Autonomous system for cross-organ investigation of ethanol-induced acute response in behaving larval zebrafish

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Ethanol is widely consumed and has been associated with various diseases in different organs. It is therefore important to study ethanol-induced responses in living organisms with the capability to address specific organs in an integrative manner. Here, we developed an autonomous system based on a series of microfluidic chips for cross-organ investigation of ethanol-induced acute response in behaving larval zebrafish. This system enabled high-throughput, gel-free, and anesthetic-free manipulation of larvae, and thus allowed real-time observation of behavioral responses, and associated physiological changes at cellular resolution within specific organs in response to acute ethanol stimuli, which would otherwise be impossible by using traditional methods for larva immobilization and orientation. Specifically, three types of chips (“motion,” “lateral,” and “dorsal”), based on a simple hydrodynamic design, were used to perform analysis in animal behavior, cardiac, and brain physiology, respectively. We found that ethanol affected larval zebrafish in a dose-dependent manner. The motor function of different body parts was significantly modulated by ethanol treatment, especially at a high dose of 3%. These behavioral changes were temporally associated with a slow-down of heart-beating and a stereotyped activation of certain brain regions. As we demonstrated in this proof-of-concept study, this versatile Fish-on-Chip platform could potentially be adopted for systematic cross-organ investigations involving chemical or genetic manipulations in zebrafish model. *Published by AIP Publishing.* [<http://dx.doi.org/10.1063/1.4946013>]

I. INTRODUCTION

Ethanol consumption is associated with a broad array of physiologic, neurological, and behavioral changes in living organism. Due to the high solubility in both aqueous and lipid environments, ethanol can easily diffuse through biological membranes and quickly affect various tissues and organs, especially the cardiovascular and the central nervous system (CNS), potentially leading to many diseases.^{1–5} Development of therapeutics to ethanol-related diseases would greatly benefit from systematic investigation of how ethanol associatively affects physiological function in the context of whole organisms.

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In the past, great efforts have been made to study ethanol-induced responses in different animals' models, including *C. elegans*,⁶ *drosophila*,⁷ zebrafish,⁸ and rodent animals.⁹ Among these models, zebrafish is the only transparent vertebrate with complex organ systems that can be optically accessed in parallel for investigations of physiology in different organs.^{10,11} For example, it was reported that acute ethanol treatment could adversely affect neural development.^{12,13} Ethanol exposure has also been shown to influence the histaminergic and dopaminergic systems and to stimulate the locomotion in larval zebrafish.^{2,14} However, little has been done to associatively study ethanol-induced acute responses in different organ systems, and to simultaneously address brain and cardiac physiology with direct links to behavioral alternations, mainly due to the lack of technology that can perform behavioral study and cellular imaging in an integrated format. Most behavioral experiments involving the use of larval zebrafish were conducted in multi-well plates,^{8,14,15} which were time-consuming and detrimental for long-term imaging. Even though some microfluidic systems were developed to study the behaviors or morphological development of zebrafish embryos, these platforms were difficult to resolve sophisticated organ-specific information at cellular level.^{16–18} On the other hand, cellular-resolution imaging of zebrafish typically requires manual or automatic manipulation and orientation of larvae using rigid gel or physical restrictions,^{19–23} which are not permissive to behavioral analysis. Even though partial restriction may be applied to allow high-resolution imaging of brain activity and observation of associated motor behaviors,^{11,24} such experiments are quite laborious and not versatile enough for high-throughput applications.

Here, we presented an autonomous system to perform cross-organ investigations of ethanol-induced acute response in awake larval zebrafish. Based on our previously published "Fish-Trap" technology,²³ the core component of this system was a variety of microfluidic chips that were designed for Fish-on-Chip studies to capture phenotypes in animal motor-function, cardiac, and brain physiology in response to acute chimerical stimulus (e.g., ethanol). The behavioral and physiological changes were then correlated to provide a systematic characterization of ethanol induced acute responses in behaving animals.

II. RESULTS

A. Devices' design

Microfluidic chips of three different designs, "Motion Chip," "Lateral Chip," and "Dorsal Chip," were introduced here to allow organ-targeted investigation of ethanol-induced acute response in behaving larval zebrafish (Figs. 1(a) and 1(b), supplementary Fig. S1).³⁸ In all designs, hydrodynamic force was used to load and immobilize zebrafish larvae (Figs. 1(c) and 1(d)), as we demonstrated previously.²³ Generally, the chips were composed of two groups of horizontal flow channels, which were connected by series of vertical trapping channels with tapering design mimicking the ergonomic structure of larvae at specific developmental stages. Once a larva was loaded into the horizontal channel, it would be automatically carried into a trapping channel by the bulk stream due to the lower flow resistance, and subsequently acted as a plug to redirect the main flow to other trapping channels (supplementary Fig. S2).³⁸ The major difference among the three designs was the shape of trapping channels, which, respectively, facilitated the observation of animal's behavior, cardiac physiology, or brain function. In the "Motion Chip," only the head of a larva was fixed by a baffle feature partially blocking the outlet of a trapping channel, while the fins and tail were allowed to move freely to allow behavioral studies (Fig. 1(a)). For organ-specific physiology study, the "Lateral chip" was used to examine cardiac function, and the "Dorsal chip" was used to image brain function in response to acute ethanol stimuli (Fig. 1(a)). In both dorsal and lateral chips, the orientation control was achieved by trapping larval tail in a plane perpendicular (dorsal) or parallel (lateral) to the horizontal plane. As shown in supplementary Figs. S1(b) and S1(c),³⁸ individual trapping channel was 9.0 mm long with a 2.1 mm wide inlet and a 100 μm wide outlet. The trapping channel has a tapering design with a sharp restricted portion (150 μm), in which only the tail of a larva can fit in. When a larva was loaded into a trapping channel by hydrodynamic flow, the tails were then automatically squeezed into the tapering portion and make the larva to assume the appropriate orientation for easy observation of the heart or

FIG. 1. A microfluidic system for high-throughput manipulation of larval zebrafish. (a) Illustrations of the core setup and the basic designs of three different chips specifically for analysis of animal behavior (Motion), cardiac physiology (Lateral), and brain function (Dorsal). Scale bar, 200 μm . (b) A representative chip loaded with trapped larvae. Scale bar, 2 mm. (c) Image sequences showing the autonomous process for larva loading and immobilization in the Motion chip. Scale bar, 1 mm (d) Simulation of velocity field showing the flow dynamics in the Motion chip.

the brain in the following experiments. Specifically, after trapping in the microfluidic chips, the larvae were released from the system without any detectable injury (supplementary Fig. S3).³⁸

B. Computer numerical control (CNC) machining based quick fabrication

In most microfluidic related investigations, soft-lithography has been a widely used fabrication method.^{25–27} However, it is not suitable for creating lab-on-chip systems involving larval zebrafish under the requirements for accommodating feature sizes of both micro and macro scales, especially in the Z-direction. Therefore, we developed a fabrication method based on high-precision CNC (Computer Numerical Control) machining, which was efficient, cost-effective, and suitable for sophisticated design with different features ranging from micrometer to millimeter scales (Fig. 2). Negative molds of the chip designs were first CNC machined on plain copper plate with 30 μm resolution (Fig. 2(b)). Polydimethylsiloxane (PDMS) was used to make the chips with flow channels by replicate molding from the copper molds (Fig. 2(b)). After curing for 12 h, the PDMS structures were released from the molds and then bonded to glass substrate with plasma treatment to form the final microfluidic chips (Fig. 2(c)), which were connected to a computer-controlled system for larvae transportation²³ (supplementary Fig. S4).³⁸ Depending on the chips that were used, a direction-switching-loop was designed to adjust the direction of the larva after loading from the reservoir to ensure a larva in a tail-forward or head-forward position before going into the chip.

C. Ethanol-induced behavioral responses of zebrafish

To analyze ethanol-induced behavioral responses in larval zebrafish, the “Motion chips” were first used. Pectoral fin beats, eye saccades, and body movements were selected as the three parameters to quantify a larva’s behavior, which were manually analyzed from video recording by visual inspection. Before the behavioral analysis, a validation test was performed to compare the behaviors of trapped larvae to that of freely behaving larvae in E3 water (supplementary Fig. S5).³⁸ It is clear that microfluidic trapping did not compromise the regular activity of larval

FIG. 2. Fabrication of microfluidic devices using CNC micromachining. (a) Schematic showing the fabrication procedures. (b) Image of PDMS cured on copper mold fabricated with CNC micromachining. Scale bar, 2 mm. (c) Image of final microfluidic chip after assembly. Scale bar, 5 mm.

zebrafish, suggesting that our system is valid and convenient for experimental analysis. After loading into the chip, the larvae (7dpf) were treated with ethanol of different concentrations (from 0.00% to 3.00% v/v),^{6,28} and their behavioral responses were recorded for 10 min (Figs. 3(a) and 3(b)). The pectoral fin beats, a type of standard locomotion that plays an important role in coordinating body axis during slow swimming,^{14,29,30} showed a steady increase from 0.15 ± 0.03 beats/s (mean $\pm$ s.e.m, $n = 10$) to 0.33 ± 0.01 beats/s (mean $\pm$ s.e.m, $n = 10$) before and after ethanol treatment at low concentrations (0%–1.5% v/v, Fig. 3(c)). However, the fin beats dropped significantly to 0.11 ± 0.03 beats/s (mean $\pm$ s.e.m, $n = 10$) when the ethanol concentration reached 3.00%. Such dynamic changes of the fin beats suggested that ethanol may alter the swimming patterns of treated larvae by impairing motor coordination.^{29,31} Similarly, the rate of eye saccades also increased at low levels of ethanol exposure and dropped significantly at the concentration of 3% (Fig. 3(d)). Even though any increase of body movements was not observed in the early period (initial couple minutes) of ethanol application (supplementary Fig. S6),³⁸ the general body movements were also reduced a lot at 3% ethanol level (Fig. 3(e)). At the same time, a special pattern of locomotion, eye nystagmus, started to appear (Fig. 3(f)). As shown from the time-dependent recordings, acute ethanol treatment actually had a stimulatory effect on fin beats and eye saccades during the couple minutes of ethanol application and gradually turned into inhibitory afterwards (Figs. 3(g) and 3(h)), which was accompanied by the appearance of nystagmus (Fig. 3(i)). These changes in the eye movements indicated that acute ethanol treatment may impair the vision function in zebrafish larvae.^{13,32}

D. Analysis of cardiac function in response to ethanol treatment

In parallel with the behavioral analysis, we investigated the effects of acute ethanol exposure on cardiac physiology in larval zebrafish using the “Lateral chips.” In these experiments, the larvae were immobilized to assume a lateral-up orientations for easy observation of the heart, which

FIG. 3. Analysis of behavioral responses in larval zebrafish using "Motion" chips. (a) Applications of ethanol stimulus in a "Motion" chip while simultaneous fluorescence imaging. After trapping larvae, a gentle positive flow was maintained in the channels throughout assay period, and ethanol was applied by switching perfusion sources (E3 water or ethanol solution). (b) Representative image showing a larva trapped within the "Motion chip." Scale bar, 200 μm . (c)–(f) Analysis of (c) pectoral fin-beat, (d) eye saccades, (e) overall body movement, and (f) eye nystagmus in larvae treated with various doses of ethanol treatment. (g)–(i) Time-dependent dynamic response, (g) pectoral fin-beats, (h) eye saccades, and (i) nystagmus in response to 3.0% ethanol treatment. The perfusion starting point was set to be "0 min." For panels (c)–(i), $n = 10$, error bars indicate standard error of the mean (s.e.m.).

was fluorescently labeled in the *cmlc2* transgenic line (Fig. 4(a)), so that the heart beating could be directly analyzed by fluorescence microscopy (Fig. 4(b), supplementary Fig. S7).³⁸ The same four levels of ethanol environment (0.00%, 0.75%, 1.50%, and 3.00%, v/v) were tested. Generally, acute ethanol treatment resulted in a slower heart rate in a dose dependent manner (Fig. 4(c)). Through real-time recording of heart beat while *in situ* ethanol stimuli in the "Lateral chip," we found that the slow-down of heart beating started at couple minutes after ethanol treatment and reached plateau in about 8 min (Fig. 4(d)). This effect was in a similar time-frame that temporally correlated with the observed behavioral changes. The representative curves of the volume change of the heart contraction-relaxation revealed that ethanol induced cardiac arrhythmias actually led to the decrease of heart rate at all the tested doses (Fig. 4(e)).

FIG. 4. Analysis of cardiac physiology using “Lateral” chips. (a) Representative image of a larva (from *cmhc2:EGFP* transgenic line) trapped within a “Lateral” chip. Scale bar, 200 μm (left), 25 μm (right). (b) Analysis of heart beat by measuring volume change of the heart during a contraction-relaxation cycle. Scale bar, 25 μm . (c) Analysis of heart rate under ethanol treatment at doses from 0.0% to 3.0%. (d) Dynamic effects on heart rate in response to the 3.00% ethanol treatment. (e) Representative volume curves showing the abnormal cardiac cycles induced by ethanol treatment. In panels (c) and (d), the perfusion starting point was set to be “0 min.” (n = 10, error bars indicate s.e.m.).

E. Regulation of brain-wide activities by ethanol treatment

To further investigate ethanol-induced local and global effects on the nervous system, we then used the “Dorsal chip” coupled with calcium imaging to analyze the ethanol-induced acute effects on brain-wide activities in detail using the *elavl3:GCaMP5G* transgenic line (Fig. 5(a)). As shown in Fig. 5(b), spiking activity from specific brain regions can be tracked at cellular resolution in this platform. Over 15 min before and after ethanol treatment, accumulated spike numbers were derived from calcium fluctuations for multiple layers along the Z-axis, which was projected to a 2D plane to form an activity density map for evaluating ethanol-induced responses (supplementary Fig. S8).³⁸ Generally, ethanol showed obvious stimulatory effects on brain activities in a dose-dependent manner (Figs. 5(c) and 5(d)). The clusters of neurons that responded to ethanol first appeared in the caudal hindbrain at low dose (0.75%) and gradually extended into cerebellum, ventral midbrain, and even forebrain as the ethanol concentration was raised to 1.5% or 3.0%, mainly covering the histaminergic and dopaminergic pathways from an anatomical point of view, which was in line with previous reports showing involvement of both dopamine and histamine in ethanol-induced response in zebrafish.^{14,33,34} Interestingly, 3.0% ethanol slowed down most of behavioral activities (except nystagmus) and heart beating, while the excitation of forebrain was the most intense at this level. Further analysis on the time-dependent dynamics showed that 3.00% ethanol actually induced a biphasic effect on the forebrain: slightly inhibitory at the initial few minutes and strong excitatory after ~6 min (Fig. 5(e)); and such excitation coincided with the abnormal nystagmus as observed in our previous behavioral studies.

III. CONCLUSION

In this study, we developed an autonomous system for quick loading and precise trapping zebrafish larvae in array format with defined orientations. The core component of the system was based on one of the three microfluidic chips (“motion,” “lateral,” and “dorsal”) designed to accommodate observation and quantification of behavioral responses and cellular imaging of

FIG. 5. Analysis of brain function using "Dorsal" chips. (a) Representative image of a larva (from *elavl3:GCaMP5G* transgenic line) trapped within a "Dorsal" chip. Scale bar, 200 μm . (b) Detection of brain-wide neuronal activity with single cell resolution by using calcium imaging. Scale bar, 100 μm (top left panel), 50 μm (bottom left panel). (c) Fluorescence traces showing the calcium fluctuation in different neurons pointed out by red circle in confocal images (bottom right panel). (d) Heat maps showing the brain-wide activation in the brains of larvae treated with different concentrations' ethanol (from 0.00% to 3.00% v/v). Each map was derived by calculating the ratio of increased (or decreased) spiking activity over a 15 min period, $n = 10$. Manual segmentations of brain regions are: LF, left forebrain; RF, right forebrain; LM, left midbrain; RM, right midbrain; LC, left cerebellum; RC, right cerebellum; LH, left hindbrain; and RH, right hindbrain. (e) Time-dependent dynamics in LF (left forebrain) and RF (right forebrain) in response to 3.00% ethanol. In panels (d) and (e), $n = 5$, standard error of the mean (s.e.m).

specific organs in larval zebrafish under acute ethanol stimulus. As our system was capable to manipulate larvae without using anesthetics or rigid gels, this enabled us to perform analysis correlating sophisticated molecular phenotypes with behavioral change in awake larvae, which was not feasible in most existing strategies.^{17,19} Using this system, we studied ethanol-induced acute responses in behaving larvae from different aspects including behavioral changes, cardiac, and neurological physiology. We found that the motor function of a larva was significantly affected by ethanol treatment, especially at a high concentration of 3%, which induced a bi-phasic effect on specific type of eye movements; and the behavioral changes were temporally

coincided with the dynamic responses in cardiac and nervous system. Notably, ethanol showed strong excitatory effects in brain regions in caudal hindbrain, cerebellum, ventral midbrain, and forebrain, which has previously been reported to be responsible for locomotion and vision related functions.^{24,35,36} We believed that this novel and versatile Fish-on-Chip platform has the potential to be widely adopted in zebrafish studies.

IV. MATERIALS AND METHODS

A. Zebrafish lines

The transgenic zebrafish lines *elavl3:GCaMP5G*, *cmlc2:EGFP*, and *flil:EGFP* were maintained and crossed using standard techniques. The larvae of 5–8dpf were used in all experiments in this study. All animal work was carried out with prior approval from the animal ethical committee of City University of Hong Kong and was in accordance with local animal care guidelines.

B. Fabrication

A negative mold of our chip design was fabricated using high-precision CNC machining on plain copper material with 30 μm resolution. Biocompatible polydimethylsiloxane (PDMS) was used to make the flow channels by replicate molding from the copper mold. After curing for 12 h, the PDMS chips containing flow channels were then bonded to glass substrate after plasma treatment to make the actual chips.

C. Health assessment

To assess the health status in larvae being trapped in the microfluidic chips for multiple sessions over a four-day-period (supplementary Figs. S3(a) and S3(b)).³⁸ In each session, the larvae were loaded into the system and being trapped for 2 h and were released afterwards. In most of the larvae, no detectable injury was detected, as evaluated by their survival rate and morphological abnormal rate. These parameters have been previously used for health assessment in zebrafish model.^{19,20,23} Functional criteria included visual confirmation of normal heart-beat and reflex response to touch stimuli. Morphological criteria included spine bending (i.e., lordosis, kyphosis, and scoliosis) and craniofacial abnormalities. The heart rate of the larvae did not change over a 2-h assessing period when trapped in a Lateral chip, as illustrated in supplementary Fig. S3(c).³⁸

D. Microscopy

Imaging was performed on a fully automated inverted fluorescent microscope (Olympus IX81) equipped with a cooled sCMOS camera (Neo, ANDOR) with a 4 $\times$ (NA, 0.16) objective (for behavioral responses recording) or a 10 $\times$ (NA, 0.4) objective (for cardiac functional analysis and brain mapping). Micro-manager 1.4 was installed to control the microscope. For confocal imaging, Leica SP8 microscope with a resonant scanner (Leica SP8) was used to perform high resolution imaging with sufficient frame rate to capture calcium dynamics in single cells.

E. Heart rate analysis

The procedures for heart rate analysis were illustrated in supplementary Fig. S7.³⁸ This method was based on imaging processing, which captured the volume change of the heart during a contraction-relaxation cycle.³⁷ First, the time-series of fluorescence images were recorded using an inverted microscope. Second, a threshold was applied to every fluorescence image to generate a binary (0 or 1) template. The threshold was determined by taking average intensities for all pixels in a reference image from non-fluorescent larvae. The total counts for non-zero pixels were then used as an indicator for the heart volume (0.42 $\mu\text{m}^2/\text{pixel}$). Third, the contraction-relaxation of a heart was then determined by monitor the time-dependent fluctuation

of heart volume that was derived in step 2, and the heart rate was derived by quantify the number of contraction-relaxation cycle in a given time.

F. Brain activities' analysis

The procedures for analyzing brain-wide activity were illustrated in supplementary Fig. S8.³⁸ Briefly, a heat map was used to intuitively display the activation level of the brain. To map brain activities, every collected frame from calcium imaging was first meshed into small regions of interest (ROI), each with a size of 2×2 pixels. The time series of fluorescence signal in each ROI was then filtered by a high pass filter to remove the interference from illumination fluctuations. The resulted trace was used to calculate and visualize the spiking activities. For spike counting, a threshold was applied to detect spikes from neural activity. The brain activities' heat map was derived by taking the percentage of increased (or decreased) spike counts over a 10-min period before and after ethanol treatment.

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