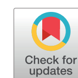
Sensors and Actuators B 268 (2018) 245–254

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Studying the time course of cardiac responses of the same zebrafish using scalable fish-dock microarchitecture



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ARTICLE INFO

Article history:
Received 16 December 2017
Received in revised form 2 April 2018
Accepted 17 April 2018
Available online 22 April 2018

Keywords:
Single-fish analysis
Water-level docking
Time-course normalization
Diphenylurea
Doxorubicin-induced cardiomyopathy

ABSTRACT

In conventional zebrafish (*Danio rerio*) studies, fishes are sacrificed at individual time points, so it is impossible to analyze the time-course responses of the same zebrafish. To tackle this problem, a PMMA-based microfluidic “Fish-dock” featuring scalable embryo loading, motility-based selection, orientation control, and drug dispensing functionalities was devised. Single zebrafish embryos were isolated and high-quality larvae were selected through an on-chip transfer mechanism for *in vivo* drug screening. Dorsal orientation of the larvae was gently and reversibly maintained by the “Fish-dock” to reduce motion artifacts during heartbeat recording. By repeatedly docking the same zebrafish at different time points, this microchip enabled parallel tracking of the cardiac function among 48 individual larvae for time-course experiments lasting over 48 h. Compared with conventional analysis method, the time-course normalized single-larva analyses revealed the similar dose dependence of diphenylurea (DPU) in treating doxorubicin (Dox)-induced cardiomyopathy, yet the drug treatment time was reduced by half compared with that conventionally required to reveal such a trend.

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1. Introduction

The zebrafish (*Danio rerio*) has been a favorite vertebrate model that bridges the gap between *in vitro* cell-based assays and *in vivo* rodent tests in biomedical and pharmacological studies [1]. In comparison with other vertebrate models, zebrafish has a distinct cost advantage in terms of its maintenance [2], while its short reproductive cycle is beneficial for the evaluation of drug toxicity [3–5], the discovery of bioactive natural compounds [6], and continuous monitoring of key developmental processes [2,4,7]. A zebrafish line with transparency and transgenic fluorescence further facilitates the physiological mapping of tissues and organs with drug efficacy and opens up the opportunity to study multi-system interactions [8]. Since the developments of the fish and human hearts utilize similar molecular strategies [9,10] and morphogenetic processes [11], the transparent zebrafish is especially useful for investigating the molecular basis of heart development and assessing the therapeutic potential of small molecules [9,12]. Owing to these advantages, zebrafish larvae are an ideal animal model for large-scale drug screening [13].

Despite the distinct advantages of a zebrafish model, there is a lack of a high-throughput, large-scale study platform that is tailored to tracing the dose responses of larvae in time-course experiments. To monitor the drug response of a particular organ, the current routine requires an individual zebrafish to be anesthetized and manually immobilized in low-melting-point agarose gel [14]. This labor-intensive practice is not only time-consuming but also introduces substantial analytical bias, which may lead to abnormal conditions of zebrafish, such as: physical damage, dehydration *etc.* [15]. Tracing the time-course responses of individual larvae is also not possible because they are sacrificed at each time point in conventional studies. Furthermore, to obtain a clear view of certain tissues of interest, current microscopic techniques require each larva to be confined within a small area and kept at a certain dorsal [16–18] or ventral [19,20] orientation, which makes the fully automated acquisition of data a great technical challenge, given the motility of living organisms.

Microfluidic chips, also known as labs on a chip, are a powerful tool to meet the challenges in large-scale zebrafish studies. Similar to when conducting zebrafish experiments on a microtiter plate [19], this approach enables an increase in the throughput by performing parallel experiments with repetitive microarchitecture. The development of zebrafish embryos has been monitored within a borosilicate glass-based microfluidic flow through a system featuring arrays of microwells. No significant difference in growth rate was observed between microfluidic and conventional approaches [21]. Subsequently, various microwell-based devices with integrated concentration gradient generators were established. The most frequently used architecture for the generation of a concentration gradient is the “pyramidal microfluidic network,” which is

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https://doi.org/10.1016/j.snb.2018.04.084
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based on controlled diffusive mixing of laminar flow streams by repeated splitting, mixing, and recombination [22,23]. The microwells have been adapted as culture chambers for single [2,24] or multiple embryos [4,5], under gradient concentrations. Although the throughput has been increased with the repetitive structure of microwells, these designs are only tailored for the study of embryos.

In addition to throughput increment, it is necessary to position larvae in a particular orientation for high-quality imaging of internal tissues. A capillary-based vertebrate screening platform (VAST) [25,26] has been developed to provide arbitrary orientation control for zebrafish larvae. Fixed or anesthetized larvae are positioned in an appropriate field-of-view and orientation by a computerized syringe pump and stepper motors, which actively rotate the larva inside a capillary tube. Although multiple angles and high-quality images of the entire larva are attainable, the single capillary design has limited scalability in screening throughput as only single fish are processed in sequential fashion. Besides, it is also difficult to integrate other functionalities, such as a gradient generator [2] or integrated electrodes [27], with the capillary-based design. In contrast to the VAST system, orientation control of a zebrafish larva has been implemented in parallel using polydimethylsiloxane (PDMS)-based microfluidic devices with various types of locally constricted microchannels that hold zebrafish in positions of interest.

A Zebrafish Entrapment by Restriction Array (ZEBRA) has been employed to achieve lateral and dorsal orientations by manually pipetting head-first or tail-first anesthetized larvae into the array [28]. While this method has opened up the possibility of orientation control through the geometric restriction of microchannels, the prerequisite of head-first and tail-first manipulation limits its potential automation. The first automatic, gel-free, and anesthetic-free platform has been successfully demonstrated by "Fish-Trap" [29] using a cell-docking principle [30] similar to that we have advocated for biological cells. The key to achieving automatic loading in Fish-Trap is to utilize the dynamic change of hydrodynamic loading force once a larva has been trapped and acted as a plug, such that other larvae are directed to unoccupied traps. However, the larva loading time should also scale linearly with sample size because the loading procedure is asynchronous and requires similar head-first or tail-first manipulation. Electroencephalographs have been recorded by an integrated zebrafish analysis platform (iZAP) through tight contact between an electrode and the head of a zebrafish restrained by a linear array of locally constricted microchannels [27]. While orientation control has also been implemented, the immobilization process takes about 30 min for 12 fish. Since all of these microfluidic designs rely on various forms of constriction to restrain the orientation of zebrafish, the elastomeric PDMS, which is notorious for its drug sorption issues, is a preferred substrate.

In this study, a PMMA-based microfluidic device with scalable sample loading, quality control, and orientation control functionalities is proposed and a concentration gradient is integrated into it to evaluate the dose dependence of a cardioprotective compound in Dox-induced cardiomyopathy using a zebrafish model. These scalable elements of larval manipulation address several key challenges in the automation of large-scale zebrafish studies. Furthermore, by time-course normalization of the data retrieved from the same larva, both the accuracy and the efficiency to reveal the dose by single-fish analysis are improved.

2. Materials and methods

2.1. Reagents

All chemicals and reagents were purchased from Sigma-Aldrich unless otherwise specified. Stock concentrations of 100 mM Dox and 50 mM DPU were prepared in dimethyl sulfoxide (DMSO). Three working solutions, namely, 50 μM Dox; 50 μM Dox and 5 μM DPU; and 50 μM Dox and 0.1 μM DPU, were freshly prepared before each experiment. A 10 mM stock solution of Resorufin and Rhodamine 110 (R110) was prepared in DMSO and two working solutions were freshly prepared: 10 μM Resorufin and R110, and 0.02 μM Resorufin and R110. The E3 medium was prepared using the following components: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$ at pH 7.2–7.3 [17]. Here, 50 × (10 mM) 1-phenyl-2-thiourea (PTU) stock was prepared in ddH_2O and diluted with E3 medium to 1× working solution before use.

2.2. Device fabrication

The device was designed using computer-aided design software (AutoCAD 2016; Autodesk, USA). A commercial 50 W water-cooling laser system (K3323; Julong Laser Systems, China) was used to cut polymethylmethacrylate (PMMA) sheets (Shengdalong Organic Glass Co., Ltd., China) with 65% power and 12 mm/s scanning speed, and a computerized numerical control (CNC) milling machine (Jingyan Instrument, China) was used to engrave microchannels (228 ± 10 μm width and 179 ± 13 μm depth) on PMMA surfaces with 10-degree V-tip carving tools. PMMA layers were cleaned by a soft brush, thoroughly rinsed, and soaked in ddH_2O for 48 h. After drying, the PMMA layers were carefully aligned and fastened in a C-clamp and the assembly was placed in a pre-heated oven for 20 min at 110 °C for thermal bonding. The whole device has six individual layers (Fig. 1a). Six outlet syringes were connected to the device through the three-way valves that were used for water-level manipulation and three inlet syringes were directly connected to the incubation chip. Inlet and outlet syringes were connected to the microchip by 1.2-m-long Teflon tubing and all of the syringes and tubing were covered by aluminum foil throughout the experimental period.

2.3. Zebrafish maintenance

Tg (*cmlc2*: GFP) transgenic zebrafish were maintained in accordance with the Zebrafish Handbook [31]. Adult fish were raised at 28 °C in an aquaculture system under a 12/12-h (light/dark) cycle and fed with newly hatched brine shrimp twice daily at 10:00 and 17:00. Male and female fish were cultured separately. Healthy one-year-old adult fish were randomly picked at a ratio of two females to three males and placed in a small tank separated by a divider overnight. The divider was removed when the light was turned on in the morning for fish mating. The fertilized eggs were collected with the help of a tea strainer and transferred to Petri dishes filled with E3 medium at 28 °C. The embryos were primarily screened at 8 hpf (hours post-fertilization) and 24 hpf based on their stages in embryogenesis under a stereomicroscope (Olympus SZX12). Pigmentation was inhibited by adding 0.2 mM PTU to the medium to facilitate fluorescence observation of the embryos and larvae. All animal experiments were conducted in accordance with ethical guidelines approved by the Institute of Chinese Medical Science, University of Macau.

2.4. Embryo loading, on-chip hatching, and spontaneous transfer

The loading tool (Fig. 1a) was attached to the incubation chip using a pair of plastic screws and the inlets and outlets were connected to two multi-channel syringe pumps (SPLab10 and SPLab12; Baoding Shenchen Precision Pump Co., Ltd.). A flow rate of 1000 $\mu L\,h^{-1}$ was used to prime the device for 30 min with E3 medium. For embryo loading, syringe pumps were paused, the device was allowed to float on a dish of E3 medium, and drops of embryos (~80 randomly picked embryos at 48 hpf) were man-

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