



High-throughput automated manipulation and imaging analysis of zebrafish larvae

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Abstract

Zebrafish (*Danio rerio*) larvae are optically transparent, small vertebrates that serve as an ideal model for in vivo high-throughput screening. However, automated high-throughput manipulation, imaging, and analysis of live zebrafish larvae remain considerable challenges. Moreover, it is critical to avoid damage to these delicate organisms because even minor injury can impair normal physiological function. To address these challenges, we developed a high-throughput manipulation platform that enables the loading of zebrafish larvae into glass tubes, enabling accurate localization and controlled rotation at a throughput of one fish every 15 s. The platform uses hydrodynamic methods, including a siphon-based system, as the primary driving mechanism to enable adaptive loading and unloading of zebrafish larvae while substantially reducing fluid pressure and minimizing potential flow-induced damage. In addition, the platform integrates machine vision to support automated control and data acquisition throughout the entire workflow. We developed a comprehensive suite of algorithms for automated image segmentation of zebrafish larval videos and three-dimensional (3D) reconstruction of their transparent structures and internal organs. Two distinct 3D reconstruction algorithm pipelines were designed: one is a computationally efficient, lightweight approach optimized for execution on conventional personal computers; the other is a high-speed rendering-based algorithm that requires large-memory computational resources to achieve reconstructions at the original image resolution. Notably, both approaches operate without requiring any additional user-defined parameter tuning. Finally, we demonstrate the platform's capability by evaluating drug-induced inhibition of zebrafish melanogenesis. This automated platform expands the utility of zebrafish as a versatile, high-throughput model system, facilitating research across diverse fields, including developmental biology, disease modeling, and pharmaceutical and toxicological evaluation.

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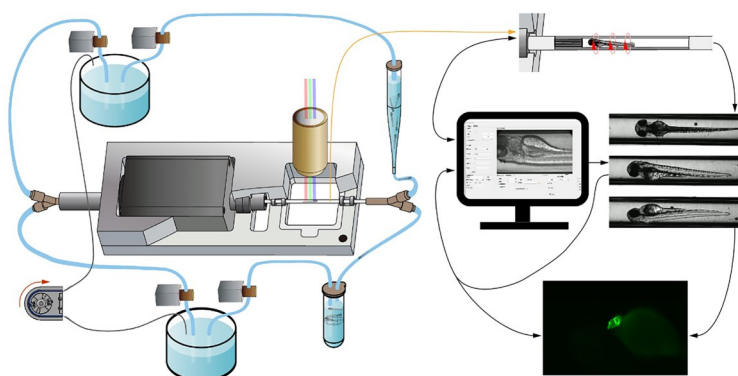
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Graphical abstract



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1 Introduction

Zebrafish offer several advantages, including high genetic homology with humans, small body size, and optically transparent embryos. These characteristics make zebrafish larvae particularly well suited for high-throughput biological studies, such as human disease modeling, drug screening, investigations of vertebrate embryonic development, and environmental toxicology research [1, 2]. High-throughput chemical screening using zebrafish provides an important complement to cell-based assays and represents a promising direction for future drug discovery [3]. However, realizing this potential requires efficient operational platforms and analytical methods because most current zebrafish-based screening approaches rely heavily on manual procedures, resulting in low efficiency.

Researchers have devoted substantial effort to developing techniques for accurate zebrafish positioning to enable efficient sequential loading and controlled orientation for acquiring informative images. For example, embedding zebrafish in agarose or gel matrices (e.g., fish capsules) can effectively immobilize larvae but offers limited flexibility in posture adjustment [4]. Microfluidic chips, while useful for loading zebrafish, cannot capture images from multiple angles [5]. Other approaches, such as syringe pump-controlled flow for loading zebrafish into glass tubes (e.g., the vertebrate automated screening technology (VAST)), suffer from drawbacks including high cost [6]. Additionally, techniques such as acoustic tweezers are complex and difficult to operate [7]. Overall, existing solutions are often time-consuming, limited in applicability, prone to causing injury, operationally challenging, or prohibitively expensive. Therefore, there is a strong need for an automated zebrafish larval manipulation and imaging analysis platform that is high-throughput, versatile,

safe, user-friendly, cost-effective, and reliable (Table S1 in the supplementary information).

Here, we present a self-driving, high-throughput zebrafish larval screening system (Fig. 1) that uses hydrodynamic methods, such as siphoning, to adaptively load zebrafish larvae into glass tubes equipped with auxiliary limiting structures for accurate positioning and posture control. This strategy enables high-throughput operation, substantially improves the success rate and flexibility of zebrafish larval loading, and minimizes physical damage by eliminating the need for external pumps. In addition, the system supports high-speed, multi-angle morphological phenotypic analysis of zebrafish larvae. We developed compatible interfaces for a wide range of microscope and camera configurations. Furthermore, we implemented an algorithm that enables rapid, multi-angle, real-time microscopic imaging of live zebrafish larvae, thereby facilitating high-content, quantitative *in vivo* phenotyping analysis. This method enables a range of tasks, including image segmentation, three-dimensional (3D) reconstruction, real-time monitoring, and quantitative analysis of zebrafish larval images. Compared with existing software [8–12], the workflows presented here require no additional user intervention or parameter tuning in the experiments described, achieving full automation from image acquisition through final evaluation. We successfully implemented automated image segmentation of zebrafish larval videos and 3D reconstruction of their transparent structures and internal organ features. Unlike existing approaches—such as surface-contour-based 3D reconstruction [13, 14], fluorescence optical sectioning [15, 16], optical projection tomography [17, 18], or implicit reconstruction [19, 20], we present an explicit 3D reconstruction approach based on high-speed rendering that uses irregular, dense point clouds to represent zebrafish 3D models. Each reconstructed model comprises approximately

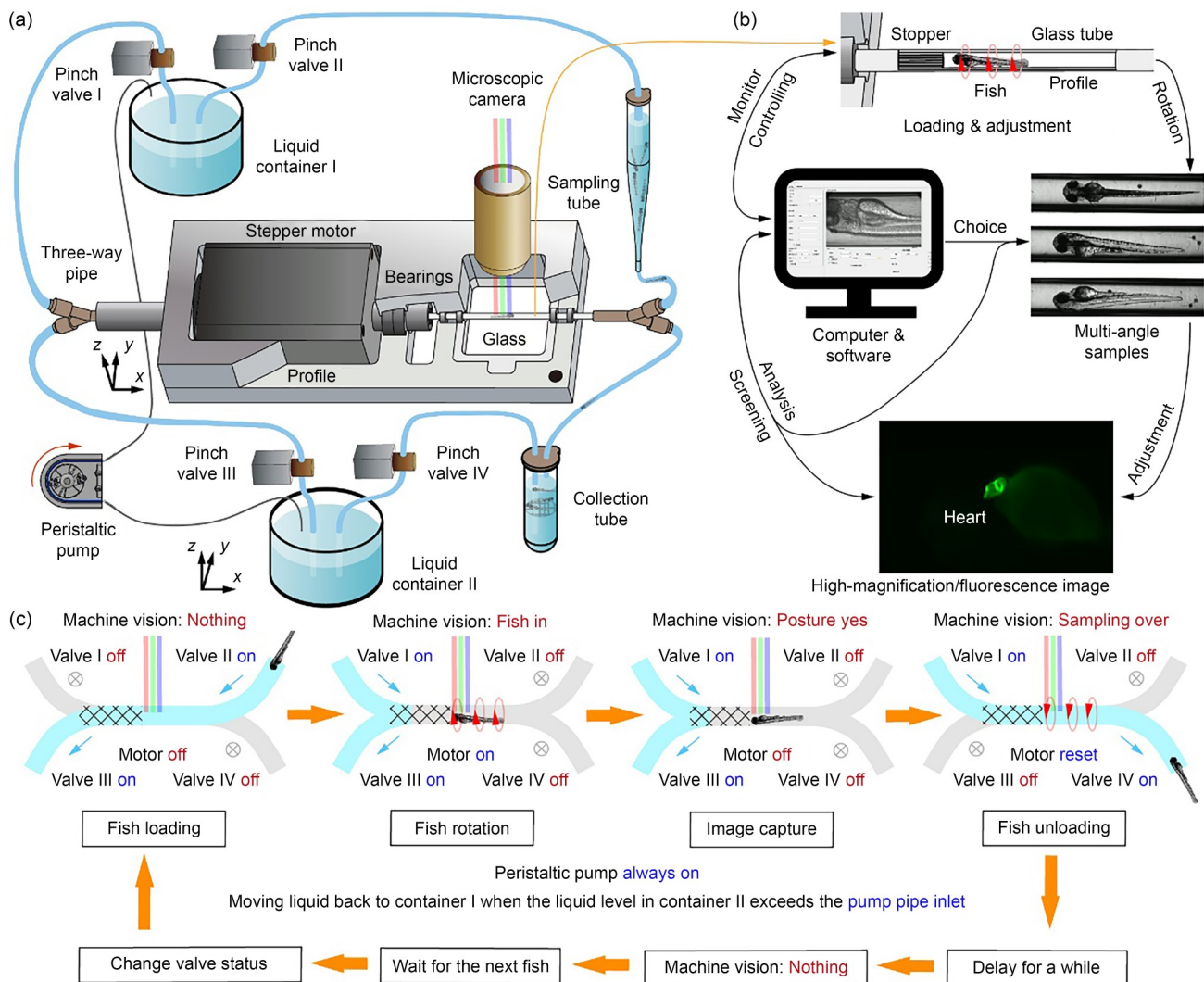


Fig. 1 Schematic of the high-throughput automated zebrafish larvae manipulation and imaging analysis platform. (a) Illustration of the experimental configuration of the self-driving, self-adaptive in vivo screening system in which zebrafish larvae are loaded individually onto a conventional optical microscope platform. (b) Operating mechanism of the zebrafish manipulation and imaging analysis platform, with representative imaging results. (c) Workflow of the system, illustrating the operation of mechanical and electrical components at different stages

12–15 million points, depending on specimen size, with each point encoding spatial position, color, and transparency—an attribute that is often neglected by conventional algorithms. Rendering such a dense point cloud on a single graphics processing unit (GPU) requires only approximately 1 s. On this basis, the point cloud features are iteratively updated by comparing rendered images with experimentally acquired images, ultimately completing the 3D reconstruction.

As a demonstration, we applied this platform to acquire comprehensive, multi-angle imaging data of melanin deposition in zebrafish larvae. Using 3D reconstruction algorithms, drug-induced inhibition of melanogenesis can be evaluated more accurately than with single-view imaging, thereby greatly enhancing the utility of zebrafish for the discovery of depigmentation agents. We believe that this high-throughput vertebrate screening system will find broad applications in

zebrafish research in the near future, including studies of organ development, disease modeling, and pharmacological and toxicological screening.

2 Results

2.1 The platform's operating mechanism and program control workflow

Our platform uses hydrodynamic forces, such as siphoning, as the primary driving mechanism instead of mechanical pumping. During operation, zebrafish larvae are transferred from a sampling tube into thin-walled glass tubes with a 0.75-mm inner diameter and fully submerged to eliminate imaging distortions caused by light refraction [21] (Fig. S1

in the supplementary information). Fluid flow is generated by the height difference between two containers, while four pinch valves regulate larval loading and unloading (Fig. 1a). A glass tube incorporating a limiting structure ensures accurate larval positioning. Machine vision enables automated system control, including larval position detection, posture recognition, and rotation adjustment via stepper motors (Figs. 1b and 1c). A microscopic camera was used to capture the information of interest, including multi-angle images, high-magnification views, fluorescence images, and full-cycle video recordings (Fig. 1b). After imaging, the previously imaged zebrafish larva was transferred into the collection tube, and the next larva was loaded. A peristaltic pump returned water to container I whenever the water level in container II exceeded the pump inlet. The system then unloaded the zebrafish and reset the motor for the next cycle (Fig. 1c). This workflow not only ensures high throughput but also considerably improves the success rate and minimizes potential damage to the larvae. The platform's self-driving and self-adaptive capabilities further enhance its flexibility and potential for secondary development.

We present the system workflow (Fig. 1c) and control process (Fig. S2a in the supplementary information). Upon startup, the system first calibrates the microscope, microscopic cameras, valves, and stepper motors to their correct initial states. Based on the equipment status, it determines whether to activate the peristaltic pump for automatic replenishment of liquid in the container. The initial siphon velocity is set by adjusting the height difference between the two water vessels, followed by opening valves II and III to initiate loading. The sampling tube is designed to prevent air bubbles from entering the thin glass tube and to increase the concentration of zebrafish per unit volume. The high density of zebrafish larvae in the sampling tube ensured efficient tube feeding, while the short transport distance increased loading speed and overall throughput, allowing the first larva to enter the thin tube rapidly—an essential factor for high-throughput operation. The system algorithm detects the larva's position using machine vision. Once a larva is detected in the glass tube, valve II closes and valve I opens to stop further loading. Simultaneously, a stopper in the tube prevents the larva from leaving the field of view. During the entire process, the zebrafish experience minimal stress (Figs. S2b and S2c in the supplementary information). Next, the algorithm uses deep learning to recognize the larva's posture and, with the assistance of the stepper motor, adjusts this posture to the correct orientation for image acquisition. After imaging is complete, the system unloads the larva by closing valve III, opening valve IV and resets the motor for the next cycle (Video S1 in the supplementary information). Upon completion of the entire imaging task, the platform's operation is concluded. Practical use has demonstrated a throughput of approximately one larva every 15 s. Furthermore, the platform's functionality is

expandable—for example, by adding additional collection tubes and corresponding valves to enable sorting capabilities.

2.2 The fluid dynamics model and computational fluid dynamics (CFD) analysis

We applied siphon theory to establish a fluid dynamics model describing zebrafish movement in the system through mathematical calculations and simulations (Figs. S2b and S2c in the supplementary information). The complete description of the motion process is provided below. When the liquid level (H) and the equivalent flow path length (L) are fixed, the presence of zebrafish in the tube alters the average flow velocity (v) by influencing the equivalent tube diameter (D), the frictional loss coefficient (λ), and the local resistance coefficient (ζ_i). Notably, the inner diameter of the glass tube is close to that of the zebrafish, which has a considerable effect on the hydrodynamic parameters. When a zebrafish enters the flow channel of the glass tube, a stopper with an auxiliary positioning function prevents it from passing through the microscope camera field, thereby avoiding failed image capture. During this process, the flow rate adaptively decreases, reducing the collision speed between the larva and the stopper and minimizing potential damage from both the impact and fluid forces. Simultaneously, the reduced flow speed considerably improves the accuracy and efficiency of the machine vision system in recognizing zebrafish larvae. Both experimental observations and fluent simulations validated this behavior (Fig. S2b in the supplementary information).

We used CFD simulations to validate and illustrate the zebrafish transport process (Fig. S2b in the supplementary information) because many changes in flow velocity occur instantaneously. A simplified flow channel model was constructed in the fluid dynamics simulation software, with cross-sectional dimensions at key locations closely matching those of the actual device. Overlapping grids and rigid-body moving meshes were used to simulate the zebrafish. To minimize grid and numerical errors, the rigid-body width in the simulation was set to 0.4 mm (Fig. S2b in the supplementary information), smaller than the actual zebrafish embryos. As a result, the impact on fluid velocity in the simulation is slightly less than in reality, but the overall behavior and trends remain accurate. The fluid-driven method proposed here operates as a constant pressure-driven system. For comparison, we also performed simulations of a pump-driven, constant flow rate approach (Fig. S2c in the supplementary information). In the constant pressure simulations, the inlet gauge total pressure was set to 650 Pa. Steady-state simulations determined that the equivalent flow velocity for the pipeline under constant flow rate input in the no-load condition (i.e., without zebrafish) was 0.095 m/s, which was then used as the input for the constant flow rate simulations. Transient simulations were subsequently performed

to calculate the forces acting on the surface of the rigid-body zebrafish model under both conditions throughout the process. The results indicate that under constant flow rate input, the pressure on the zebrafish surface rises sharply at the start of transport and when the larva reaches the stopper. Additionally, the maximum pressure increases as the zebrafish navigates bends in the tube. In contrast, under constant pressure input, pressure variations are much smoother, and after reaching the stopper, the pressure remains lower than in the constant flow rate condition until the fluid flow ceases. Because force is the primary source of injury, under constant flow rate input, the maximum instantaneous pressure experienced by the zebrafish can approach the peak output of the motor rotor, far exceeding the pressure the larvae can safely withstand. The handling of zebrafish by laboratory personnel using a Pasteur pipette also relies on air pressure. Compared with this manual approach, the constant pressure input method proposed here produces both lower maximum liquid flow rates and lower instantaneous pressures. As a

result, the system effectively prevents injury to the zebrafish. In conclusion, the constant pressure input method not only simplifies the device structure but also provides a safer environment for larval transport, preventing direct damage from sudden pressure spikes and substantially reducing the adverse effects associated with the stopper.

2.3 Zebrafish position detection, pose recognition, and rotational adjustment methods

Machine vision was used to enable fully automated control, data acquisition, and processing throughout the entire workflow. In the high-throughput system, zebrafish enter the glass tube in just a fraction of a second—often in one or two camera frames (Fig. 2a). To detect their positions, we analyzed pixel intensity changes between the initial image of each cycle and subsequent frames after the fish appeared. The image processing algorithm identifies the zebrafish's location by

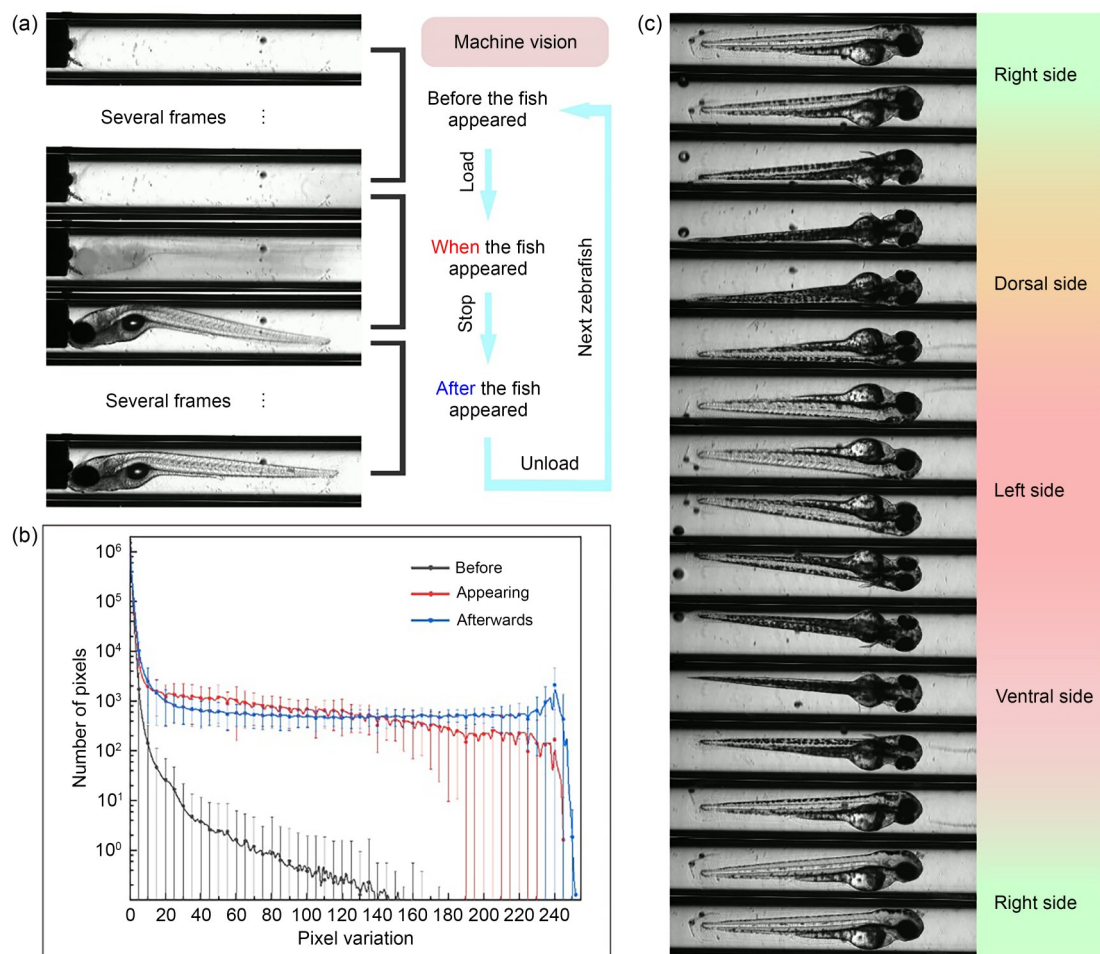


Fig. 2 Zebrafish position detection and pose recognition method. (a) Process of zebrafish entering the glass tube under machine-vision monitoring. (b) During tube entry, the number of pixels exhibiting each degree of pixel-value change relative to the initial value at the start of each cycle was counted. In this plot, one data point is displayed for every five measurements. Data are expressed as mean $\pm$ standard deviation ($n=40$). (c) Images of zebrafish in different postures

monitoring these pixel value variations, allowing the system to track the state of the glass tube in real time. When abrupt changes in pixel values occurred—indicating the presence of a zebrafish—the system detected the larva's position and responded by opening pinch valves I and III while closing the remaining valves, thereby confining the zebrafish to the stopper in the glass tube. During entry, the number of pixels exhibiting each degree of intensity change was counted relative to the initial frame at the start of each cycle. A pixel change range of 60–160 was found to be optimal for accurately determining the zebrafish's position (Fig. 2b). Timely position detection and valve actuation were critical for preventing system failures and ensuring the safety of the larvae.

The deep learning method [22] was used to automatically identify the zebrafish's posture, enabling the platform to autonomously adjust larvae for optimal image capture (Fig. 2c). Several convolutional neural networks were evaluated, and the deep residual network (ResNet18) [23] was selected for training the zebrafish recognition algorithm. The model was trained to recognize the lateral posture of the zebrafish as the target orientation. Using our platform, we captured a total of 11,643 images of zebrafish in various postures, which were divided into training, validation, and test datasets. To prevent overfitting, images collected at different times and from multiple experiments were included in the training set. To further prevent overfitting, zebrafish images collected at different times and from separate experiments were used during training. Additionally, to ensure the reliability of the results, the training, validation, and test datasets were completely non-overlapping: each zebrafish image captured at a given time was included in only one dataset, meaning that images in the validation and test sets were never seen during training. A four-class classification framework was used for posture recognition in this study. In the classification framework, lateral postures were assigned label 1 as the first category; ventral and dorsal postures were assigned label 0 as the second category; all other postures comprised the third category; and images containing no zebrafish were designated as the fourth category to prevent misidentification of empty glass tubes. The method achieved a recognition accuracy of 96.02%, with an average processing time of 0.02 s per image. Subsequently, a pose control experiment was performed in which the program performed real-time posture recognition and controlled the stepper motor to rotate the glass tube and adjust the zebrafish orientation accordingly. In 200 trials, pose control targeting the lateral posture succeeded 198 times with an accumulated rotation angle of less than 180°, and 2 times with an angle between 180° and 360°. These results demonstrate that zebrafish posture recognition and rotation adjustment were successfully accomplished. Building on this foundation, we also developed a method to predict zebrafish posture at any rotation angle during imaging by determining specific orientations, achieving a maximum

angular prediction error of approximately 7° (Table S2 in the supplementary information). The integration of this platform with image processing and deep learning substantially enhances its potential for real-time monitoring and analysis of zebrafish larvae.

2.4 Zebrafish image segmentation and lightweight 3D reconstruction highlighting internal organ features

We developed a comprehensive suite of algorithms for zebrafish image segmentation (Fig. 3) and 3D reconstruction (Fig. 4), both of which are common tasks in zebrafish image analysis. Image segmentation [24], also known as foreground extraction, is a critical step in identifying zebrafish structures. Our platform captures background images during zebrafish unloading and motor resetting. For static images, segmentation is performed using background subtraction, binarization, morphological operations such as opening and closing, and bitwise operations including masking (Fig. 3a). However, video processing presents additional challenges owing to motion blur and physical vibrations. Applying the same static-image approach to video frames—i.e., subtracting a matched background frame from the original frame—produces foreground images containing substantial erroneous information. These issues are clearly visible in the binarized image (Fig. 3b). To mitigate the effects of motion blur, our approach incorporates the frames immediately preceding and following the matched background frame. Specific image features, such as the position of the glass tube wall, are then used to align the background image by adjusting it upward or downward based on pixel disparities, ensuring accurate alignment with the original frame. This step effectively compensates for positional deviations caused by physical vibrations. Additionally, because pixel difference calculations are inherently integer-based, the background image is further translated incrementally relative to the feature-aligned background, generating a sequence of adjusted background images (Fig. 3b). Each of these adjusted backgrounds is subtracted from the target frame, and a pixel-by-pixel comparison is performed to select the minimum value, producing the final foreground segmentation. This method demonstrates strong generalization, eliminating the need for repeated parameter tuning or debugging across different image datasets.

For 3D reconstruction, camera parameter determination was the first step [13, 14]. Nearly all platform parameters were fixed, except for the position of the rotation axis, which remained variable owing to the microscope's configuration (Fig. 4a). Accordingly, the program determined the rotation axis position (I) by calculating the location of the glass tube wall. To reconstruct a 3D model of the zebrafish—including transparent structures and internal organs such as the eyes—we applied a low threshold when binarizing the previously

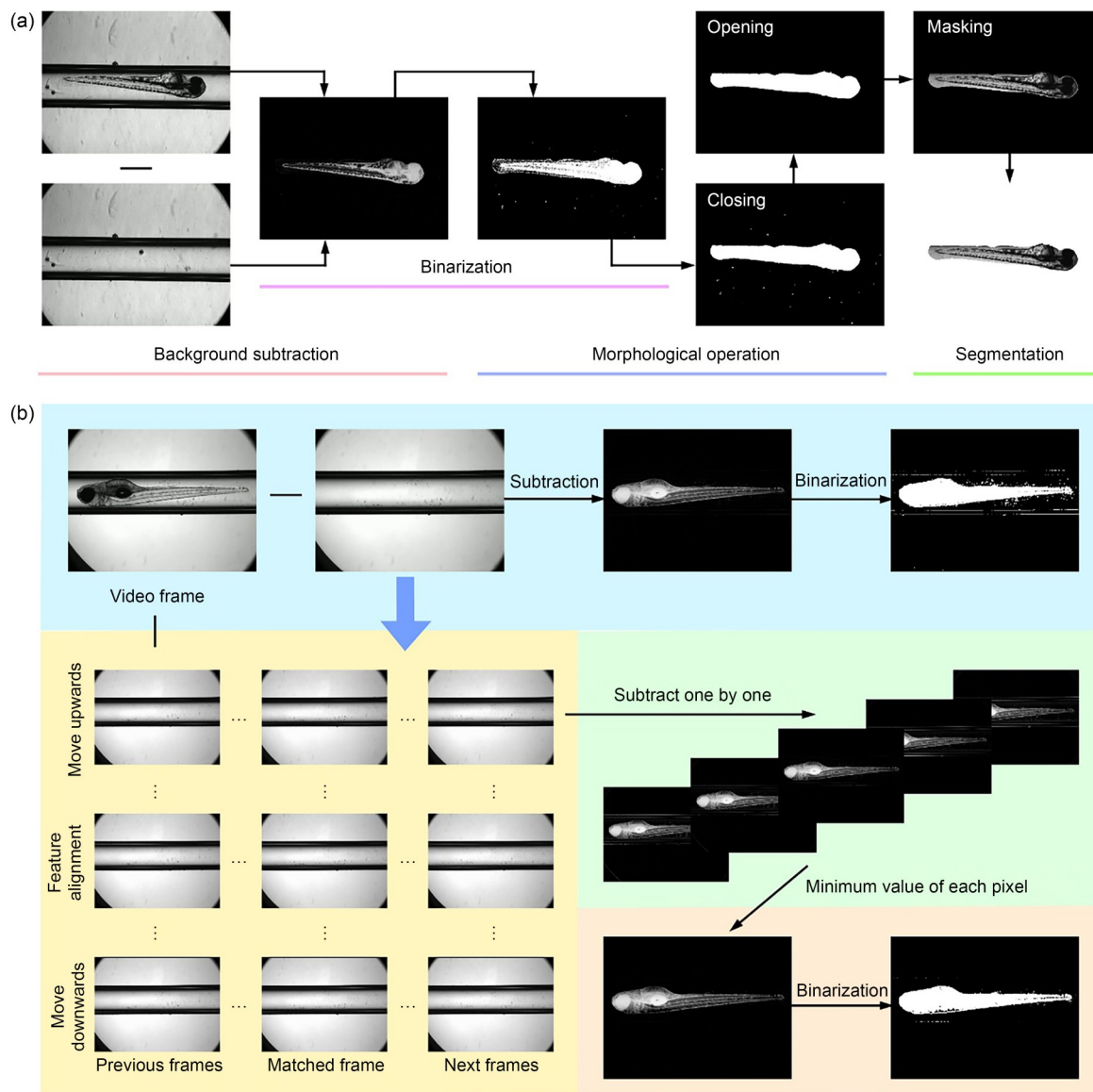


Fig. 3 Automatic zebrafish image and video frame segmentation method. (a) Automated zebrafish image segmentation process for static images. (b) Optimized solutions to key challenges in automated zebrafish video frame segmentation. In the optimized algorithm, the background frame—after feature matching with the zebrafish frame—is augmented using a series of background images, including the preceding and following frames, as well as translations

segmented images. While this approach may introduce some noise, it does not adversely affect the reconstruction. After performing a simple closing operation, we initialized a 3D voxel grid based on the two-dimensional (2D) silhouette and back-projected the 2D profiles into this framework to complete a lightweight 3D reconstruction (i.e., shape calculation) with a resolution of 14 μm (Fig. 4b). Noise was excluded during this computation. Subsequently, higher binarization thresholds were applied to the segmented images to generate 2D silhouettes for reconstructing internal organ features in 3D (i.e., color calculation) (Fig. 4c; Video S2 in the supplementary information). Additional view projections of the 3D reconstructions are shown in Fig. 4d. This

lightweight reconstruction method can be executed on personal computers; however, its fidelity is limited by computational constraints. Nevertheless, it provides a robust foundation for the high-speed rendering-based 3D reconstruction algorithms presented later in this study.

2.5 Quantification of drug-induced inhibition of melanogenesis in zebrafish during the hatching period

Melanogenesis, the biological process of melanin production, is highly conserved between zebrafish and humans, making zebrafish an excellent model for studying pigmentation

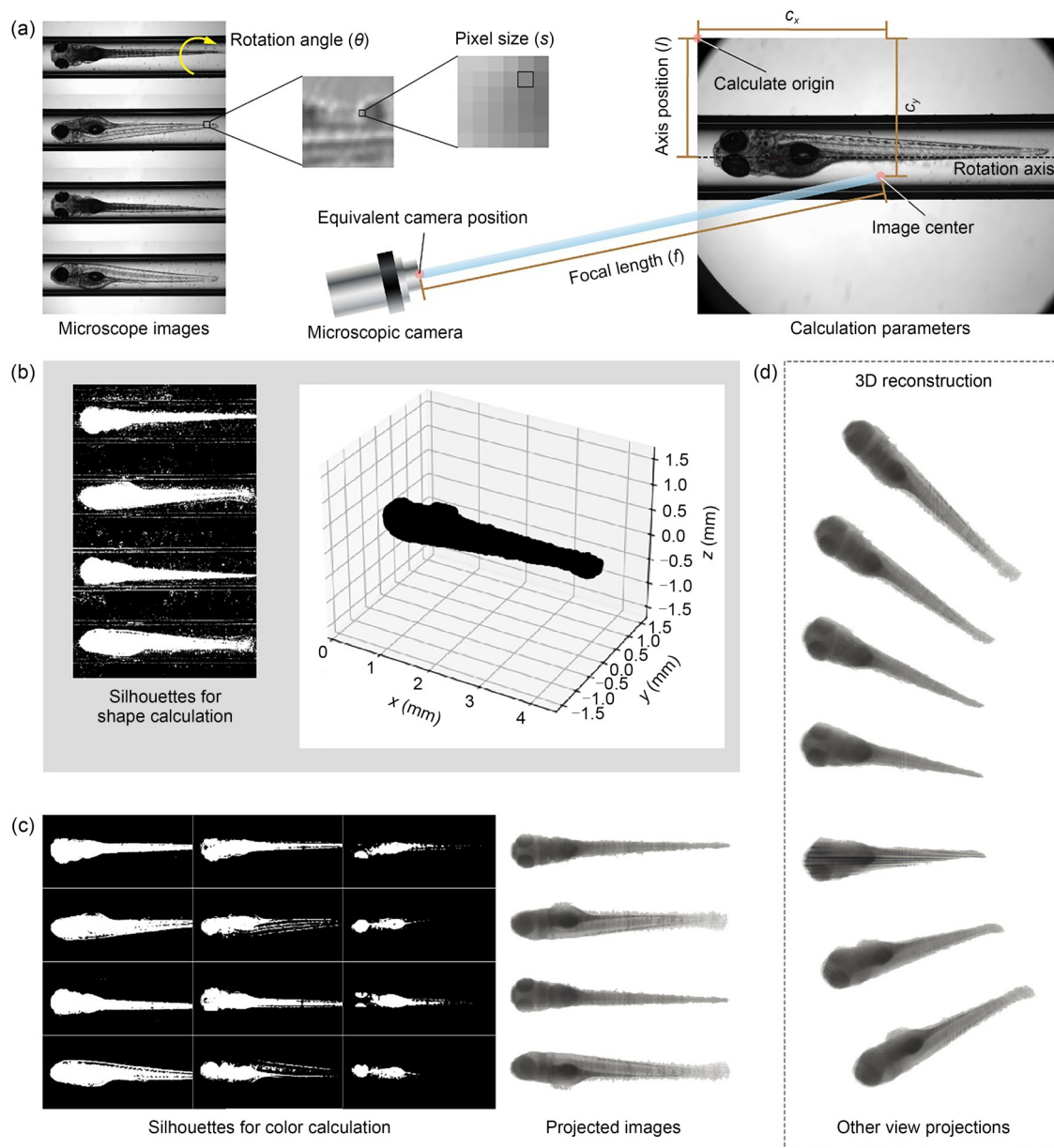


Fig. 4 Method and results of lightweight 3D reconstruction of zebrafish highlighting internal organ features. (a) Camera parameters determined during the 3D reconstruction process. (b) Shape calculation for reconstructing the external morphology of zebrafish. (c) Color calculation for reconstructing internal organ features. (d) Additional view projections of the lightweight 3D reconstruction results

disorders [25]. Dysregulated melanin synthesis contributes to various skin conditions, including melasma and post-inflammatory hyperpigmentation, underscoring the need to identify compounds that can modulate melanogenesis [26]. Although zebrafish have been widely used for *in vivo* evaluation of depigmenting agents, traditional methods require substantial manual intervention at multiple steps, including handling, imaging, and data acquisition [27, 28]. These approaches are influenced by factors such as embryo orientation, imaging angle, lighting conditions, and the accuracy of manual analysis, while also being time-consuming and labor-intensive. Using the widely studied depigmenting agent

1-phenyl-2-thiourea (PTU) [29], we demonstrate our automated high-throughput platform for evaluating the efficacy of depigmenting agents in zebrafish with high accuracy and sensitivity.

The inhibitory effects of PTU on melanogenesis were evaluated during the zebrafish hatching period (48–72 hours post-fertilization (hpf)), a phase characterized by rapid melanin synthesis and widespread melanin distribution throughout the body (Fig. 5). To assess the sensitivity and reliability of our platform in detecting subtle phenotypic changes, 2 days post-fertilization (dpf) larvae were treated for 3 d with a concentration gradient of PTU (0.0197, 0.0394, 0.0788, 0.1577,

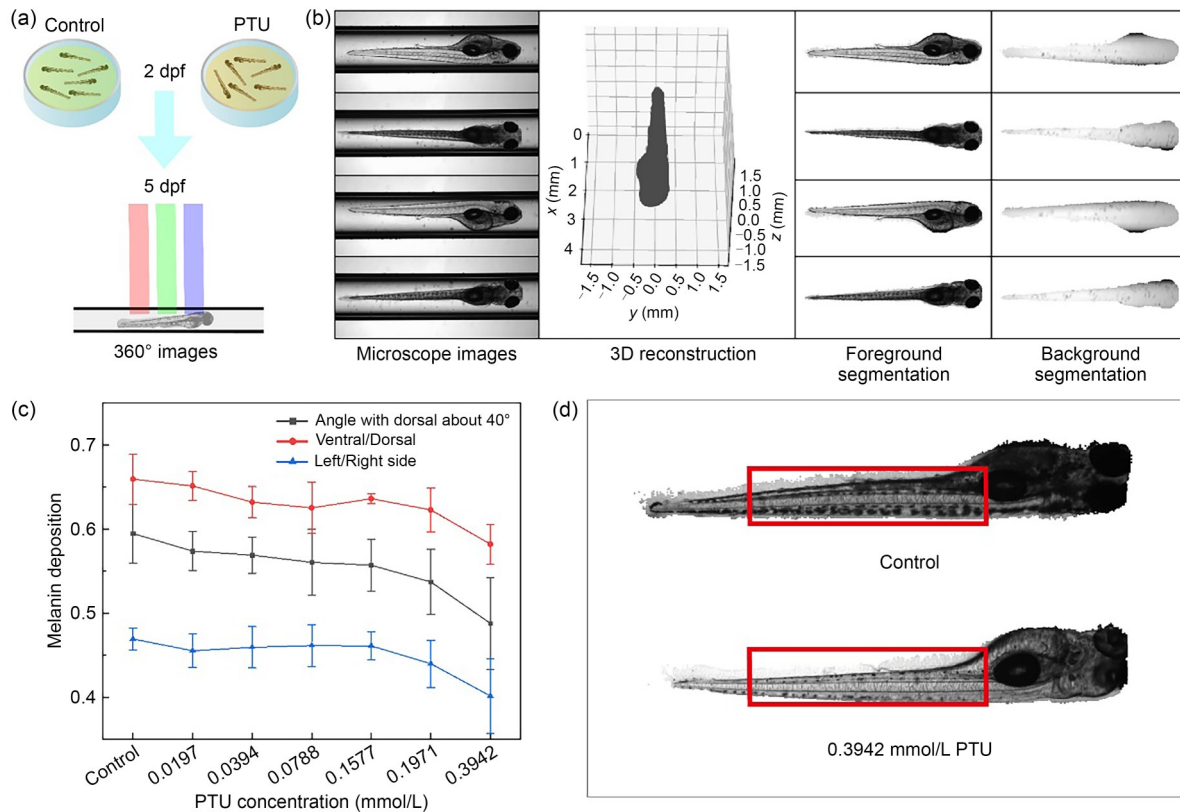


Fig. 5 Quantitative evaluation of drug-induced inhibition of melanogenesis in zebrafish during the hatching period. (a) Zebrafish larvae were raised in egg water containing varying concentrations of 1-phenyl-2-thiourea (PTU) at 2 dpf and cultured for 3 d for 360° imaging analysis. (b) Foreground and background extraction method based on 3D reconstruction for melanin evaluation. (c) Comparison of melanin deposition assessment results from images taken at different angles, with the optimal angle around 40° from the dorsal side. Data are expressed as mean±standard deviation ($n=24$). (d) Visual representation of zebrafish images at approximately 40° from the dorsal, providing the optimal display of melanin deposition during the hatching period

0.1971, and 0.3942 mmol/L) and imaged at 5 dpf. A control group was maintained in egg water without PTU (Fig. 5a). Studies of zebrafish development indicate that melanin production during this period is primarily concentrated in the dorsal region [30]. To capture optimal observations, 360° imaging was performed, ensuring clear visualization of melanin deposition. These multi-view images also facilitated subsequent lightweight 3D reconstruction. The resulting 3D projections were used for multi-view foreground and background segmentation, enabling accurate quantification of melanin deposition (Fig. 5b).

For analysis, images captured from multiple angles were segmented to quantify melanin deposition. The melanin deposition value (Fig. 5c) correlates positively with both the amount and depth of melanin, and is calculated as the ratio of the difference between the total background segmentation pixel value and the total foreground segmentation pixel value to the total background segmentation pixel value. For each treatment group, four larvae were randomly selected, and three frames per angle were analyzed for statistical comparison. Common single-view perspectives, such as ventral, dorsal, or lateral postures, were insufficient to clearly

distinguish intergroup differences, particularly in groups treated with lower PTU concentrations (Fig. 5c). For statistical analysis, three frames before and after each angle were selected, resulting in six frames per angle per group when considering symmetry. We found that images captured at approximately 40° from the dorsal view most effectively highlighted intergroup differences (Fig. 5c), consistent with previous observations that melanogenesis predominantly occurs in the dorsal region during the hatching period [30]. This 40° dorsal angle provided the clearest assessment of melanin quantity, size, and depth (Fig. 5d). Statistical analysis of multi-view images enabled our platform to accurately demonstrate the dose-dependent inhibitory effect of PTU on melanin formation, offering an accurate and rapid approach for leveraging zebrafish in the discovery of depigmenting agents.

2.6 3D reconstruction using high-speed rendering and spatial analysis of melanogenesis in zebrafish

Lightweight 3D reconstruction algorithms are limited in producing high-accuracy 3D models, primarily because they

cannot accurately capture both the color and transparency of zebrafish structures. In images obtained via background subtraction, nontransparent regions of the zebrafish often lose too much background color, whereas in images obtained through foreground segmentation, transparent regions retain excessive background color (Fig. 6a). Therefore, neither method can accurately represent zebrafish color and transparency. Achieving higher-quality 3D reconstruction requires accurate acquisition of the true feature information of zebrafish. To accomplish this, it is first necessary to understand the physical principles underlying the imaging of 3D objects in the real world. The imaging of 3D objects can be conceptualized as a layered, weighted superposition of colors, where the weighting parameters are determined by the transparency

of each layer, with the background image forming the final layer (Fig. 6b). Because this superposition process is difficult to invert directly, we used forward modeling rendering combined with a backpropagation algorithm to perform 3D reconstruction via gradient descent (Fig. 6d). During forward modeling, dense point clouds—initially obtained from lightweight 3D reconstruction—are used to represent the 3D model. To enable multi-angle rendering, we used irregular dense point clouds to eliminate the anisotropy inherent in regular point cloud models (Fig. 6c; Fig. S3 in the supplementary information). Using a large-memory computing system, high-speed rendering-based 3D reconstruction was achieved in approximately 200 iterations (Fig. S4 in the supplementary information). The resulting

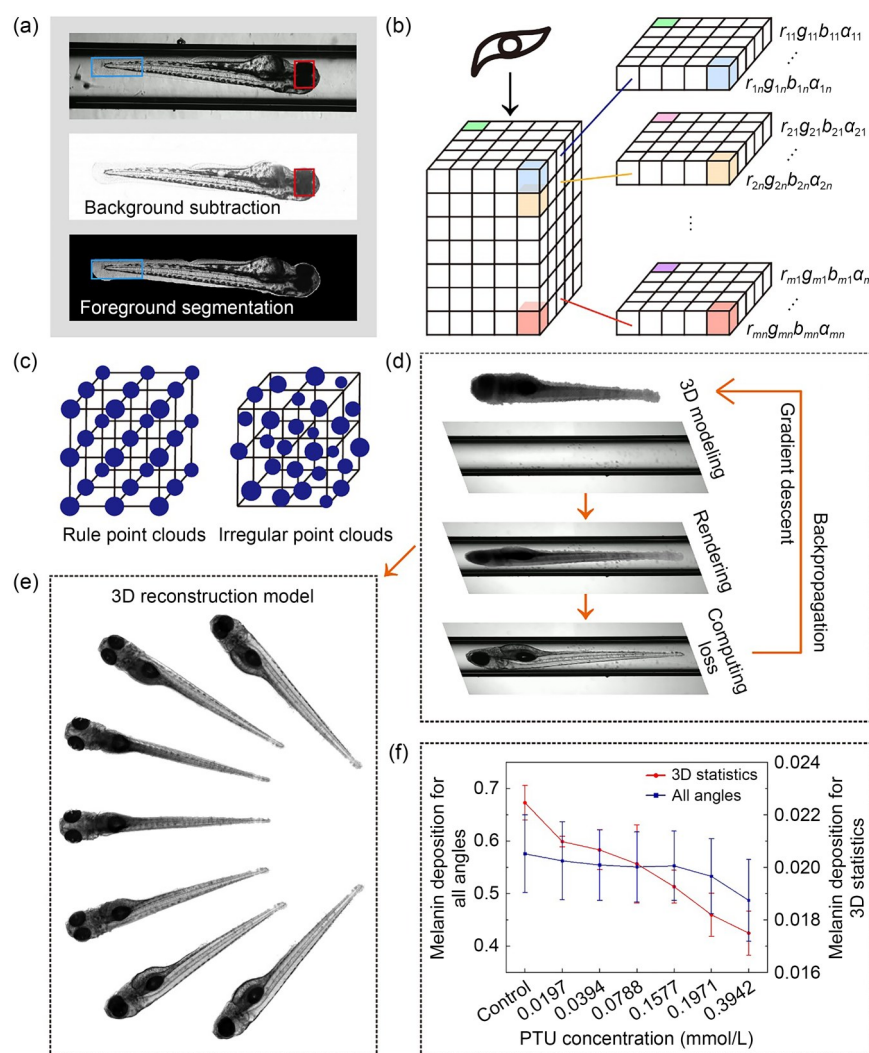


Fig. 6 High-speed rendering-based 3D reconstruction methods, results, and melanin-related applications. (a) Limitations of background subtraction and foreground segmentation methods for acquiring zebrafish larvae feature data. (b) Computer simulation of the optical imaging process in the real physical world, where r , g , and b represent color values and α represents transparency values. The subscripts indicate both the layer number and the order of the voxel within that layer. The voxel model presented here comprises m layers, with each layer consisting of n voxels. (c) Irregular point clouds used to eliminate the anisotropy of regular point clouds. (d) Main workflow of 3D reconstruction based on high-speed rendering. (e) Various view projections of the 3D reconstruction results. (f) Comparison of melanin deposition assessment in zebrafish using full-angle images versus directly using reconstructed 3D models. Data are expressed as mean $\pm$ standard deviation ($n=308$ for all angles; $n=4$ for 3D statistics)

3D reconstructions are shown in Fig. 6e and Videos S2 and S3 (supplementary information).

In Sect. 2.5, we identified an optimal viewing angle that improves upon traditional observation methods. However, fixing zebrafish at an accurate angle remains challenging for researchers without access to the specialized tools developed in this study. Even small angular variations can introduce fluctuations in the statistical results. Moreover, although images captured at approximately 40° from the dorsal view provide improved visualization, they still do not fully reveal all melanin deposits, limiting the efficiency of extracting biological information from zebrafish. The problem of overlapping melanin particles cannot be completely resolved simply by adjusting the imaging angle. The novel high-speed rendering-based 3D reconstruction algorithm enables direct evaluation of drug-induced inhibition of melanogenesis in zebrafish using 3D data. Compared with analyses based on images from multiple angles or a single perspective, extracting measurements from reconstructed 3D models more accurately captures the dose-dependent inhibitory effect of PTU on melanin formation. This approach provides a more efficient and accurate method for leveraging zebrafish in the discovery of depigmenting agents (Fig. 6f).

3 Discussion

In summary, zebrafish represent a highly valuable model organism owing to their numerous advantages. In this study, we focused on advancing high-throughput, automated systems for zebrafish manipulation and imaging analysis. We developed a self-driving, self-adaptive zebrafish larval screening platform that ensures high throughput, considerably improves both the success rate and flexibility of zebrafish loading and imaging, and minimizes the risk of injury. Furthermore, we implemented algorithms capable of rapid, multi-angle, real-time imaging of live zebrafish larvae, enabling comprehensive quantitative phenotyping through fully automated segmentation and 3D reconstruction. Importantly, these image processing algorithms require no manual parameter adjustments; the platform automatically adapts and performs analysis once the data are acquired. Collectively, this system expands the utility of zebrafish as a model for high-throughput screening of depigmenting agents and other applications.

In our study, zebrafish embryos are relatively inactive during early developmental stages, allowing them to be loaded and observed without anesthesia. However, anesthesia becomes necessary at later stages, particularly after 5 dpf, to prevent movement during imaging and ensure accurate path selection. Using our high-speed rendering-based 3D reconstruction algorithm, we successfully mapped biological information, such as melanin, from images captured at multiple angles onto a 3D model. While the theoretical reconstruction

accuracy of this method can still be improved, we aim to further optimize both the platform and algorithms to enhance their practicality for broader zebrafish research applications and to explore the potential benefits of batch explicit 3D reconstruction. Future efforts include standardizing the production of custom components to improve accessibility, ease of use, and compatibility with larger zebrafish, as well as developing automated image processing software tailored for diverse research applications through integration of existing algorithms [31, 32].

In conclusion, this high-throughput vertebrate screening system offers considerable potential for a wide range of applications in zebrafish research, including developmental studies, disease modeling, and drug screening. Its scalability and flexibility provide a foundation for transformative advances in zebrafish biology and beyond. Furthermore, its powerful 3D data acquisition capabilities offer substantial scientific and engineering value.

4 Methods

4.1 Equipment design, fabrication, and automated operation

Non-standard components of the platform were designed using SolidWorks 2016 (Fig. S5a in the supplementary information) and fabricated via stereolithography 3D printing using photosensitive resin (8200Pro, WeNext Technology Co., Ltd., China). Among these, the stopper was handcrafted from a metal tube and wire (Fig. S5b in the supplementary information) because attempts using various advanced manufacturing methods [33–36] failed to reliably produce this component. Standard components include a thin-walled borosilicate glass tube with an inner diameter of 0.75 mm and a wall thickness of 0.125 mm (B100-75-10, Sutter Instrument Co., Ltd., USA), a stepper motor with a hollow shaft of 5 mm outer diameter, and bearings with an inner diameter of 1 mm and an outer diameter of 3 mm. Additional components include bearings with an inner diameter of 5 mm and an outer diameter of 8 mm, a high-transparency BK7 or K9 optical glass plate (18 mm×17 mm×1 mm), flexible silicone tubing with a 1 mm inner diameter, electromagnetic pinch valves for silicone tubing, Y-shaped plastic connectors (Y-016, Nanjing Runze Fluid Control Equipment Co., Ltd., China), and a micro peristaltic pump (BJ-RZ1030, Nanjing Runze Fluid Control Equipment Co., Ltd.) used to maintain liquid level differences during long-duration experiments. All components were assembled according to our schematic diagram (Fig. 1a) and positioned under an inverted biological microscope (NIB900, Ningbo Yongxin Optics Co., Ltd., China) for inspection and imaging via a microscope camera (MTR3CMOS, Hangzhou ToupTek Photonics Co., Ltd., China). Connections between

the silicone tubing and both the sampling and collection tubes were sealed to prevent leaks (Fig. 1a). The platform was controlled using a computer running Python 3.11, with communication to Arduino Uno and relays to operate the various components.

4.2 Fluid dynamics model calculations and CFD simulations

The fluid velocity can be approximately described by the following formula:

$$v = \sqrt{\frac{2gH}{1 + \lambda \frac{L}{D} + \sum \zeta_i}}, \quad (1)$$

where v is the average liquid flow rate, g is the acceleration owing to gravity, H is the liquid level difference, λ is the equivalent frictional loss factor, L is the equivalent runner length, D is the equivalent inner diameter, and ζ_i is the coefficient of local resistance. When H and L are fixed, the presence of zebrafish in the tube alters v by affecting D , λ , and ζ_i . Specifically, when a zebrafish is trapped in the flow channel, D effectively increases while λ and ζ_i decrease. These changes in equivalent parameters collectively lead to an increase in the average flow rate v . This conclusion was validated through CFD simulations performed using Ansys 19.1 (Fig. S2b in the supplementary information).

We used the fluid flow (Fluent) module in Ansys Workbench for CFD simulations. First, the 2D geometry of the flow channel and the zebrafish was created using SpaceClaim and DesignModeler to reduce computational complexity. The cross-sectional dimensions at key locations in the channel closely matched those of the actual device; for example, the inner diameter of the glass tube is 0.75 mm. To prevent grid and calculation errors caused by the proximity of the dynamic mesh to the channel walls, the zebrafish model in the simulation was assigned a width of 0.4 mm, which is smaller than the actual zebrafish embryos. In the second step, a detailed mesh with an element size of 0.01 mm was generated for the model using overlapping grids and the triangle method. The viscous model was set to the standard k -epsilon model in Fluent. The Motion UDF was used to define the motion attributes of the dynamic mesh zones. Steady-state simulations were then performed to obtain equivalent values for constant pressure and constant flow rate inputs. With a constant pressure input of 650 Pa, the steady simulations indicated that the equivalent flow velocity under no-load conditions for the constant flow rate input is 0.095 m/s; this value was subsequently used as the input condition for the constant flow rate simulations. Finally, transient simulations were performed to describe variations in flow velocity and pressure under both input conditions.

4.3 Preparation of zebrafish larvae

Our experiments used zebrafish from wild-type stocks with the AB genetic background, maintained according to standard practices. Embryos were obtained through natural spawning, and all experiments were completed prior to 5 dpf in accordance with the guidelines of the Animal Ethics Committee of the Laboratory Animal Center, Zhejiang University, China. Zebrafish larvae at 2 dpf were raised in egg water containing PTU at concentrations of 0.0197, 0.0394, 0.0788, 0.1577, 0.1971, and 0.3942 mmol/L and cultured for 3 d. A control group was maintained in egg water without PTU for comparison.

4.4 Algorithms for detecting zebrafish positions

The machine vision system monitored the state of the glass tube to detect the zebrafish's position by analyzing pixel values. During the process of zebrafish entering the tube, the number of pixels with each degree of change relative to the initial image captured at the beginning of each cycle was counted. A range between 60 and 160 was determined to be the optimal threshold for accurately identifying the zebrafish's position (Fig. 2b). The algorithm was implemented in Python 3.11 using OpenCV 4.9. At the start of the loading process, the microscope camera captures an initial reference image, after which the system continuously monitors the status of the glass tube in real time. For each captured frame, the difference between the current image and the initial reference image was calculated. The resulting difference image was then binarized: pixels with values below 60 or above 160 were set to 0, while those in the range of 60–160 were set to 1. The number of pixels with a value of 1 was then counted. If the count was on the order of 10^3 or lower, it indicated that the zebrafish had not yet entered the tube. Counts of 10^5 or higher suggested that the zebrafish was entering or had already entered the tube. Based on these observations, a threshold of 10,000 was established as the decision criterion. Once a zebrafish entered the glass tube, the computer program automatically controlled the valves through relays to manage the flow and positioning of the larva.

4.5 Algorithms for zebrafish pose recognition and rotational adjustment

A deep learning approach was used to automatically identify the posture of zebrafish. The model was trained using 11,643 images of multiple zebrafish in various postures, which were divided into training (7523 images), validation (2062 images), and test (2058 images) sets. To ensure that the sets were independent, images from the same zebrafish appeared in only one set. Data augmentation techniques, including

random rotations (0° – 10°), random flips, random brightness adjustments (0–0.1), random cropping, and normalization, were applied to improve the model's robustness to complex and variable real-world conditions and to prevent overfitting. During dataset annotation, the target lateral posture was assigned a label of 1 as the first category. Ventral and dorsal postures were labeled as 0, representing the second category, while other postures were assigned to the third category. Images without zebrafish were designated as the fourth category to prevent misidentification of empty glass tubes. Using this four-class classification framework, the algorithm achieved a recognition accuracy of 96.02% on the validation set, with an average processing time of 0.02 s per image. Experiments were performed on a system equipped with an Intel Core i5-9400F central processing unit (CPU) at 2.90 GHz and a 16 GB NVIDIA GeForce GTX 1650 GPU. It is important to note that zebrafish posture is inherently a continuous variable (Fig. 2c). For example, an image with a posture corresponding to a label of 0.95 could reasonably be annotated as either the first category ("lateral") or the third category ("other"). The observed 3.98% misclassification rate arises from this ambiguity, but it does not considerably affect practical applications. When a three-class classification approach is adopted, excluding images in the third category ("other"), recognition accuracy increases to 100%, and the training and validation loss values decrease substantially (Fig. S6 in the supplementary information). This demonstrates the strong capability of the used neural network for zebrafish posture classification. In this three-class approach, the deep learning model is trained only on images labeled as 1 and 0, corresponding to lateral and ventral/dorsal postures. Intermediate postures falling between these categories can be classified based on confidence values, providing guidance for decision-making.

In the pose control experiment, we used the four-class labeling approach, assisted by confidence scores for decision-making. Zebrafish were loaded into the field of view 200 times with randomized initial poses, and the target pose was set to lateral. A stepper motor rotated the glass tube and the zebrafish while real-time posture recognition was performed. When the confidence score for the target pose reached its maximum and exceeded 0.9, the stepper motor was stopped, and five additional real-time frame checks were performed. If the confidence in the target pose remained above 0.9, the pose control was considered complete; otherwise, rotation and recognition continued. A trial was deemed successful if the zebrafish ended in the lateral posture at the completion of pose control; otherwise, it was considered a failure. In this experiment, pose control succeeded 198 times when the accumulated rotation angle was less than 180° , and 2 times when the rotation angle ranged between 180° and 360° . The stepper motor was controlled via Arduino Uno using our custom software.

We further developed a method to predict zebrafish posture at any angle during rotational recording by identifying specific postures. In the classification algorithm, specific postures are divided into two scenarios: lateral posture and ventral/dorsal posture. To expedite judgment, the algorithm automatically selects which specific posture to use based on the initial posture. For example, if the initial posture is lateral, the algorithm selects ventral/dorsal as the target posture; if the initial posture is neither lateral nor ventral/dorsal, the posture that appears first is chosen as the specific posture. Once a specific posture is recognized, the algorithm records the number of consecutive frames with a confidence score exceeding 0.99, allowing determination of the optimal frame for that posture. For example, if the algorithm records frames 15–20 as having a confidence score greater than 0.99 for the ventral/dorsal posture, the optimal frame for predicting this posture is estimated to be frame 17.5, corresponding to frame 17 or 18. This minor discrepancy arises from the camera frame rate. In this experiment, a full rotation of the zebrafish comprised 77 frames, resulting in an angular increment of 4.68° per frame. Using this method, the algorithm can predict the optimal frame for any angle. For convenience in annotation, the optimal frame for the subsequent specific posture—lateral posture—is predicted as 17.5 plus 19.25 frames. Rounding to the nearest integer or half frame yields frame 37. When compared with manually labeled optimal frames for specific postures, the maximum deviation observed across 30 replicate experiments with different zebrafish was 1.5 frames, corresponding to an angular error of 7.01° (Table S2 in the supplementary information). These results indicate that the optimal frame for identifying the first specific posture can be determined in half a rotation of the zebrafish. Subsequently, the algorithm can rotate the zebrafish to any desired angle in a full rotation, with the entire process requiring no more than 540° of rotation.

4.6 Image segmentation for static images and video frames

For static images, in the image segmentation process described here (Fig. 3a), a threshold value of 10 was used for binarization. The kernel size selected for the morphological closing operation was 15×15 , while the kernel size for the morphological opening operation was 10×10 .

For video frames, the segmentation process differed owing to motion blur and physical vibrations. In this study (Fig. 3b), the input videos consisted of a zebrafish rotation video and an empty glass tube reset video, both automatically recorded by the platform. Therefore, each frame had a corresponding background frame. Based on this background frame, a series of adjusted background images was generated. The first step involved selecting the preceding and subsequent frames of the background frame. A threshold value of 40 was then

applied to binarize these three images to calculate the positions of the glass tube in each frame. The three background images were first translated to align with the target frame. Following this feature alignment, each background image was additionally shifted upward by one pixel and downward by one pixel, completing the construction of the adjusted background image series. The target frame was then subtracted from each of these adjusted background images individually. Finally, a pixel-by-pixel comparison was performed, and the minimum value at each pixel was selected to generate the final result, achieving effective foreground extraction.

4.7 3D reconstruction of zebrafish

For 3D reconstruction, the relationship between a 2D image point (a_n) and its corresponding 3D coordinate (A_n) is as follows:

$$a_n = K [R_n | T] A_n, \quad (2)$$

where K is the camera intrinsic matrix, R_n is the rotation matrix, and T is the translation vector. The relationship between K and camera parameters is

$$K = \begin{bmatrix} f_x & 0 & c_x \\ 0 & f_y & c_y \\ 0 & 0 & 1 \end{bmatrix}, \quad (3)$$

where f_x and f_y are the focal lengths, and c_x and c_y are the image centers. The relationship between R_n and the rotation angle (θ) is

$$R_n = \begin{bmatrix} 1 & 0 & 0 \\ 0 & \cos \theta & -\sin \theta \\ 0 & \sin \theta & \cos \theta \end{bmatrix}. \quad (4)$$

The relationship between T and camera parameters is

$$T = \begin{bmatrix} T_x \\ T_y \\ T_z \end{bmatrix} = \begin{bmatrix} 0 \\ l s \\ f s \end{bmatrix}, \quad (5)$$

where l is the position of the rotation axis, f is the focal length, and s is the pixel size. Because the rotation axis is parallel to the X -axis, T_y can be approximated as the coordinate of the rotation axis. The values of f_x , f_y , and f were set to 200,000, and c_x and c_y were set to 0 because image distortion in the microscope images was negligible. The variable θ corresponds to the rotation angle of the zebrafish. The value of l is calculated automatically by the program, while s is determined through calibration (Fig. 4a).

Lightweight 3D reconstruction algorithms can be processed on standard personal computers, such as our system equipped with an Intel Core i5-9400F CPU at 2.90 GHz, 24 GB DDR4 memory at 2667 MHz, and a 16 GB NVIDIA GeForce GTX 1650 GPU. Based on the foreground extraction of video frames, a low threshold value of 2 was applied

to binarize the extracted foreground for reconstructing the transparent parts of the zebrafish. Although this may introduce some noise, it is manageable. After performing a morphological closing operation with a 9×9 kernel, a 3D voxel grid was initialized, and the 2D silhouettes were back-projected into this 3D framework to complete the shape reconstruction (Fig. 4b). Owing to the limitations of available computing resources, the spacing between adjacent voxels in the initialized 3D grid was set to five times the distance between adjacent pixels in the original images. Calibration showed that 1 mm corresponds to 352 pixels in the image, giving a pixel resolution of $1/352$ mm. Therefore, the resolution of the 3D reconstruction was $1/70$ mm, or $14 \mu\text{m}$. After completing the shape calculation, the same steps were repeated to calculate color, adjusting the threshold for binarization in increments of 25 for each iteration (Fig. 4c). The combined shape and color calculations produced a complete 3D reconstruction of the zebrafish, including its transparent components and internal organs, such as the eyes (Video S2 in the supplementary information).

Three-dimensional reconstruction algorithms based on high-speed rendering require large-memory computing resources, such as our system equipped with $8 \times$ Intel 8535 32-core CPUs at 2.6 GHz, 32×32 GB error-correcting code (ECC) DDR4 memory modules at 3200 MHz, and an 80 GB NVIDIA A800 SXM4 GPU. To enhance computational efficiency, we first compute the zebrafish contour and generate a dense point cloud within it. The average distance between points is set to half the pixel size of the original images, and points are randomly reordered in the cloud to eliminate anisotropy caused by perspective variations (Fig. 6c; Fig. S3 in the supplementary information). Projections of this irregular dense point cloud are then calculated at different angles using the imaging formula (Figs. 6b and 6d):

$$C_i = c_{1i} \alpha_{1i} + c_{2i} \alpha_{2i} (1 - \alpha_{1i}) + c_{3i} \alpha_{3i} (1 - \alpha_{1i}) (1 - \alpha_{2i}) + \dots, \quad (6)$$

where C_i is the color value of a specific RGB channel for a given pixel in the projected image. The parameters c_{1i} and α_{1i} correspond to the color and transparency of the first spatial layer projected onto that pixel. Subsequent parameters follow the same principle, with the final layer corresponding to the background image at the respective angles.

Using dense point clouds, the color and transparency of each spatial voxel are represented as the average values of all points contained in it. The projected results of the point cloud are then compared with the actual image frames to compute the reconstruction error. Three-dimensional reconstruction is completed within 200 iterations using backpropagation and gradient descent. The root mean square error (RMSE) serves both as the loss function for computation and as a quantitative measure of reconstruction error. After 200 iterations, the RMSE decreases to approximately 0.06 (where 0 indicates no error), while the structure similarity index measure

(SSIM), which quantifies reconstruction fidelity, increases to approximately 0.80 (where 1 indicates perfect fidelity). To improve computational efficiency, every seven consecutive frames are grouped together, with each iteration processing one such group. As a result, the reconstruction error curve exhibits periodicity with a period of 11 (Fig. S4 in the supplementary information). Using a single GPU, completing 200 iterations required approximately 25 min, with each iteration performing 7 renderings, totaling 1400 renderings, each taking roughly one second. In practice, to ensure convergence, the number of iterations was set to 500. This high-speed rendering 3D reconstruction method can also autonomously remove noise. During the pre-processing image alignment step, alignment can be achieved using only the prominent features of the glass tube, simplifying the procedure compared to lightweight 3D reconstruction. This process yielded a 3D model capturing detailed anatomical and transparent structures of the zebrafish (Fig. 6e; Videos S2 and S3 in the supplementary information).

4.8 Quantitative evaluation of melanogenesis in zebrafish during the hatching period

The calculation method for zebrafish melanin deposition (R , Fig. 5c) is as follows:

$$R = (p_{\text{back}} - p_{\text{fore}}) / p_{\text{back}}, \quad (7)$$

where p_{back} is the total pixel value of the background segmentation and p_{fore} is the total pixel value of the foreground segmentation. Whether using individual images or 3D reconstruction results for the quantitative evaluation of melanogenesis in zebrafish, the final calculation can be expressed using this formula, despite minor differences between the two approaches.

When using images to calculate melanin deposition, background information cannot be completely removed from the acquired foreground images (Fig. 6a). Therefore, during image segmentation, it is necessary to segment both the zebrafish foreground and the corresponding background. We accomplished this using the lightweight 3D reconstruction method. Specifically, the 3D reconstruction result was projected into a 2D mask, and background and foreground were segmented through masking operations. For this purpose, only the shape calculation of the 3D reconstruction was required. To improve accuracy, the kernel size for the morphological closing operation in the reconstruction algorithm was set to 5×5. For each group, including the control and various concentrations of PTU (FishBio-C08156, Shanghai FishBio Co., Ltd., China), four zebrafish were randomly selected for multi-angle video recording using the platform. The glass tube rotated each zebrafish once in 5.5 s, while the camera recorded at 14 frames/s yielding 77 images at

different angles. For each zebrafish, three frames before and three frames after each angle were selected for calculation and statistical analysis. Using the 3D reconstruction results for melanin quantification is even simpler: the spatial proportion of melanin in the zebrafish 3D model can be calculated directly through a weighted summation based on melanin color intensity.

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Author contributions YNL performed the theory analysis, computational fluid dynamics simulation, device fabrication, biological experiments, 3D reconstruction, and wrote the paper with input from all the authors. YNL and SW contributed to biological experimental design and subsequent bio-sample preparation and processing. YNL, XYZ, and HNJ developed the control programs and image processing algorithms. YW, YS, LZ, and YH provided guidance and contributed to the experimental design and data analysis throughout the project.

Declarations

Conflict of interest YH is a deputy editor of *Bio-Design and Manufacturing* and was not involved in the editorial review or the decision to publish this article. YNL and YH are inventors on a patent application CN116735590A submitted by Zhejiang University. SW, XYZ, HNJ, YW, YS, and LZ declare that they have no competing interests.

Ethical approval Our experiments used zebrafish from wild-type stocks with the AB genetic background, maintained according to standard practices. Embryos were obtained through natural spawning, and all experiments were completed prior to 5 dpf in accordance with the guidelines of the Animal Ethics Committee of the Laboratory Animal Center, Zhejiang University.

Data availability All data supporting the findings of this study are available from the corresponding authors upon reasonable request. The source data underlying Figs. 2b, 5c, and 6f, as well as Figs. S2c, S4, and S6 in the supplementary information are provided with this paper.

Code availability All computational code and related video data used in this study are available from the corresponding authors upon reasonable request.

Use of generative AI tools During the preparation of this manuscript, the authors used DeepSeek only to polish the language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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