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Research article

A high-throughput system for drug screening based on the movement analysis of zebrafish

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ABSTRACT

Zebrafish is a highly advantageous model animal for drug screening and toxicity evaluation thanks to its amenability to optical imaging (i.e., transparency), possession of organ structures similar to humans, and the ease with which disease models can be established. However, current zebrafish drug screening technologies and devices suffer from limitations such as low level of automation and throughput, and low accuracy caused by the heterogeneity among individual zebrafish specimens. To address these issues, we herein develop a high-throughput zebrafish drug screening system. This system is capable of maintaining optimal culturing conditions and simultaneously monitoring and analyzing the movement of 288 zebrafish larvae under various external conditions, such as drug combinations. Moreover, to eliminate the effect of heterogeneity, locomotion of participating zebrafish is assessed and grouped before experiments. It is demonstrated that in contrast to the experimental results without pre-selection, which shows ~20 % damaged motor function (i.e., degree of attenuation), the drug-induced variations among zebrafish with equivalent mobility reaches ~80 %. Overall, our high-throughput zebrafish drug screening system overcomes current limitations by improving automation, throughput, and accuracy, resulting in enhanced detection of drug-induced variations in zebrafish motor function.

1. Introduction

Zebrafish, with its transparent nature and easily reproducible genome, has become a vital model organism in biomedical research. It has found extensive applications in genetics, biology, pharmacology, toxicology, and ecology [1–4]. Moreover, zebrafish drug

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toxicity evaluation technology has received GLP certification from regulatory bodies such as the FDA (Food and Drug Administration) and EMA (European Medicines Agency), ensuring compliance with standards for materials entering clinical trials [5,6]. These factors have set the stage and propelled the rapid advancement of zebrafish drug screening technology and equipment.

Drug screening based on the movement behavior of zebrafish larvae represents a significant research area [7–9]. However, current methods for drug screening using zebrafish larvae encounter several challenges. For instance, Chen et al. demonstrated that despite attempts to conduct rapid drug screening by monitoring changes in zebrafish larvae swimming distances, experimental throughput was limited by the use of a single 24-well plate [10]. Zimmermann et al. employed 48-well plates for imaging zebrafish larvae, yet relied on external commercial software for behavioral tracking to facilitate drug screening [11]. Similarly, Li et al. placed zebrafish larvae in 24-well plates and utilized commercial recording devices and analysis software to capture movement data [12]. Overall, current drug screening research based on zebrafish larval behavior faces challenges such as limited experimental throughput, low automation levels, and inadequate drug screening experimental systems [13–16]. While some studies have demonstrated high-throughput analysis of zebrafish larvae, they often overlook issues related to high-throughput imaging and environmental stability [15,17]. Additionally, variations in locomotor function among individual zebrafish may influence drug screening outcomes [18–22]. Currently, specific methods to address these challenges remain elusive.

In this study, we propose an automated drug screening system, specifically designed for high-throughput analysis of zebrafish larvae movement. The system is capable of accommodating 12 well-plates under well-controlled environmental conditions, i.e., at a temperature of 28 °C. Through automated image acquisition and analysis, the motion of 288 zebrafish is simultaneously tracked, enabling real-time preliminary screening of zebrafish motor function. Our results demonstrate that, without pre-selection, drug-induced motor function impairment (i.e., degree of attenuation) leads to approximately a 20 % difference between the drug-treated zebrafish (copper sulfate and ethanol) and the control groups. However, when evaluating zebrafish with equivalent mobility, this distinction increases up to ~80 %. Therefore, the proposed system is suitable for drug screening and evaluation studies using zebrafish as a model animal, achieving a fine balance between accuracy and high-throughput according to specific experimental requirements.

2. Material and method

2.1. System configuration

The system is designed using SolidWorks software and produced using 3D printing (CR-3040, CREALITY and UTSLAC600, UnionTech, China). The software controlling the system is developed using MATLAB software. The camera is sourced from Yichuang Electronic Imaging manufacturer (China), the light source is purchased from MFRD Optical Technology manufacturer (China), PCR sealing film sourced from Bohui Laboratory Consumables (China). The environment control unit maintaining a temperature of 28 °C is custom-made as described elsewhere [23].

2.2. Zebrafish culture

The wild-type AB strain zebrafish [24] at four days' post-fertilization are cultured in E3 medium (NaCl: 0.29 g/L, KCl: 0.012 g/L, CaCl2: 0.036 g/L, MgSO4: 0.04 g/L, pH 7.4). Subsequently, they are transferred to a 28 °C incubator for further cultivation. At the age of 4–5 days, zebrafish larvae are individually placed into the wells of our 24-well plate, i.e., one fish per well. The plate is then positioned in the automated high-throughput system for drug-screening tests. The zebrafish drug experiments mentioned in this paper comply with the ARRIVE guidelines, the U.K. Animals (Scientific Procedures) Act and associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Research Council's Guide for the Care and Use of Laboratory Animals. As zebrafish have not yet differentiated by sex at this stage, gender has no influence on the research results described in this paper.

2.3. System operation

To operate the system, we firstly, turn on the control unit and set the parameters for the experimental environment (Fig. S1a). Secondly, use a pipette to inject zebrafish and culture medium into the well plate (Fig. S1b). Place one fish in each well and ensure that the culture medium volume is at 2 ml. The control unit monitors the temperature inside the box and controls the heating fan accordingly to maintain a stable temperature of 28 °C. Once the environmental conditions inside the high-throughput testing box are stable, well plates are carefully placed in position (Fig. S1c). To minimize the impact of environmental changes on zebrafish behavior, the zebrafish are cultured for 5 h in the testing box before the behavioral analysis experiment.

After the system are stabilized, we connect the computer (i.e., the imaging and analysis software) to the testing box (Fig. S1d), which synchronizes the camera and light source to collect images sequence in the format of TIF or JPG, or video files (AVI) at a frame rate of 15 frames per second (Fig. S1e). In this study, zebrafish larvae were cultured under dark conditions, while the collection of motion behavior images was conducted under light conditions. Following a 10-min exposure to the drug, we analyzed their movement.

2.4. Data analysis

The image sequence or video file are analyzed using a customized MATLAB program. Movement of individual zebrafish is tracked by virtually locking the centroid of the zebrafish head. Their trajectory can then be obtained and plotted. This study explores the effect of drugs on behavior by capturing 1000 frames (67 s) of images, with a frame interval time of 0.067 s. The movement ability of the fish

is measured by calculating their swimming distance every 0.13 s (2 frames). The distanced moved by larvae is calculated using the following formula:

$$s = |\vec{r}_{0.13} - \vec{r}_0|$$

where s was the distance of larvae within 0.13s(2 frames); $\vec{r}_0$ was the position vector of zebrafish larvae at the initial time (t_0); $\vec{r}_{0.13}$ represents the position vector of zebrafish larvae at the end time ($t_{0.13}$).

2.5. Drug preparation

To prepare 1.3 mg/ml copper sulfate (M1) and 4 mg/ml copper sulfate (M2), solid copper sulfate is weighed and dissolved in the zebrafish culture medium, followed by thorough shaking to ensure complete dissolution. For 0.1 ml/ml ethanol (M3) and 0.2 ml/ml ethanol (M4), a specific volume of anhydrous ethanol is added to the zebrafish culture medium, and then thoroughly shaken until complete dissolution is achieved.

3. Result and discussion

3.1. Construction of a high-throughput zebrafish drug screening system

This paper develops an automated system for high-throughput analysis of zebrafish larvae movement and drug screening. Design of the system is shown in Fig. S2. It mainly consists of two parts: the testing box and the control unit (Fig. S2a). The testing box is 60 cm × 60 cm x 40 cm (length x width x height) in dimension, and fully sealed to ensure stable environmental conditions. The testing box can accommodate maximum 12 plates (24-well), and thus allows throughput of 288 zebrafish. Presently, equipment designed for studying zebrafish larval behavior typically exhibits low throughput. For example, Clift et al. demonstrated a single experimental processing capacity of 20 zebrafish larvae [18], while Li et al. showed a single experimental processing capacity of 24 zebrafish larvae [12]. In contrast, the testing chamber within the automatic drug screening system developed in this study can accommodate up to 12 trays, with each tray containing 24 wells. Consequently, the system enables simultaneous study of hundreds of fish specimens.

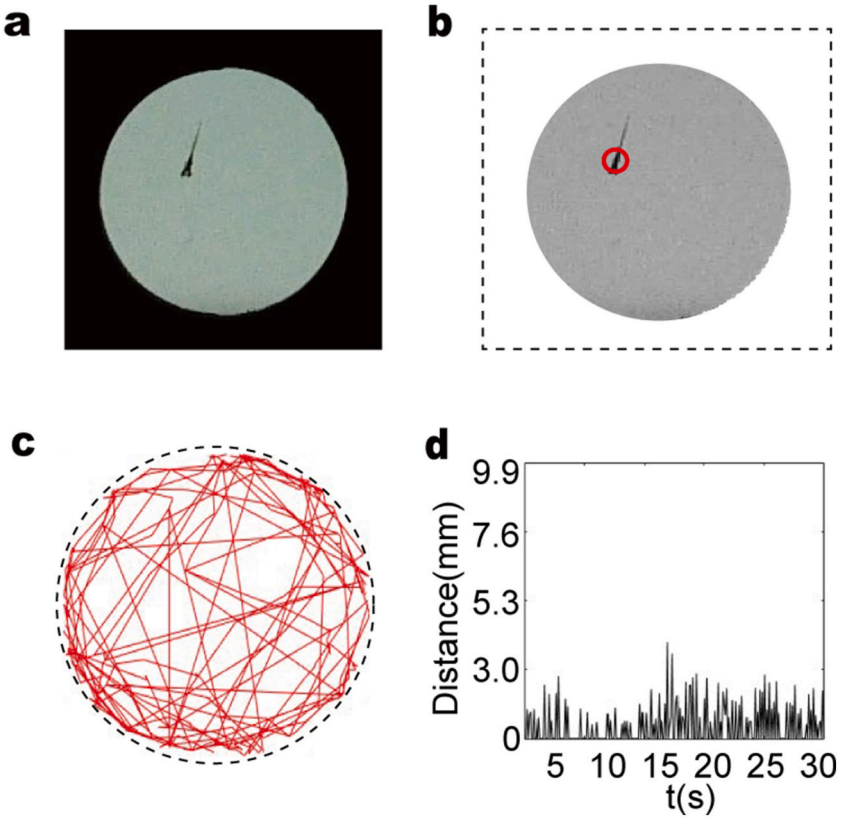

Fig. 1. Analysis of zebrafish larvae movement. (a). Original captured images, the actual diameter of the single hole is 16 mm. (b). Image processing, the pixel size of the aperture is 300 pixels. (c). Diagram illustrating the swimming trajectory of zebrafish. (d). Statistical analysis of the zebrafish swimming distance within 30 s over the experiment time-span.

To monitor zebrafish behavior, each well plate is equipped with a fixed-focus camera with a focal length of 13 cm and a field of view area of 140 × 100 mm at the top, and a light source at the bottom (Fig. S2b, Fig. S2c). To obtain image sequence or video of zebrafish movement, all 12 cameras are connected to the computer, and controlled using a customized Matlab program. The lightning and temperature within the testing box are controlled by connecting the lighting source to the Matlab program and a heating fan to the control unit, respectively. The sensor chip, which are mounted at the rear of the testing box, monitors the overall environmental conditions. Many devices designed to study zebrafish larval behavior often overlook the critical factor of maintaining environmental stability, which can significantly affect experimental precision [19–21]. In contrast, our system has established a stable experimental environment characterized by enclosed surroundings and consistent regulation of temperature and humidity levels (Fig. S2). This approach effectively mitigates the potential influence of environmental fluctuations on the behavior of zebrafish larvae.

The image acquisition and analysis unit of our system comprises a computer, an image acquisition program, and an image analysis program. In darkness, when the image acquisition program controls the camera to capture images, the light source is automatically activated. Following a 100-ms delay, the camera starts to capture an image. Our system controls up to 12 cameras to simultaneously monitor the behavior of 288 zebrafish.

3.2. Analysis of zebrafish behavior

To demonstrate high throughput capacities of the system, we conduct real-time analysis of the movement of 288 zebrafish larvae in 12 plates under dark conditions (Fig. 1a). Bright field images of the zebrafish indicate the image quality is consistent and clear enough for real-time tracking zebrafish behavior, e.g., trajectories and conformation. To locate individual zebrafish, we firstly convert the original RGB image into grayscale, and then to binary by applying a global threshold (Fig. S3). The noises are removed by performing pixel filling to smooth the edges. These processes result in a binary image with a radius of 150 pixels. In the binary images, the point representing the zebrafish is defined as "1″, while other points are designated as "0" (Fig. S3). Trajectories of zebrafish can then be obtained by connecting the centroids of the bright pixels (i.e., "1″) of image sequence (Fig. 1b, Fig. S4b).

After optimizing the images, we use software to track the movement of zebrafish larvae (Video S1), represented using a two-dimensional Cartesian coordinate system. The circular hole (the circular culture dish) is considered the origin of the x-axis and y-axis, with the leftmost and bottommost edges serving as the zero points, respectively (Fig. 1c, Fig. S4c). We conducted real-time tracking of zebrafish for 30 s as an illustrative example. The swimming distance within a defined duration is calculated by converting pixels to physical measurements, with the vertical axis (y) representing millimeters and the horizontal axis (t) representing seconds (Fig. 1d, Fig. S4d). The automatically acquired track (Fig. 1c) may differ from the fish's actual swimming path (Video S1). However, our comparison of automatic and manual tracking methods showed no significant differences between them.

Supplementary video related to this article can be found at doi:10.1016/j.heliyon.2024.e36495

Currently, in most behavioral studies of zebrafish, it is necessary to manually collect images of the zebrafish and then use software to analyze the images for tracking zebrafish movement and obtaining data [25,26].In contrast, our system integrates hardware and software components to enable the automated acquisition of zebrafish behavioral images, automatic tracking of zebrafish, and seamless generation of swimming trajectory images and swimming distance data through an internally developed image processing module and a dedicated swimming distance estimation algorithm, thereby achieving a high level of automation. Consequently, the system exhibits a sophisticated degree of automation, empowering operators to effortlessly conduct high-throughput drug screening experiments.

3.3. Drug screening tests

The system automatically collects the swimming distances of each fish. For instance, the swimming distance of zebrafish in the first

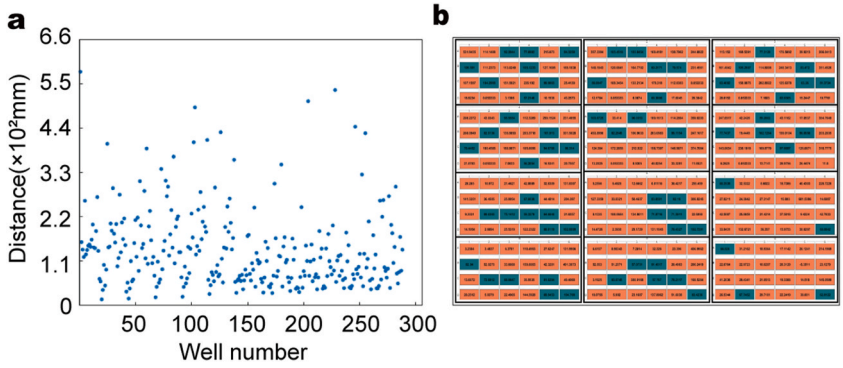

Fig. 2. Pre-screening of zebrafish swimming ability. (a). Scatter plot of the swimming distance of 288 zebrafish within 1000 frames (i.e., 67 s). (b). Automatic screening can be used for drug screening experiments on zebrafish. Green indicates the location of suitable zebrafish. While, orange indicates the eliminated ones. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

well of the first plate (1:1A) is 531.9435 pixels. Fig. 2 illustrates the swimming distances of the 288 fish, with each data point representing the moving distance of individual zebrafish over 1000 frames. The swimming distance of most zebrafish range from 0 mm to 590 mm, and centered between 53.3 and 106.7 mm (1000–2000 pixels) (Fig. 2a). This indicates significant heterogeneities in swimming abilities among individual zebrafish, with maximum distances reaching 580 mm and minimum distances as low as 10 mm, which may affect the results of drug screening tests targeting at motor functions.

Previous studies mostly rely on the averaged value of a group of zebrafish (e.g., the moving speed and distance) [27]. Clark, K J. et al. used a large number (96) of zebrafish, which are divided into four groups [28]. They evaluate the swimming ability of each group by measuring the proportion of exercise time per unit time (activity rate). Although this method partially addresses the issue of individual differences, it does not consider the effect of heterogeneity in zebrafish motor function. To address this issue, our system rapidly assesses the swimming ability of each zebrafish prior to administration (refer to Fig. S5 for the flowchart). For example, in this study, the system automatically analyzes and selects zebrafish with swimming distances ranging from 53.3 to 106.7 mm (1000–2000 pixels) for subsequent drug screening experiments. To streamline subsequent experimental procedures, the system automatically color-codes the well plates that meet the criteria as green (e.g., 1:3A, 2:4D), and the well plates that do not meet the criteria as orange (e.g., 1:1A, 2:1A). This color marking enables swift and accurate identification of suitable individual zebrafish during the drug administration process (Fig. 2b).

Fig. 3b illustrates the effect of different drugs on zebrafish motor function. Firstly, the system automatically selects 67 suitable zebrafish for experimentation and allocates them to five different groups: a control group (Co), a 1.3 mg/ml copper sulfate group (M1), a 4 mg/ml copper sulfate group (M2), a 0.1 ml/ml ethanol group (M3), and a 0.2 ml/ml ethanol group (M4). Drugs are then added to the designated well sites as shown in Fig. 2b. Following a 10-min exposure to the selected drug, we analyzed their movement. To assess the impact of the drugs on zebrafish motor function, we utilize a decay rate formula: D (degree of decay) = ((S0–S)/S0) × 100 %, where S0 represents the swimming distance of zebrafish prior to drug administration, and S represents the swimming distance of zebrafish after drug administration. Our results show varying degrees of impaired motor function compared to the untreated control group (Fig. 3b), which is dose-dependent (ethanol/copper sulfate) [29–32]. We conducted a two-tailed test to analyze the statistical significance. The test found significant differences between Prescreening groups and Non prescreening groups under the same drug conditions (Fig. 3; M1: $p < 0.01$; M2: $p < 0.01$; M3: $p < 0.01$; M4: $p < 0.01$).

Among a group of 288 zebrafish in total, 67 zebrafish are selected for drug screening experiments. Pre-screening of zebrafish enables the experimental samples to possess consistent motor abilities, eliminating the influence of heterogeneities on the outcomes. By comparing the results with and without pre-screening, it is evident that zebrafish with equivalent mobility can better reflect the drug-induced damage in motor function. For example, without pre-screening, the decay degree caused by 0.1 ml/ml ethanol is 22 % (Fig. 3a). In contrast, with pre-screening, the decay degree caused by ethanol is 78 % (Fig. 3b). Current high-throughput drug screening experiments involving zebrafish larvae often overlook the individual heterogeneity present in zebrafish larval swimming abilities [33–35]. This device mitigates the impact of individual heterogeneity in zebrafish on experimental results through pre-screening (refer to Fig. S5 for the flowchart). Prior to the experiment, the device automatically assesses the movement behavior of zebrafish samples and selects samples with similar swimming abilities for research (Fig. 2). Our research findings demonstrate that, in comparison to the control group without pre-screening, the zebrafish larval population subjected to pre-screening exhibits notable advantages in experimental outcomes.

The high-throughput drug screening system employed in this study ensures rigorous experimental precision by maintaining environmental stability within enclosed settings, thereby minimizing potential fluctuations. Compared to existing systems [13–15], its capacity to accommodate multiple trays with wells allows for simultaneous testing of numerous specimens, significantly enhancing throughput. Moreover, its capability to handle multiple trays with apertures facilitates concurrent testing of a large number of samples, thereby markedly improving throughput. Automation is achieved through integrated hardware and software for seamless image acquisition, zebrafish tracking, and comprehensive data analysis, thereby optimizing efficiency in behavioral studies. Furthermore, the system records zebrafish trajectories and positional data at multiple time points, facilitating analysis of various parameters based on

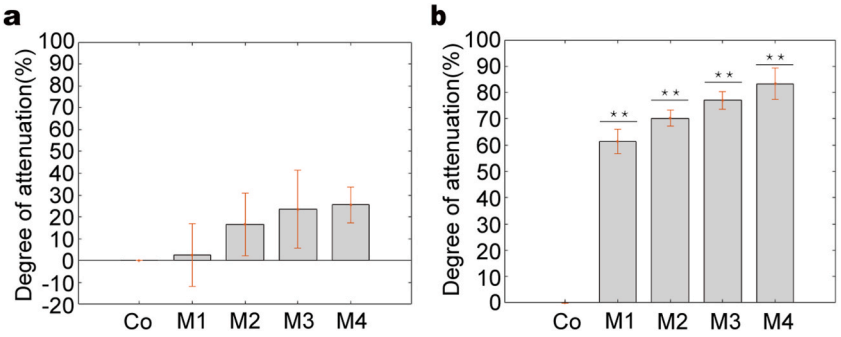

Fig. 3. Effect of different drugs on zebrafish motor function with and without prescreening. (a). Without prescreening, drug-induced attenuation in zebrafish motor function is approximately 20 %. (b). With prescreening, mobility of zebrafish in the control group (Co) is ~80 % higher than the ones treated by drugs including M1 = 1.3 mg/ml copper sulfate; M2 = 4 mg/ml copper sulfate; M3 = 0.1 ml/ml ethanol; M4 = 0.2 ml/ml ethanol. *$p < 0.05$, **$p < 0.01$.

positional changes. To address individual heterogeneity in zebrafish swimming abilities, the system incorporates automated pre-screening of samples, ensuring experimental consistency without compromising throughput.

4. Conclusion

In this study, we present an automated system for high-throughput drug screening using zebrafish, which enables real-time monitoring and analysis of zebrafish larvae movement. It is demonstrated that the system effectively collects the swimming data of 288 zebrafish. The data is then automatically grouped based on the motion abilities of individual zebrafish, which eliminates the effects of heterogeneity and improves the accuracy of drug screening experiments. When compared to existing technologies, this system has significant advantages. Therefore, the proposed system holds considerable potential for its application in high-throughput zebrafish behavior monitoring and drug screening research.

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Ethics statement

The zebrafish experiments were conducted according to the ethical guidelines of Northwest University. All experimental protocols were approved by the Experimental Animal Management and Ethics Committee of Northwest University, and the ethical code was NWU-AWC-20200302Z.

Informed consent statement

Not applicable.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Xinkai Zhao: Writing – original draft, Software, Resources, Investigation. **Ziyu Li:** Writing – original draft, Visualization, Software. **Bingbing Cao:** Visualization, Resources, Investigation. **Yichao Jin:** Resources, Investigation, Conceptualization. **Wenxing Wang:** Visualization, Conceptualization. **Jing Tian:** Writing – review & editing, Conceptualization. **Liang Dai:** Writing – review & editing, Methodology. **Dan Sun:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration. **Ce Zhang:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36495.

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