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A lateral-immobilization zebrafish microfluidic chip-based system for *in vivo* real-time evaluation of antithrombotic agents

Lijuan He[a], Hongxia Du[a], Yi Yang[a], Zhihua Guan[a], Jinjin Li[a], Honglin Li[a,c], Xudong Lin[b,*], Lili Zhu[a,*]

[a] *Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science & Technology, Shanghai 200237, China*
[b] *Guangdong Provincial Key Laboratory of Sensor Technology and Biomedical Instrument, School of Biomedical Engineering, Shenzhen Campus of Sun Yat-sen University, Shenzhen 518000, China*
[c] *Innovation Center for AI and Drug Discovery, East China Normal University, Shanghai 200062, China*

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ABSTRACT

Thrombosis remains a major global health concern mainly characterized by high rates of morbidity and mortality. Animal models serve as an indispensable tool to understand the underlying pathogenesis of thrombosis and assess the efficacy of novel antithrombotic drugs. Currently, zebrafish has emerged as a valuable model organism for thrombosis research. However, the traditional method of studying zebrafish thrombosis requires a laborious and time-consuming procedure, including anesthesia and manual immobilization of zebrafish. In this study, based on hydrodynamic force, a lateral-immobilization zebrafish microfluidic chip (LIZMC) was designed to evaluate the cardiovascular system of multiple larvae within a single microscope field of view. Specifically, coupling with microscope imaging, real-time monitoring of the peripheral blood circulation in the tail of phenylhydrazine (PHZ)-induced zebrafish thrombosis was enabled. Furthermore, the reliability of LIZMC for *in vivo* evaluation of antithrombotic agents in zebrafish was verified using aspirin. Collectively, this novel LIZMC-based system can be used for *in vivo* zebrafish thrombosis studies and rapid screening of antithrombotic agents.

Thrombosis is a major trigger for death and disability worldwide, with myocardial infarction, thromboembolic stroke, and venous thromboembolism as key thrombotic cardiovascular disease (CVD) events [1–3]. As the population ages, the global burden of thrombosis is likely to increase. Antithrombotic drugs can be classified into three types: antiplatelet agents, anticoagulants, and fibrinolytic agents [4]. Nevertheless, all antithrombotic drugs are associated with an increased risk of bleeding, especially in elderly patients [5,6]. More studies are needed to better understand the pathophysiology of thrombosis, as well as to discover more candidate agents to treat thrombosis. Therefore, the establishment and evaluation of thrombosis model are crucial for developing safer and more effective antithrombotic drugs.

Over the last decade, the utilization of zebrafish as a screenable vertebrate model has revolutionized the scope of genetic investigations of complex diseases, owing to its high fecundity, speedy embryonic development, optical transparency, and extensive functional similarities with mammalian species [7–9]. Currently, several zebrafish thrombosis models have been established, including ferric chloride-induced, laser-induced, and phenylhydrazine (PHZ)-induced thrombosis [10–12]. However, traditional manual methods used for handling zebrafish exhibit instability and low throughput [13,14]. First, the amount of tedious manual operations, such as plate administration, anesthesia, and gel immobilization may result in low throughput and high time costs. Second, since zebrafish larvae are transparent, it is difficult to accurately determine their fixed position with the naked eye. Third, the use of traditional fixing tools such as forceps to repeatedly adjust the zebrafish's position may cause damage to the larvae. The advent of microfluidic technology and the consequent emergence of "Lab-on-a-chip" technology present a promising tool for the study of zebrafish biology [15–17]. Many microfluidic chips have been developed to study zebrafish development and behavior [18–20], but there is currently a dearth of chips focused on the studies of the blood circulation in zebrafish.

In this study, a novel lateral-immobilization zebrafish microfluidic chip (LIZMC) was developed for *in vivo* real-time monitoring of peripheral blood circulation in the tail of zebrafish (Fig. 1). The

* Corresponding authors.
E-mail addresses: linxd37@mail.sysu.edu.cn (X. Lin), zhulfl@ecust.edu.cn (L. Zhu).

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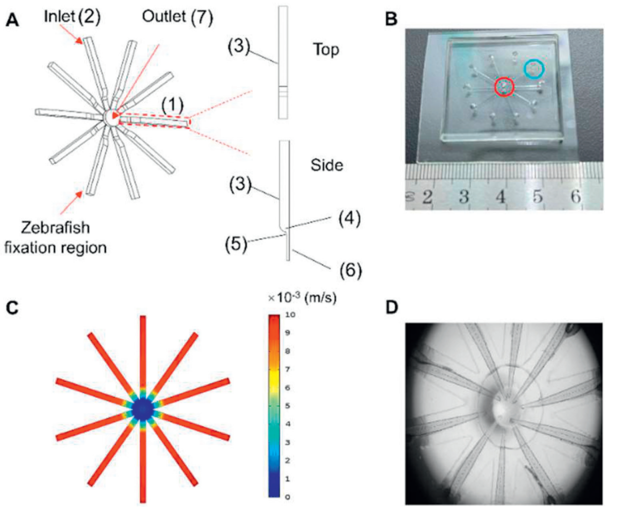

Fig. 1. Structure of the zebrafish LIZMC. (A) Composition of the LIZMC: (1) individual monomer; (2) inlet; (3) main channel; (4) head fixed chamber; (5) height gradient area; (6) tail fixed chamber; (7) outlet. (B) An image of the LIZMC. Red circle: outlet; Blue circle: inlet. (C) Simulation results of the flow dynamics within a chip after larvae loading. (D) An image of ten zebrafish larvae fixed in the LIZMC.

LIZMC consists of ten channels, and achieves automated loading and precise capturing of zebrafish larvae. The larvae at 3 days post fertilization (dpf) can be loaded into the LIZMC and laterally immobilized by fluid flow dynamics. The LIZMC is continuously perfused with the culture solution (or the solution containing drugs), which is delivered by an automatic injection pump through the shunt chip at a slow flow rate (Fig. S1 in Supporting information). Peripheral blood circulation in the tail of zebrafish, such as erythrocytes and platelets aggregation, can be observed in real-time by a microscope. This innovative system thus has potential applications for *in vivo* zebrafish thrombosis studies.

The LIZMC for studying the cardiovascular system of zebrafish is composed of ten individual monomers (1) and an outlet (7), as depicted in Fig. 1A. Each monomer is comprised of five parts: an inlet (2), a main channel (3), a head fixed chamber (4), a height gradient area (5), and a tail fixed chamber (6). The inlet diameter of the LIZMC is 1.6 mm, as indicated by the blue circle in Fig. 1B. The solution, therefore, flows out through the outlet, which is shown by the red circle in Fig. 1B. The main channel (3) has a width of 700 µm and a height of 710 µm, while the height of head fixed chamber (4) decreases from 710 µm to 270 µm. Moreover, the height of the height gradient area (5) is reduced from 270 µm to 180 µm, and the height of the tail fixed chamber (6) is 180 µm. The head fixed chamber, height gradient area, and tail fixed chamber are combined to form a half-funnel shape. The overall appearance of LIZMC resembles a spoke. The tail of the larvae firstly enters the chip, and then the flowing solution brings the larvae lateral-immobilized to a fixed position in the LIZMC based on the hydrodynamic principle and the tapered microstructure design (Fig. 1C). As a result, the tails of ten zebrafish larvae are observed under the microscope (Fig. 1D). In summary, this system allows the simultaneous processing of multiple zebrafish larvae without causing physical damage, and enables the automatic orientation and immobilization of larvae.

In order to assess the applicability of the LIZMC, we investigated the survival quality of zebrafish larvae cultured in the LIZMC. The heartbeat of zebrafish cultured in the LIZMC for 24 h was monitored and analyzed (Fig. 2A). The resulting data demonstrated that the heart rate of zebrafish cultured in the chip was approximately 175 beats/min, which was consistent with the previously reported results for zebrafish larvae at 4 dpf [21,22]. Touch-evoked escape behaviors can be used to test the motility of zebrafish. After being cultivated in the LIZMC for 24 h, zebrafish larvae swam rapidly away from the initial position in response to touch, which was consistent with the escape response of larvae cultured in plates (Fig. 2B). Moreover, the survival rate of zebrafish cultured in the LIZMC for 48 h was 100%. Therefore, the LIZMC might have no effect on the survival quality of zebrafish and the long-term cultivation of zebrafish larvae in LIZMC could be achieved.

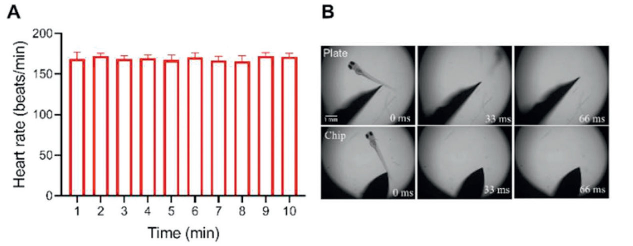

Fig. 2. Evaluation of zebrafish viability in the LIZMC. (A) Heart rate detection of zebrafish cultured in the LIZMC for 24 h. Bars represented mean ± SEM. (B) Touch-evoked responses of zebrafish cultured in the LIZMC and plates.

Subsequently, we utilized PHZ as a thrombosis inducer to establish the zebrafish thrombosis model in the LIZMC. PHZ is known to externalize phosphatidylserine (PS) on red cells and thrombocytes, and generate superoxide radicals, which cause oxidative damage to the lipid membrane of erythrocytes and thrombocytes rapidly adhere to the endothelial surface, leading to thrombosis [10]. To directly observe the aggregation of erythrocytes and platelets, we used the transgenic line *Tg(gata1:dsRed)* and the thrombocyte-labeling line *Tg(−6.0itga2b:EGFP)* to study the cardiovascular system of zebrafish. Firstly, 3 dpf zebrafish larvae were loaded into the LIZMC and laterally immobilized by fluid flow dynamics. Then 0.5 µmol/L PHZ solution was delivered to the LIZMC by an automatic injection pump through the shunt chip with the speed of 60 µL/min. Zebrafish were cultured in the LIZMC for a duration of 12 h, and then the degree of erythrocytes aggregation and the number of circulating platelets were detected by fluorescence microscope. Compared to the control group, the larvae treated with 0.5 µmol/L PHZ for 12 h showed a significant reduction in caudal blood flow (Videos S1 and S2 in Supporting information). The accumulation of erythrocytes in the caudal vessel was observed in the PHZ-treated group, indicating the formation of thrombosis (Fig. 3 and Fig. S2 in Supporting information). In addition, PHZ-treated larvae had a notable decrease in the number of circulating platelets in the peripheral blood circulation (Fig. 4, Fig. S3 and Videos S4 and S5 in Supporting information), which was presumably due to cumulative platelets attachment to injured endothelial cells. To summarize, we detected that PHZ led to erythrocytes aggregation and the reduction of circulatory platelets in zebrafish, which was consistent with the reported literature [23]. It was proposed that PHZ induced thrombosis possessed features of both venous and arterial thrombosis [23]. Based on these representative pathological changes of thrombosis, we decided to use the LIZMC-based PHZ-induced zebrafish thrombosis model for further study.

To evaluate whether the LIZMC-based zebrafish thrombosis model is suitable for assessing antithrombotic agents, we confirmed its effectiveness with the common antithrombotic drug, aspirin (Asp). All zebrafish were cultured in the LIZMC for 12 h with or without drug treatment. Compared with the PHZ-treated model group, the group of PHZ-induced zebrafish with Asp protection showed that the accumulation of erythrocytes decreased (Fig. 3, Fig. S2) and the caudal blood flow increased (Videos S2 and S3 in Supporting information). Moreover, the peripheral platelet circulation was significantly increased following Asp treatment (Fig. 4, Fig. S3, Videos S5 and S6 in Supporting information). The above

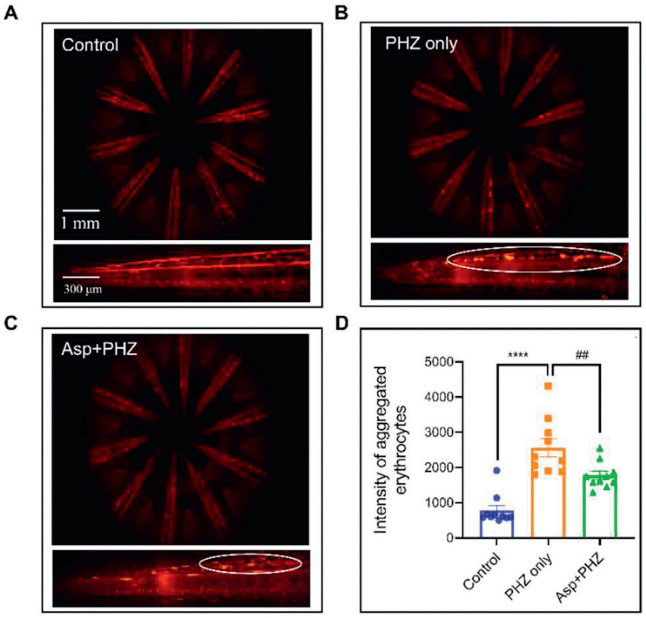

Fig. 3. Effect of drug treatment on the LIZMC-based PHZ-induced *Tg(gata1a:DsRed)* zebrafish thrombosis model. (A) Erythrocytes in control larvae. (B) Erythrocytes in PHZ treated larvae. (C) Erythrocytes in PHZ treated larvae with Asp protection. (D) Quantification analysis of the intensity of aggregated erythrocytes. Aggregated erythrocytes were circled in white. Bars represented mean ± SEM, $n = 10$. $^{\#\#}P < 0.01$. $^{****}P < 0.0001$.

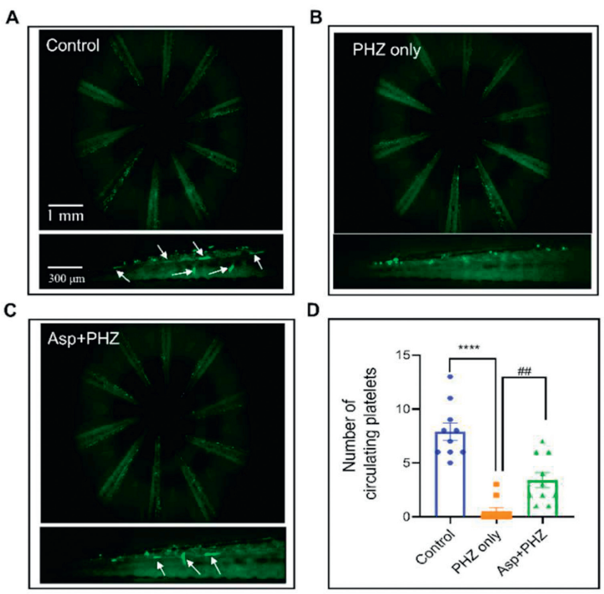

Fig. 4. Effect of drug treatment on the LIZMC-based PHZ-induced *Tg(−6.0itga2b:EGFP)* zebrafish thrombosis model. (A) Platelets circulation in control larvae. (B) Platelets circulation in PHZ treated larvae. (C) Platelets circulation in PHZ treated larvae with Asp protection. (D) Quantification analysis of circulating platelets. White arrows point to circulating platelets. Bars represent mean ± SEM, $n = 10$. $^{\#\#}P < 0.01$, $^{****}P < 0.0001$.

results validated that Asp protection significantly restored the circulation of erythrocytes and platelets in PHZ-treated zebrafish, and inhibited the endogenous thrombus formation, which were consistent with the reported literatures [23,24]. Overall, our findings suggested that the LIZMC-based system could be a powerful tool for assessment of antithrombotic agents.

In this study, taking advantage of microfluidics technology, we developed a novel system for real-time monitoring of peripheral

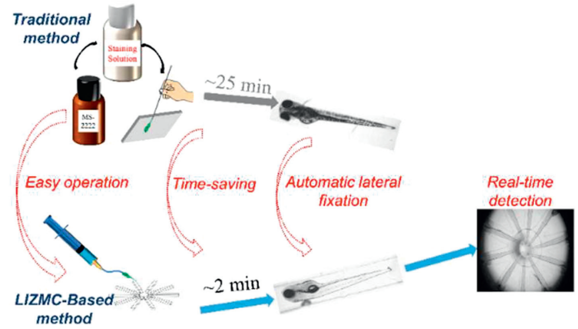

Fig. 5. Advantages of the LIZMC-based method compared to the traditional method.

blood circulation in the tail of zebrafish, as well as the evaluation of antithrombotic agents. In comparison to traditional experimental methods, the LIZMC-based PHZ-induced zebrafish thrombosis model has several advantages (Fig. 5): (1) It is simple and easy operation without plate administration, anesthesia and agarose immobilization procedures. (2) It is time-saving to immobilize zebrafish. The immobilization time of one zebrafish larva using the LIZMC is approximately 2 min, however, the traditional method probably needs 25 min to complete the fixation of one zebrafish larva. (3) It achieves automated loading and lateral fixation of zebrafish larvae, which is beneficial for observation of tail peripheral blood circulation. (4) It can be used to real-time monitor peripheral blood circulation in the tail of zebrafish and evaluate the cardiovascular system of multiple larvae within a single microscope field of view.

Declaration of competing interest

The authors declare that they do not have any known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.109013.

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