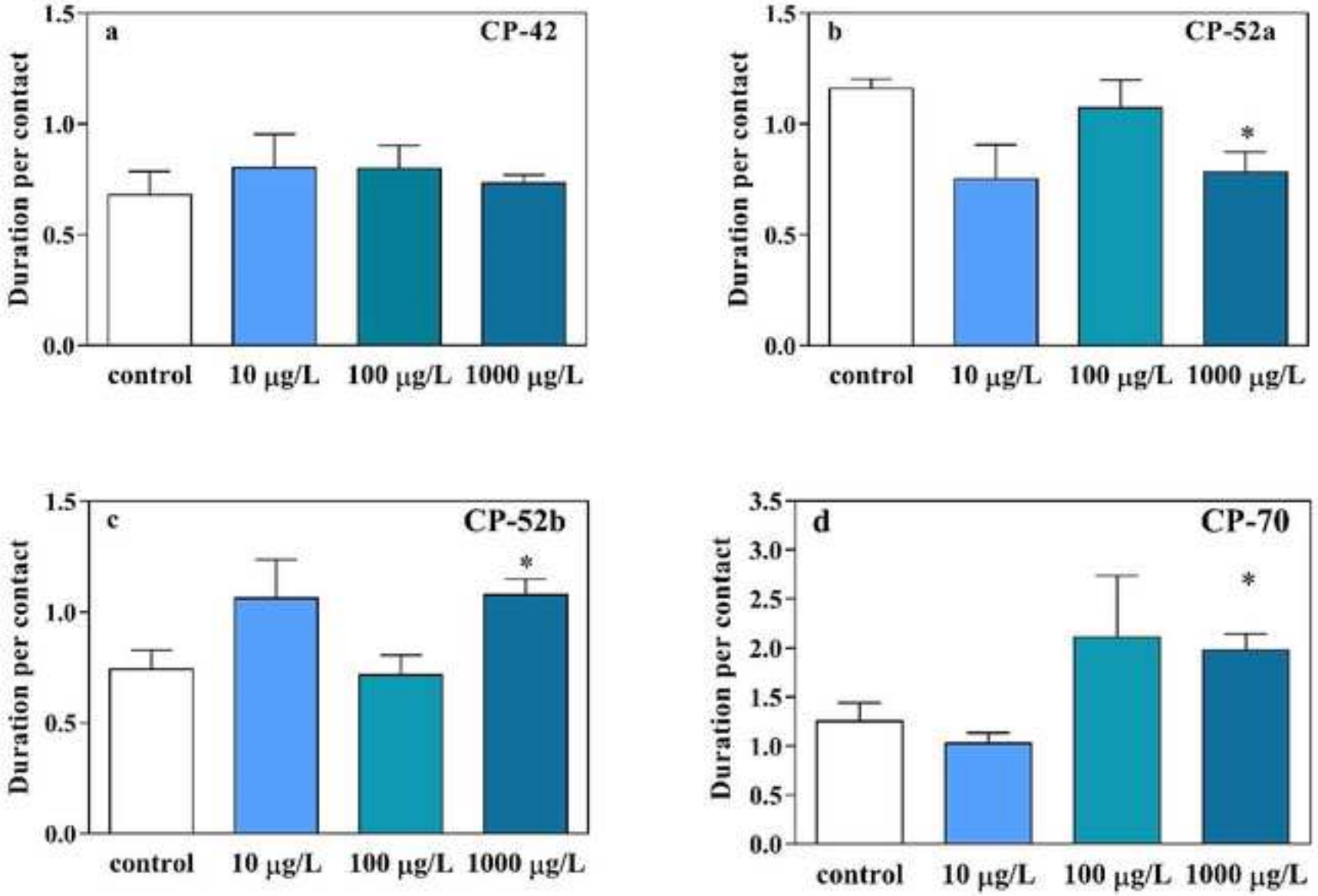

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
48-well-microplate	Corning	3548	Embyros housing
6-well-microplate	Corning	3471	Embyros housing
BDE-47	AccuStandard	5436-43-1	Pollutant
DMSO	Sigma	67-68-5	Cosolvent
Microscope	Olympus	SZX 16	Observation instrument
Pipette	Eppendorf	3120000267	Transfer solution
Zebrabox	Viewpoint	ZebraBox	Behavior instrument
Zebrafish	Shanghai FishBio Co., Ltd.	Tubingen	Zebrafish supplier
ZebraLab	Viewpoint	ZebraLab	Behavior software

Response to editor and reviewers' comments

1. As your article contains detailed, step-by-step, descriptions of software usage, the inclusion of supplemental screen capture or screenshots for the software usage would greatly expedite the scripting and production. You can either take screenshots of the software GUI or use screen capture software (https://www.jove.com/video/5848/screen-capture-instructions-for-authors?status=a7854k). Please include the manuscript number in these supplemental files and number the files in order of appearance: JoVE60818R1_screenfile1, etc.

Answer： Thanks for the comment. We have added a screen capture of protocol 5.1 (**JoVE60818R1_screenfile1)**.

2. The editor has formated the manuscript to match the journal's style. Please retain the formatting.

Answer: Thanks for the editor's work. We retain the formatting.

3. Please address all the specific comments marked in the manuscript.

Answer: Thanks for the comments. We have addressed all the specific comments and please see the revised manuscript listed below.

4. All of the reviewers' comments are not addressed. Please see my comments marked in the manuscript.

Answer: Thanks for the comments. We have addressed all the reviewers' comments and please see the revised manuscript listed below.

5. We need Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. Please include the figures which you had included previously. Please include reprint permission for the figures published before.

Answer: Thanks for the comments. We have added the previous figures and also got the reprint permission.

6. Please ensure that the highlighted steps form a cohesive narration and are in line with the manuscript title. The highlights should not be more than 2.75 pages including headings and spacings.

Answer: Thanks for the comment. It is OK.

7. Once done, please proofread the manuscript for any spelling or grammar issues.

Answer: Thanks for the comment. We have revised the manuscript. Note that the manuscript below with marks addressed all the comments of editor and reviewers, but it is not the final version for that we have corrected some spelling or grammar mistakes later.

TITLE:

Studying Neurobehavioral Effects of Environmental Pollutants on Zebrafish Larvae

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KEYWORDS:

Neurobehavioral effects; Environmental pollutants; Zebrafish larvae; Neurotoxicity; ~~–~~Light stimuli~~-Zebrabox~~; Behavior test

SUMMARY:

A detailed experimental protocol is presented in this paper for the neurobehavioral toxicity evaluation of environmental pollutants using zebrafish larvae model, including the exposure process, neurobehavioral indicators test and so on.

ABSTRACT:

In the recent years, more and more environmental pollutants have been proved to have neurotoxicity, especially at the early development stages of organisms. Zebrafish larvae is a preeminent model for the neurobehavioral study of environmental pollutants. Here a detailed experimental protocol is provided for the neurotoxicity evaluation of environmental pollutants using zebrafish larvae, including the embryos collection, the exposure process, neurobehavioral indicators and the test process and so on. Also, the condition of culture, exposure process and the environment of experiment are discussed to ensure the success of the whole assay. The protocol has been used in the development of psychopathic drugs and research on environmental neurotoxic pollutants, and also can be optimized to make corresponding studies or be helpful for mechanistic studies. The protocol presented is hoped to provide a bigger picture

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to reveal similar neurotoxicity effects of various neuro toxicants or pollutants, and also to demonstrate a clear operation process of studying neurobehavioral effects on zebrafish larvae.

INTRODUCTION:

Recent years more and more environmental pollutants have been proved to have neurotoxicity[1-4]. However, the in vivo assessment of neurotoxicity of environmental pollutants is not as easy as that of endocrine disruption or developmental toxicity. Early exposure to environmental pollutants especially at the environmental relevant dose has been a hot topic and attracts increasing attention on the toxicity studies[5-8]. Thus, an animal model, fit for the neurotoxicity studies during the early development after exposure to environmental pollutants, is being found.

As a vertebrate, zebrafish develop faster than other species after fertilization. Actually larvae come out from the chorion about 2 days post fertilization (dpf), and develop a behavior which can be observed such as swimming and turning from 3-4 dpf[9-13]. Meantime, larvae do not need to be fed because the nutrient gotten from the chorion is enough for them to live until 7 dpf[14]. Early behavior of larvae can be tracked, quantified and analyzed automatically using behavior instruments[15-18]. In addition, high-throughput tests can also be perfomed. Thus zebrafish larvae serve as a preeminent model for the neurobehavioral study of environmental pollutants[19]. Herein, a protocol is offered using a high-throughput monitoring enclosure, Zebrabox, to study the neurobehavioral toxicity of environmental pollutants on zebrafish larvae under light stimulation.

Our lab has studied the neurobehavioral toxicity of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47)[20,21], 4'-Hydroxy/Methoxy-2,2',4,4'-tetrabromodiphenyl ether (6-OH/MeO-BDE-47)[22], deca-brominated diphenyl ether (BDE-209), lead, and commercial chlorinated paraffins[23] using the presented protocol. Actually many labs also used the protocol to study the neurobehavioral effects of other pollutants on larvae or adult fish[24-27]. Neurobehavioral protocol was used to help provide mechanistic support that low-dose exposure to bisphenol A and replacement bisphenol S induced precocious hypothalamic neurogenesis in embryonic zebrafish[27]. In addition, some researchers optimized the protocol to make corresponding studies. A recent study eliminated the toxicity of amyloid beta (Aβ) in a facile, high-throughput zebrafish model using casein coated-gold nanoparticles (βCas AuNPs). They observed that βCas AuNPs in systemic circulation translocate across the blood brain barrier of zebrafish larvae and sequester intracerebral Aβ42 eliciting toxicity in a nonspecific, chaperone-like manner, which was evidenced by behavioral pathology[28].

Locomotion, path angle, and social activity are three neurobehavioral indicators used to study the neurotoxicity effects of zebrafish larvae after exposure to pollutants. Locomotion means the swimming distance of larvae and can represent if the movement ability is damaged by exposure to pollutants. Path angle and social activity are two more high-grade behaviors which have a closer relationship with the function of the brain and the central nervous system[29]. The path angle refers to the angle of the path of animal motion relative to the swimming direction[30]. Eight angle classes from -180° ~ +180° are set in the system. To simplify the comparison, 6 classes in the final outcome were defined as routine turn (-10° ~ 0°, 0° ~ +10°), average turn (-10° ~ -90°,

+10° ~ +90°) and responsive turn (-180°~ -90°, +90°~ +180°) according to our previous studies[21,22]. The distance shorter than 0.5 cm between two larvae recorded by the system could be defined as a valid social "contact", and the two-fish social activity is fundamental of group shoaling behavior.

Therefore, the protocol presented is hoped to provide a bigger picture to reveal similar neurotoxicity effects of various neuro toxicants or pollutants, and also to demonstrate a clear operation process in studying neurobehavioral effects on zebrafish larvae. The protocol will benefit researchers that are interested in studying the neurotoxicity of environmental pollutants.

PROTOCOL:

The protocol is in accordance with guidelines approved by the Animal Ethics Committee of Tongji University.

1. Zebrafish embryos collection

1.1. Put two pairs of healthy adult zebrafish (Tubingen) into the spawning box on the night beforehand, keeping the sex ratio at 1:1.

1.2. Remove the adult fish back to the system 30-60 min after the light is on at 8:00 am the next morning.

1.3. Remove the embryos out of the spawning box.

1.4. Rinse the embryos with system water.

1.5. Transfer the embryos into a glass Petri dish with 9 cm diameter and enough system water.

1.6. Observe the embryos under the microscope and select healthy embryos for later exposure.

NOTE: The healthy embryos are usually transparent with light golden color under the microscope. The unhealthy embryos are usually pale and with flocs under the microscope.

2. Preparation before exposure

2.1. Prepare the Hanks' solution according to the guidelines of the zebrafish book[31].

NOTE: The Hanks' solution includes 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 4.2 mM $NaHCO_3$.

2.2. Dilute the Hanks' solution to 10% Hanks' solution using sterile water for the preparation of other solutions.

2.3. Add 1 mL DMSO into 999 mL of 10% Hanks' solution to make the control solution (10% Hanks

solution including 0.1% DMSO).

NOTE: The next steps take BDE-47 as an example for the configuration of the exposure solution.

2.4 Dissolve the neat standard (5 mg) of BDE-47 in 1 mL of 100% DMSO to make the concentration of the mother exposure solution at 5 mg/mL.

2.5 Vortex the 5 mg/mL solution for 1 min to make BDE-47 dissolved in the DMSO completely.

2.6 Transfer 10 µL 5 mg/mL solution to a 12 mL brown glass bottle.

2.7 Add sterilized water to 10 mL to make the concentration of BDE-47 exposure solution at 5 mg/L and DMSO ratio at 0.1%, then vortex for 1 min.

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Commented [A6R5]: This is 5 mg/L, which is diluted 1000 times from 5 mg/mL in Step 2.4. They are different.

2.8 Transfer 10 µL and 100 µL 5 mg/L solution into two 100 mL brown glass bottles respectively.

2.9 Add 10% Hanks' solution including 0.1% DMSO (prepared in Step 2.3) to 100 mL to make the final concentrations of BDE-47 exposure solutions at 5 µg/L and 50 µg/L respectively.

2.10 Store the prepared solutions in brown glass bottles under 4 ℃ in the refrigerator.

3. Embryos exposure

3.1 Transfer about 50 embryos into each of the three glass Petri dishes with 6 cm diameter at 3-5 h post fertilization (hpf).

3.2 Use a pipette with a 1 mL pipette tip to blot the system water around the embryos.

3.3 Use a pipette to transfer the three exposure solutions (control, 5 µg/L, 50 µg/L) into the three glass Petri dishes respectively.

3.4 Shake the glass Petri dishes gently one by one to make the embryos scattered in the bottom dispersedly.

3.5 Put the glass Petri dishes into the light incubator under 28.5 ℃.

3.6 Renew half of the exposure solutions one by one every 24 h until 5 dpf.

3.7 Check dead embryos of every group on 1 dpf & 2 dpf, and calculate the death rate.

3.8 Check incubated embryos of every group on 2 dpf & 3 dpf, and calculate the hatchability.

3.9 Check the deformity of larvae every day after they come out from the chorion, and calculate the deformity rate of every group.

NOTE: The deformity indicators include pericardial cyst, spinal curvature, tail curvature, etc[32].

4. Preparation before behavior test

4.1 Prepare a 48-well-microplate for locomotion & path angle test and three 6-well-microplates for social activity test on 5 dpf morning.

4.2 Transfer 800 µL of the exposure solution into every well of the 48-well-microplate.

NOTE: Every group (the control solution, 5 µg/L, and 50 µg/L group) has 16 wells.

4.3 Use a pipette with a 1 mL pipette tip to transfer 200 µL exposure solution with one larva from the glass Petri dish into one well of the 48-well-microplate.

4.4 Transfer 4 mL exposure solution into every well of the 6-well-microplate.

NOTE: Every group uses one 6 well microplate.

4.5 Use a pipette with a 1 mL pipette tip to transfer 200 µL exposure solution with two larvae into each well of the 6-well-microplate.

NOTE: Every group has 6 repeating groups.

4.6 Turn on the air conditioner of the test room and set the temperature at 28 °Cthe same as the zebrafish room two hours before the test.

NOTE: Behavior test is usually performed in the afternoon.

5. Behavior test

5.1 Locomotion & path angle test

5.1.1 Click on the launcher icon on the computer desk to open the software (see **Table of Materials**) which controls the high-throughput monitoring enclosure to start the Program.

5.1.2 Choose the "**Tracking, Rotations, Path Angles**" module to enter the operating interface.

5.1.3 Transfer the prepared 48-well-microplate in Step 4.3 to the recording platform and pull down the cover.

5.1.4 Click the "**File**" "**Generate Protocol**" button in turn in the software to begin generating a new protocol.

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Commented [A10R9]: We have added the software in the table of materials.

5.1.5 Input “**48**” in the “**Location count**” position and click the “**OK**” button.

5.1.6 Click the “**Parameters**” “**Protocol Parameters”** “**Time**” button in turn in the software. Set the experiment duration for 1 h and 10 min and set the integration period to 60 s[33].

5.1.7 Draw the detected areas.

5.1.7.1 Select the elliptical shape and draw the first circle around the first top left well.

5.1.7.2 Select the circle, click the “**Copy**” “**Top-Right Mark**” “**Paste**” button in turn, and use the mouse to drag the copied circle to the top-right well.

5.1.7.3 Select the circle, click the “**Copy**” “**Bottom Mark**” “**Paste**” button in turn, and use the mouse to drag the copied circle to the bottom-right well.

5.1.7.4 Click the “**Build**” “**Clear marks**” button in turn.

NOTE: The system will automatically draw every other well of the plate. The newly created areas should be perfectly fitting for each well between the actual fish and its reflection on the side of the well.

5.1.8 Click the “**Draw Scale**” button, draw a calibration line on the screen (a diagonal path or parallel to the side of the microplate), enter its length and set the “**Unit**”. Then click the “A**pply to group**” button.

5.1.9 Set the detection threshold at 16-18 to allow the detection of the animal.

5.1.10 Set the animal color at “**Black**” in the software.

5.1.11 Input inactive/small and small/large speeds at 0.5 cm/s and 2.5 cm/s respectively.

5.1.12 Set the path angle classes. Input “-90, -30, -10, 0, 10, 30, and 90” to make 8 path angle classes from -180 to 180.

5.1.13 Set the light conditions

5.1.13.1 Click the “**Parameters**” “**Light driving**” “**Uses one of the 3 triggering methods below**” “**Enhanced stimuli**” button in turn to set the light conditions.

5.1.13.2 Choose the “**Edge**” button, then set a dark period of 10 min, followed by three cycles of alternating 10 min light and dark periods.

5.1.14 Save the protocol and turn down the light of the test room.

Commented [A11]: Is there a specific reason for the chosen timings.

Commented [A12R11]: Actually the first 10 minutes is for the adaption of larvae. Three alternating light and dark circles are used for the light stimuli. We have added a reference here.

5.1.15 Make the larvae acclimated in the dark for 10 min and click the "**Experiment**" "**Execute**" button in turn, then choose the folder where the experiment files are saved.

5.1.16 Click the "**background**" "**start**" button in turn to start the test.

NOTE: Steps from 5.1.1 to 5.1.16 could be seen in the supplemental file JoVE60818R1_screenfile1.

5.1.17 Click the "**Experiment**" "**Stop**" button in turn to stop the experiment in the software when the test ends.

NOTE: The system shows the data tested when the system stops. The data includes the tracked distance at three speed classes and path angle numbers at eight angle classes of every minute. Take the locomotion test in our study for an example, we would calculate the total distance in every light period (10 min) and compare the difference between the control group and the treatment groups.

5.1.18 Transfer the 48-well-microplate back to the light incubator for other experiments.

5.2 Social activity test

5.2.1 Click on the launcher icon on the computer desk to open the software which controls the Zebrabox to start the Program.

5.2.2 Choose the "**Social Interactions with Zebrabox**" module to enter the operating interface.

5.2.3 Transfer the prepared 6-well-microplate (control group) in Step 4.4 to the recording platform and pull down the cover.

5.2.4 Click the "**File**" "**Generate Protocol**" button in turn in the software to begin generating a new protocol.

5.2.5 Input "**6**" in the "**Location count**" position and click the "OK" button.

5.2.6 Click the "**Parameters**" "**Protocol Parameters**" "**Time**" button in turn in the software. Set the experiment duration at 1 h and 10 min and set the integration period at 60 s.

5.2.7 Draw the detected areas.

5.2.7.1 Select the elliptical shape and draw the first circle around the first top left well.

5.2.7.2 Select the circle, click the "**Copy**", "**Top-Right Mark**" "**Paste**" button in turn, and use the mouse to drag the copied circle to the top-right well.

5.2.7.3 Select the circle, click the "**Copy**" "**Bottom Mark**" "**Paste**" button in turn, and use the

mouse to drag the copied circle to the bottom-right well.

5.2.7.4 Click the "**Build**" "**Clear marks**" button in turn.

NOTE: The newly created areas should be perfectly fitting for each well and between the actual larvae and its reflection on the side of the well.

5.2.8 Click the "**Draw Scale**" button, draw a calibration line in the screen (a diagonal path or parallel to the side of the microplate), enter its length and set the "**Unit**". Then click the "**Apply to group**" button.

5.2.9 Set the detection threshold at 16-18 to allow the detection of the animal.

5.2.10 Click the "**Black animal**" button in the software.

5.2.11 Choose the "**Distance Threshold**" button and input "**5**" in the software.

5.2.12 Set the light conditions

5.2.12.1 Click the "**Parameters"** "**Light driving**" "**Uses one of the 3 triggering methods below**" "*Enhanced stimuli*" button in turn to set the light conditions.

5.2.12.2 Choose the "**Edge**" button, then set a dark period of 10 min, followed by three cycles of alternating 10 min light and dark periods.

5.2.13 Save the protocol and turn down the light of the room.

5.2.14 Make the larvae acclimated in the dark for 10 min and click the "**Experiment**" "**Execute**" button in turn, then choose the folder where the experiment files are saved.

5.2.15 Click the "**background**" "**start**" button in turn to start the test.

5.2.16 Click the "**Experiment**" "**Stop**" button in turn to stop the experiment in the software when the test ends.

NOTE: the system shows the data tested when the system stops.

5.2.17 Transfer the 6-well-microplate (control group) back to the light incubator for other experiments.

5.2.18 Transfer the 6-well-microplate (5 μg/L and 50 μg/L group) to the recording platform and repeat the steps from 5.2.4 to 5.2.17 by ordinal.

6. Data analysis

Commented [A13]: Fish or larvae?

Commented [A14R13]: larvae

Commented [A15]: Please include steps as asked by reviewer 2 to show what happens after the data collection. Why specific endpoints. How is the analysis performed? etc in brief.

Commented [A16R15]: We have added related information.

6.1 Open the Excel file in the locomotion & path angle results.

6.2 Select the three distance columns (**inadist**, **smldist**, **lardist**), and sum up them.

NOTE：The data of i**nadist**, **smldist** and **lardist** means different distance recorded by the system in different speed classes (inactive/small/large) respectively.

6.3 Sum up every ten minutes' distance of every well, calculate the average distance of 16 wells in every light duration, and compare the data of the three groups under light stimuli.

6.4 Sum up every ten minutes' angle number of every well in every light duration from **cl01** to **cl08** in turn, and compare the data of the three groups under light stimuli.

NOTE： The data of columns from **cl01** to **cl08** means different path angel numbers recorded by the system in different path angel classes (-180~-90, -90~-30, -30~-10, -10~0, 0~10, 10~30, 30~90, 90~180) respectively.

6.5 Open the Excel file in the social activity results.

6.6 Select the **contct** and **contdur** columns, sum up every ten minutes' social times and duration of every well.

6.7 Calculate the average social times and duration of one group in every light duration, and compare the data of the three groups under light stimuli.

REPRESENTATIVE RESULTS:

Here, we describe a protocol for studying neurobehavioral effects of environmental pollutants using zebrafish larvae under light stimulation. The definition of locomotion, path angle, and social activity has been introduced in the first part (see Introduction). The setup of the microplate in locomotion & path angle test and the images of the software are shown below. In addition, our own research results are presented as examples. Two studies present the locomotion and path angle effects of BDE-47 and 6-OH/MeO-BDE-47 exposure. The third study present social effects of commercial chlorinated paraffins.

The setup of the 48-well-microplate and the movement locus of the larvae in the locomotion & path angle test

Three groups including one control group and two treatment groups are used in the protocol. Thus by using a 48-well-microplate, the system could realize high-through test of locomotion & path angle in one microplate, for every group can have 16 animals. In **Figure 1**, one larva is treated with the control solution, 5 μg/L solution and 50 μg/L solution in each well of the first, middle and last two rows respectively.

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If using previously published results, please include the reprint permission and attach it to the editorial manager.

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Figure 1 also shows all movement locus of the larvae in the locomotion & path angle test. The system can track the locomotion of larvae and calculate the swimming distance at different speed classes. Meantime, the system can also calculate the path angle numbers of larvae at different path angle classes. Researchers can analyze the data recorded by the system in their own ways.

[Figure 1 here.]

The 6-well-microplate in the social activity test

Figure 2 shows a 6-well-microplate in the social activity testing process. Every well has two larvae, and the system can record the distance between the two larvae during the whole testing process. The system can finally perform the social activity numbers and duration in the set testing time (one minute in the present protocol).

[Figure 2 here.]

BDE-47 exposure induced locomotion effects of zebrafish larvae at 5 dpf

From **Fig. 3**, pronounced hypoactivity was elicited by the highest concentration group of BDE-47 during the dark period. However, BDE-47 exposure during the light periods did not induce observed changes.

[Figure 3 around here.]

6-OH/MeO-BDE-47 exposure induced path angle effects of zebrafish larvae at 5 dpf

From **Fig. 4**, the high concentration group of 6-OH-BDE-47 performed less routine turns and average turns at 5 dpf. However, more responsive turns were induced by 6-MeO-BDE-47 exposure groups.

[Figure 4 around here.]

CPs exposure induced social activity effects of zebrafish larvae at 5 dpf

As shown in **Fig. 5**, the results showed that the social behaviors of zebrafish larvae were influenced by three CP products. The social activity was stimulated by CP-70 and the short-chain CP-52b. The long chain CP-52a shortened the duration per contact of larvae.

[Figure 5 around here.]

FIGURE AND TABLE LEGENDS:

Figure 1: The setup of the 48-well-microplate and the movement locus of the larvae in the

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locomotion & path angle test (A1-A8, B1-B8: the control group； C1-C8, D1-D8: the 5 μg/L group; E1-E8, F1-F8: the 50 μg/L group; the black color tracking line means inactivity/small movements, the green color tracking line means normal movements, and the red color tracking line means large movements)

Figure 2: The 6-well-microplate in the social activity test (Every well has two larvae, the yellow line means the distance between two animals is shorter than 0.5 cm, and the red line means the distance between two animals is longer than 0.5 cm)

Figure 3: Effects of BDE-47 exposure on locomotion of larval zebrafish at 5 dpf. Locomotion (distance moved, cm) was recorded in alternating periods of darkness and light for a total duration of 70 min. Solid and open bars at the bottom indicate dark and light periods, respectively. Data are presented as mean ± SEM (*$p < 0.05$ compared with control). This figure has been modified from Zhao et al.[17] with permission.

Figure 4: Effects of 6-OH/MeO-BDE-47 on the path angle of larval zebrafish during dark period. Data are presented as the mean ± SEM (*$p < 0.05$ compared with control). This figure has been modified from Zhang et al.[18] with permission.

Figure 5: The average social duration per contact in different light/dark periods of different groups. (a) CP-42 (b) CP-52a (c) CP-52b (d) CP-70 The data are presented as the mean ± SEM (*$p<0.05$ compared with the control). This figure has been modified from Yang et al.[19] with permission.

DISCUSSION:

In this work a detailed experimental protocol is provided for the neurotoxicity evaluation of environmental pollutants using zebrafish larvae model. Zebrafish would go through the process from embryos to larvae, which means that the care of embryos and larvae are both of vital important. It should be known that any operation which would influence the development of embryos and larvae has the possibility to influence the final result. Here the condition of culture, exposure process and the environment of experiment are discussed to ensure the success of the whole assay.

For the condition of culture, it is widely known that zebrafish are lived in a stable temperature around 28 ℃, the same for the new embryos and larvae. In this work a light incubator which could set the light condition automatically and also could keep the stable temperature is used to house the embryos and larvae. The embryos do not come out from the chorion at 1 dpf and 2 dpf, so the process of renewing the exposure solution should avoid damage the unhatched embryos, especially at the first two days. Meantime, the ratio of DMSO in the solution should be under 0.1%, and the exposure solution should be recovered to 28 ℃before renewed.

Before the exposure process, the selected embryos are also the key factor for the success of the experiment. The criterion is choosing healthy and synchronously developmental embryos in

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2.Please include the study Weichert et al as asked by reviewer 2. This can be included as a modification/limitation of the present study which will be evaluated later.
3.Please cite papers which use 0.1% DMSO and present this detail in the discussion as a comparison to your protocol.

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every group, to guarantee the accuracy of later toxicity assessment. Meantime, zebrafish can live without food during the first 7 days after fertilization, so do not feed the embryos or larvae during the whole process for that any food would induce unobserved influence to the final result. Another point is that the exposure solution is suggested to be prepared when needed.

During the process of behavior test, it is essential to offer the larvae enough time to adapt to the environment of the high-throughput monitoring enclosure. Before the test, every step of the tested protocol should be checked carefully including the light condition, testing time, and so on. Meantime, the testing room should be kept quiet and dark strictly, in order to avoid any disturbance to the animals.

Actually the presented protocol here offers a fundamental frame to study the neurobehavioral toxicity of environmental pollutants. There are also other types of behaviors studying neurobehavioral effects, such as color-preference test, bottom dwelling test, light/dark preference test, etc. However, adult zebrafish are mainly used in these tests, not fit for high-throughput test. In addition, Weichert et al. payed attention on the behavior of spontaneous tail movements, which could be videotaped and quantified just after 24 h exposure. Meantime, the evaluation of neurobehavioral toxicity also includes mechanism studies on the function of the brain and the central nervous system. The fundamental neurobehavioral indicators are introduced here and actually we can development more complex indicators using behavior instruments. Taken in all, the development of new neurobehavioral indicators, accompanied with mechanism study, is suggested in future studies.

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DISCLOSURES

The authors have nothing to disclose.

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