inventions

MDPI

Article

Development of a Simple ImageJ-Based Method for Dynamic Blood Flow Tracking in Zebrafish Embryos and Its Application in Drug Toxicity Evaluation

Fiorency Santoso [1,2,†], Bonifasius Putera Sampurna [2,†], Yu-Heng Lai [3], Sung-Tzu Liang [2], Erwei Hao [4,5,*], Jung-Ren Chen [6,*] and Chung-Der Hsiao [1,2,7,*]

1 Master Program in Nanotechnology, Chung Yuan Christian University, Chung-Li 32023, Taiwan; fiorency_santoso@yahoo.co.id
2 Department of Bioscience Technology, Chung Yuan Christian University, Chung-Li 32023, Taiwan; boni_bt123@hotmail.com (B.P.S.); stliang3@gmail.com (S.-T.L.)
3 Department of Chemistry, Chinese Culture University, Taipei 11114, Taiwan; lyh21@ulive.pccu.edu.tw
4 Guangxi Key Laboratory of Efficacy Study on Chinese Materia Medica, Guangxi University of Chinese Medicine, Nanning 530200, China
5 Guangxi Collaborative Innovation Center for Research on Functional Ingredients of Agricultural Residues, Guangxi University of Chinese Medicine, Nanning 530200, China
6 Department of Biological Science & Technology, College of Medicine, I-Shou University, Kaohsiung 82445, Taiwan
7 Department of Chemistry, Chung Yuan Christian University, Chung-Li 32023, Taiwan
* Correspondence: ewhao@163.com (E.H.); jrchen@isu.edu.tw (J.-R.C.); cdhsiao@cycu.edu.tw (C.-D.H.)
† These authors have equally contributed to this paper.

Received: 9 September 2019; Accepted: 21 October 2019; Published: 1 November 2019

Abstract: This study aimed to develop a simple and cost-effective method to measure blood flow in zebrafish by using an image-based approach. Three days post fertilization (dpf) zebrafish embryos were mounted with methylcellulose and subjected to video recording for tracking blood flow under an inverted microscope equipped with a high-speed CCD camera. In addition, Hoffman lens was used to enhance the blood cell contrast. The red blood cell movement was tracked by using the TrackMate plug-in in the ImageJ image processing program. Moreover, Stack Difference and Time Series Analyzer plug-in were used to detect dynamic pixel changes over time to calculate the blood flow rate. In addition to blood flow velocity and heart rate, the effect of drug treatments on other cardiovascular function parameters, such as stroke volume and cardiac output remains to be explored. Therefore, by using this method, the potential side effects on the cardiovascular performance of ethyl 3-aminobenzoate methanesulfonate (MS222) and 3-isobutyl-1-methylxanthine (IBMX) were evaluated. MS222 is a common anesthetic, while IBMX is a naturally occurring methylxanthine. Compared to normal embryos, MS222- and IBMX-treated embryos had a reduced blood flow velocity by approximately 72% and 58%, respectively. This study showed that MS222 significantly decreased the heart rate, whereas IBMX increased the heart rate. Moreover, it also demonstrated that MS222 treatment reduced 50% of the stroke volume and cardiac output. While IBMX decreased the stroke volume only. The results are in line with previous studies that used expensive instruments and complicated software analysis to assess cardiovascular function. In conclusion, a simple and low-cost method can be used to study blood flow in zebrafish embryos for compound screening. Furthermore, it could provide a precise measurement of clinically relevant cardiac functions, specifically heart rate, stroke volume, and cardiac output.

Keywords: blood flow tracking; ImageJ; zebrafish; IBMX; MS222; cardiovascular function assessment

1. Introduction

Angiogenesis and organogenesis are critical events during embryonic development. Nutrient and oxygen transportation, waste product removal, gene expression control, and endothelial cell behavior all depend on blood circulation [1]. Blood cells and the blood vessel network are constantly subjected to the mechanical forces generated by heartbeat. Consequently, there is a strong correlation between heartbeat, blood flow, and cardiovascular morphogenesis. Cardiovascular morphogenesis can be seen as a manifestation of the evolving interactions between hemodynamic forces, heart function and the gene expression network activated in endothelial cells via mechanotransduction [2].

Establishment of the blood circulatory system starts at 24–26 h post-fertilization (hpf) in zebrafish. Initially, blood circulates in a single circulatory loop. The vascular tracts and blood flow pattern of zebrafish are similar to those of other vertebrates [3]. Screening protocols for the effects of drugs or other toxic compounds have been well established using animal models, including zebrafish [4]. Zebrafish, one of the lower vertebrates is best suited for high-throughput screening compared to the other animals [5]. With respect to pharmaceutical products, cardiotoxicity is one of the chief reasons for their retraction from the market in the last century [3]. A recent study showed that the cardiotoxic effects of drugs in zebrafish are similar to those in humans. Cardiotoxic drugs may elicit several adverse effects such as cardiomyopathy, arrhythmia, QT prolongation, bradycardia, depressed circulation, and abnormal atrium and ventricle beating ratio [4]. Heartbeat and blood flow are highly correlated and the flow velocity and perfusion of the blood could be affected by various chemical compounds. In a previous study, local blood perfusion rate was reduced in the zebrafish larvae when exposed to 2,3,7,8-tetrachlorodibenzo-*p*-doxin (TCDD) [6]. Although the heart rate did not change, severe pericardial edema was observed in fish exposed to TCDD. Blood flow was nearly absent in the mesencephalic vein of fish exposed to TCDD after 96 h. These results indicated that drugs could alter blood circulation without affecting the normal heartbeat. The corresponding result on heartbeat and blood flow velocity might be in accordance with each other or, could have different manifestations [6].

Ethyl 3-aminobenzoate methanesulfonate (Tricaine methanesulfonate, MS222), is the most common anesthetic used to sedate and/or euthanize zebrafish during experimental procedures. However, it is reported that MS222 may cause respiratory acidosis, cardiac depression, cardiac failure, and death after prolonged exposure and/or at high doses [7]. A previous study showed that zebrafish incubated in 160 ppm MS222 had a reduced heart rate of 97 bpm [8]. On the other hand, 3-isobutyl-1-methylxanthine (IBMX), one of the most toxic methylxanthines, has been reported to have potent cardiac toxicity and cause severe hemodynamic perturbations [9]. It was also found to have epileptogenic effects in animal studies, although in human studies, an indication of epilepsy was not found [10]. In addition to heart rate, the effect of drug treatments on other cardiovascular function parameters, such as blood flow velocity, stroke volume, and cardiac output remains to be explored.

In previous studies, several methods have been proposed to calculate blood flow in zebrafish and other animal species. However, the currently available methods have several drawbacks, such as high instrument cost (high-speed CCD, single/two-photon confocal microscopy or Doppler tomography) [1,11–14], the requirement to use special transgenic fish lines with blood fluorescence labeling [1], and reliance on complicated software scripts (summarized in Table S1). In addition to ImageJ open-source software, there are two third-party commercial software modules available: MicroZebraLab (Viewpoint, Lyon, France) and DanioScope (Noldus, Wageningen, The Netherlands). However, the high cost of commercial software solutions limits the accessibility of routine cardiovascular studies. Thus, the objective of this study was to design a simple and cost-effective method to assess drug or chemical toxicity on cardiovascular function. An inverted microscope combined with a high-speed CCD was used for video acquisition, without the need for fluorescence probes or transgenic fish line. The result from blood flow analysis can support the cardiotoxic effects and/or give new insights to previously unidentified side effects. This method can also provide precise measurements of relevant cardiac functions, specifically heart rate, stroke volume, and cardiac output.

2. Results

2.1. Basic Principle and Experimental Protocol for Blood Flow Tracking and Velocity Measurement in Zebrafish Embryos Using ImageJ-Based Methodology

In this study, blood flow tracking and velocity measurements were performed using a simple and cost-effective setup, consisting of an inverted microscope and high-speed CCD camera to capture the images. Initially, the captured video recording was in mp4 format, which was then converted to avi format using the VirtualDub software before it can be further processed by the open-access software ImageJ application. This application was then utilized to perform blood cell tracking and velocity measurements. Subsequently, the red blood cell movement was tracked by using the TrackMate plug-in in the ImageJ application to establish the corresponding pixel change relative to blood flow velocity. For blood flow patterns, we selected a region of interest (ROI) in the dorsal aorta (DA) or the posterior cardinal vein (PCV) and used the Stack Difference and Time Series Analyzer plug-in in the ImageJ application to detect the dynamic pixel changes over time. Finally, the blood flow pattern was obtained by curve fitting. The overall scheme is summarized in Figure 1.

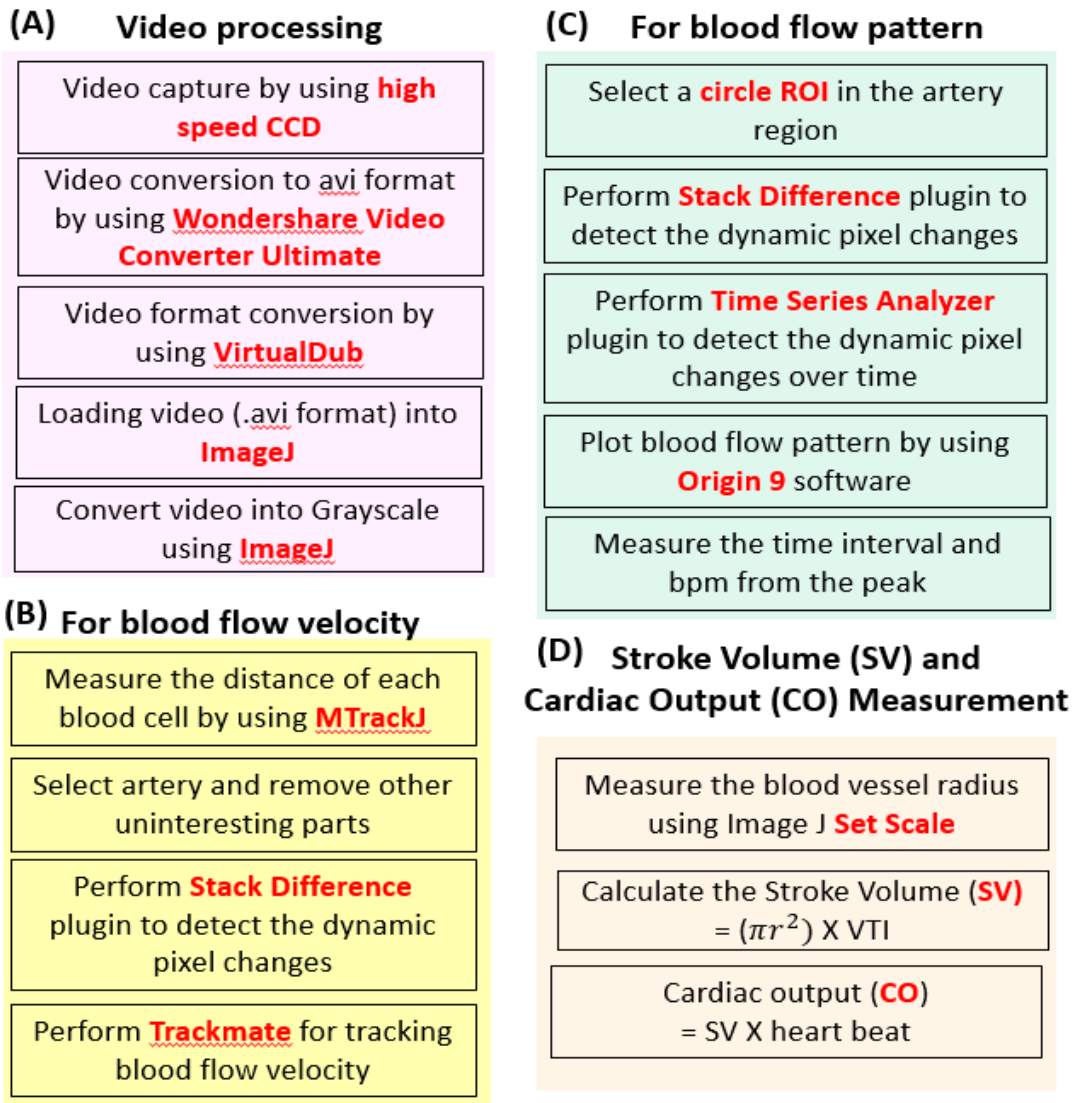

Figure 1. The basic principle for blood flow measurement in zebrafish embryos. (**A**) The experimental scheme for video processing. (**B**) The experimental pipeline of blood flow velocity analysis, blood flow pattern analysis (**C**), stroke volume, and cardiac output measurement (**D**).

Initially, the position of zebrafish was adjusted to the anterior-to-left position under the inverted microscope (Figure 2A). The blood flow video was captured by high-speed CCD camera mounted on the inverted microscope (Figure 2B) at a rate of 200 frames per second (fps). To enhance the contrast of the object without staining, Hoffman lens was applied (Figure 2C). In image processing, digital filters are often used to suppress the high frequencies in the image to smooth the image, or the low frequencies to enhance or detect edges in the image. The derivative Laplacian filter, a 2-D isotropic measure of the 2nd spatial derivative of an image, highlights the edges with rapid changes in the image sequence. Gaussian smoothing filter is a linear filter that can blur the images and reduce noise for edge detection. Combining the Gaussian filter with the Laplacian filter is called Laplacian of Gaussian (LoG) segmentation [15]. LoG segmentation is the algorithm used by TrackMate plug-in to track single blood cell motion. Blood cell velocity can be calculated according to the x and y coordinates. Pattern recognition of ROI may not always be done with tracking blood alone since the red blood cells would leave the ROI area in a short period of time. Therefore, another plugin, Time Series Analyzer was used to analyze the pattern in the DA. In principle, Time Series Analyzer is based on the dynamic pixel change. During high blood flow velocity (heart pumping), the dynamic pixel change intensity would be higher, and higher peak rhythm was formed. On the contrary, on low blood flow velocity, the lower peak rhythm was formed (Figure 2D). The typical velocity pattern for both artery (highlighted by red color) and vein (highlighted by blue color) obtained by the dynamic pixel change method could be seen in Figure 2E. Finally, the combination of these methods was successfully used to perform blood velocity tracking (detailed protocol can be found in the Supplementary Data).

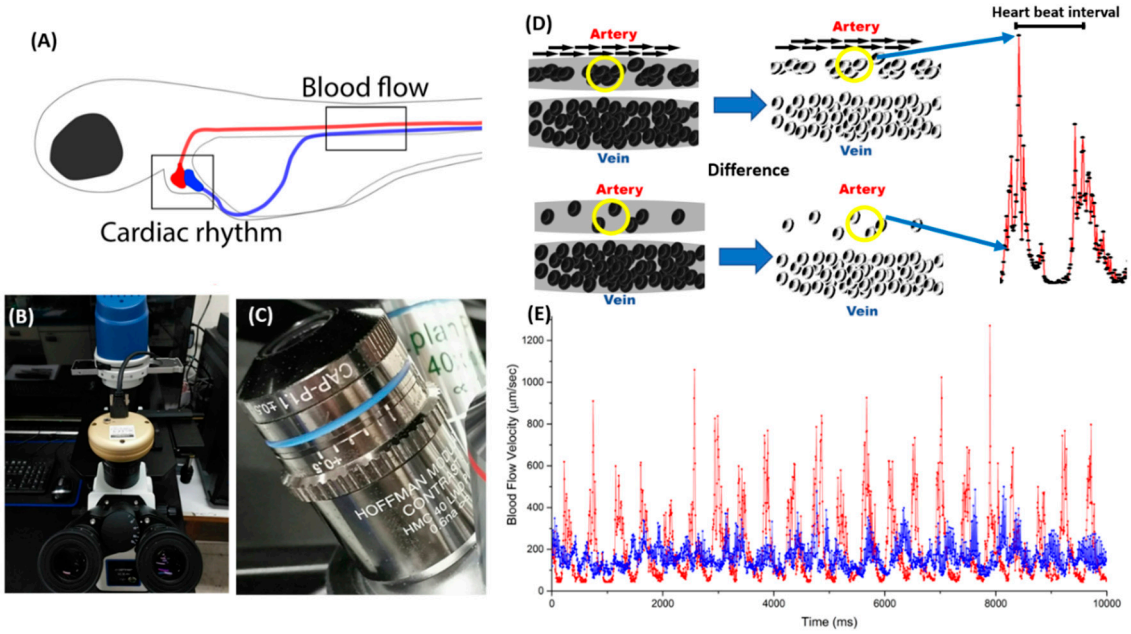

Figure 2. The instrumental framework for blood flow measurement in zebrafish embryos. (**A**) The area to detect heartbeat and blood flow is highlighted in black. (**B**) The inverted microscope, high-speed CCD and (**C**) Hoffman objective lens was used to record blood flow. (**D**) The dynamic pixel changes in the selected region-of-interest (ROI) of the artery or vein were calculated. (**E**) The typical velocity pattern for both artery (red color) and vein (blue color) were obtained using dynamic pixel change methodology.

2.2. Measurement of Blood Vessel Radius in 72hpf Zebrafish Embryos

In order to measure cardiovascular performance, specifically stroke volume and cardiac output, it is necessary to measure the radius of the blood vessel. Consequently, five points were chosen from the anterior to posterior positions of the DA and PCV as indicated in Figure 3A to perform radius measurement. By using five sampling points for radius measurement, no significant difference between the successive sampling points of both the DA (Figure 3C) or in PCV (Figure 3D) was found. This

result suggests that the average radius length could be used to carry out cardiovascular performance measurements. The average radius of the DA was 7.3 ± 0.2 µm whereas the PCV has an average radius of 9.6 ± 0.2 µm (Figure 3B) in 72 hpf zebrafish embryos.

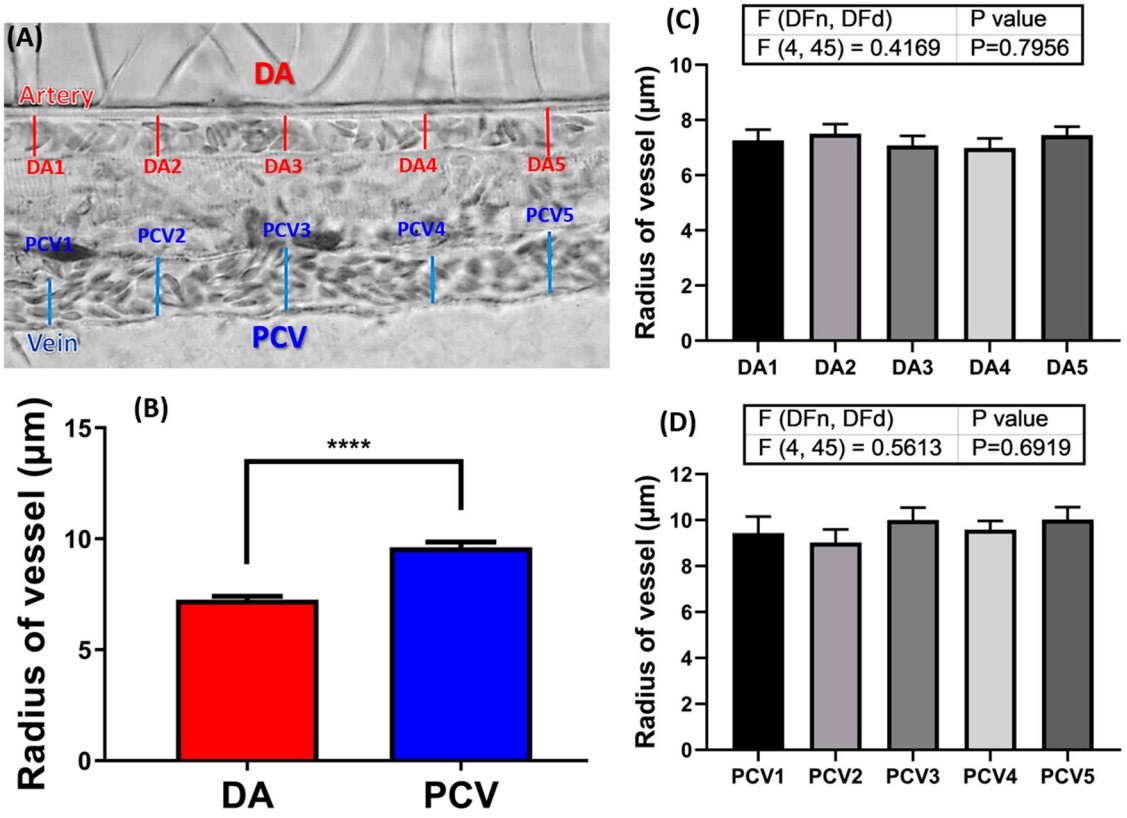

Figure 3. Measurement of the blood vessel radius in zebrafish embryos aged at 72 h-post-fertilization. (**A**) The radius of the dorsal aorta (DA, red color) and posterior cardinal vein (PCV, blue color) were measured at five points from anterior to posterior positions. (**B**) Comparison of the average radius of the DA and PCV in zebrafish embryos. Comparison of five sampling points from anterior to posterior position as indicated in (**A**) for either in (**C**) DA or in (**D**) PCV. Data were compared using one-way ANOVA test and post hoc test of Tukey analysis. The level of significance was set at a p value < 0.05.

2.3. Cardiovascular Performance in Zebrafish Embryos on 1–5 Days Post Fertilization (dpf)

The velocimetry profiles for zebrafish from 1 to 5 dpf are shown in Figure 4A–C and Video S1. Blood was ejected from day 1 and incremented until day 3. By day 4, there was a significant reduction in blood flow velocity with a subsequent increase on the fifth day. The highest blood flow velocity was observed on day 3. Blood flow velocity was higher in the DA than in the PCV. The observations are consistent with a previous study that used a confocal microscope for quantitative measurement of cardiovascular performance in zebrafish embryos and larvae [14]. There are two possible explanations for the reduction in velocity during ontogeny. First is the growing size of the ventricular-bulbar valve and the ongoing production of blood cell which increased the hematocrit and second, there was a motion immediately after systole after 4-dpf fish. This could be mistaken for retrograde flow, however, this motion occurred due to the closure of the valve pushing red blood cells back into the ventricle. This phenomenon was not present in the 3 dpf fish. The motion is a true retrograde flow caused by the force applied to the ventricular-bulbar valve as the ventricle expand [16].

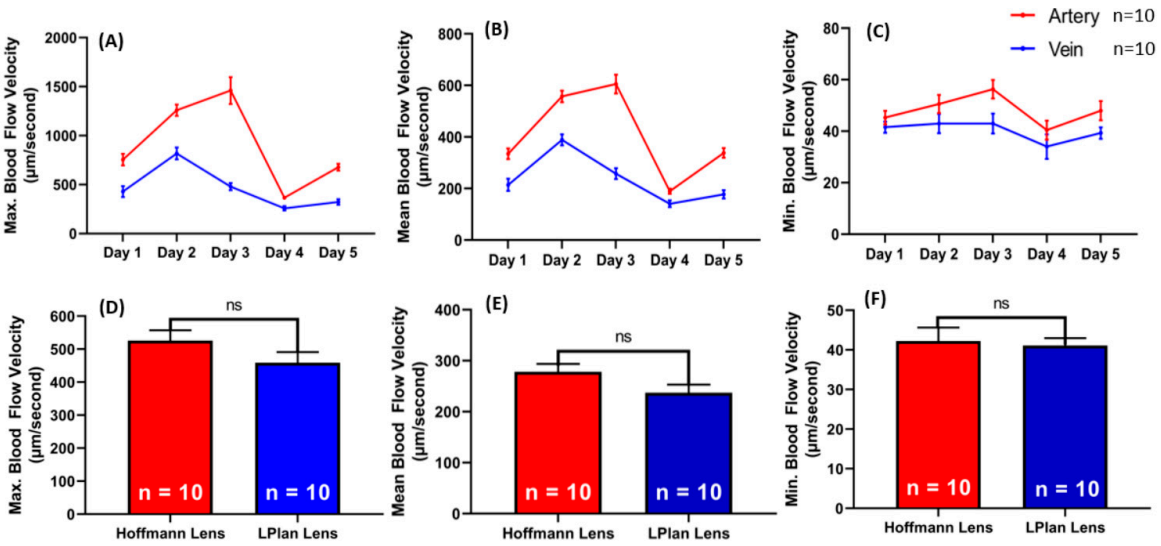

Figure 4. Comparison of blood flow velocity in zebrafish embryos. (**A**–**C**) Comparison of maximum, mean, and minimum blood flow velocity for the dorsal aorta (DA, red color) and posterior cardinal vein (PCV, blue color) in zebrafish embryos aged 1 to 5 dpf. (**D**–**F**) Comparison of the blood flow velocity in the DA using Hoffmann lens (red color) and non-Hoffmann lens (blue color).

2.4. Blood Flow Velocity Comparison in Zebrafish Embryos Using Hoffmann Lens and Non-Hoffmann Lens

In this study, Hoffmann lens was used to capture the blood flow image. Hoffman lens was used to magnify and enhance the contrast of the blood cell image, however, it is more expensive compared to a regular lens. The differences in blood velocity using Hoffmann lens and non-Hoffmann lens were then determined. Compared to the regular object lens, the Hoffmann lens provided a clear blood cell image and video recording thereby providing more stereo details. However, there was no significant difference between the recorded maximum, mean, and minimum blood flow velocity by the Hoffman and non-Hoffman lens, indicating that the non-Hoffmann lens could also be used to measure the blood flow velocity by using this ImageJ-based method (Figure 4D–F, Video S2).

2.5. Functional Assessment of Cardiovascular Performance in Zebrafish Embryos Before and After Exposure to Drugs

Primary DA is a pair of longitudinal vessels whose anterior ends are directly connected to the embryonic heart via the outflow tract. The pattern of blood flow in DA follows the beating of the heart [17]. The results of this study showed that blood flow displayed more dynamic fluctuations in DA rather than in PCV. The maximum, mean, and minimum blood flow velocity of normal zebrafish embryo calculated from DA were 1460 ± 138, 605 ± 36, 56 ± 3 μm/s, respectively. By contrast, the maximum, mean, and minimum blood flow velocity calculated from PCV were 479 ± 36, 258 ± 21, 43 ± 3 μm/s, respectively. The maximum velocity in DA was approximately 3.5-fold higher than in PCV (Figure 5A–C). The findings are comparable to a previous study which used expensive laser scan confocal microscopy technology [1,18].

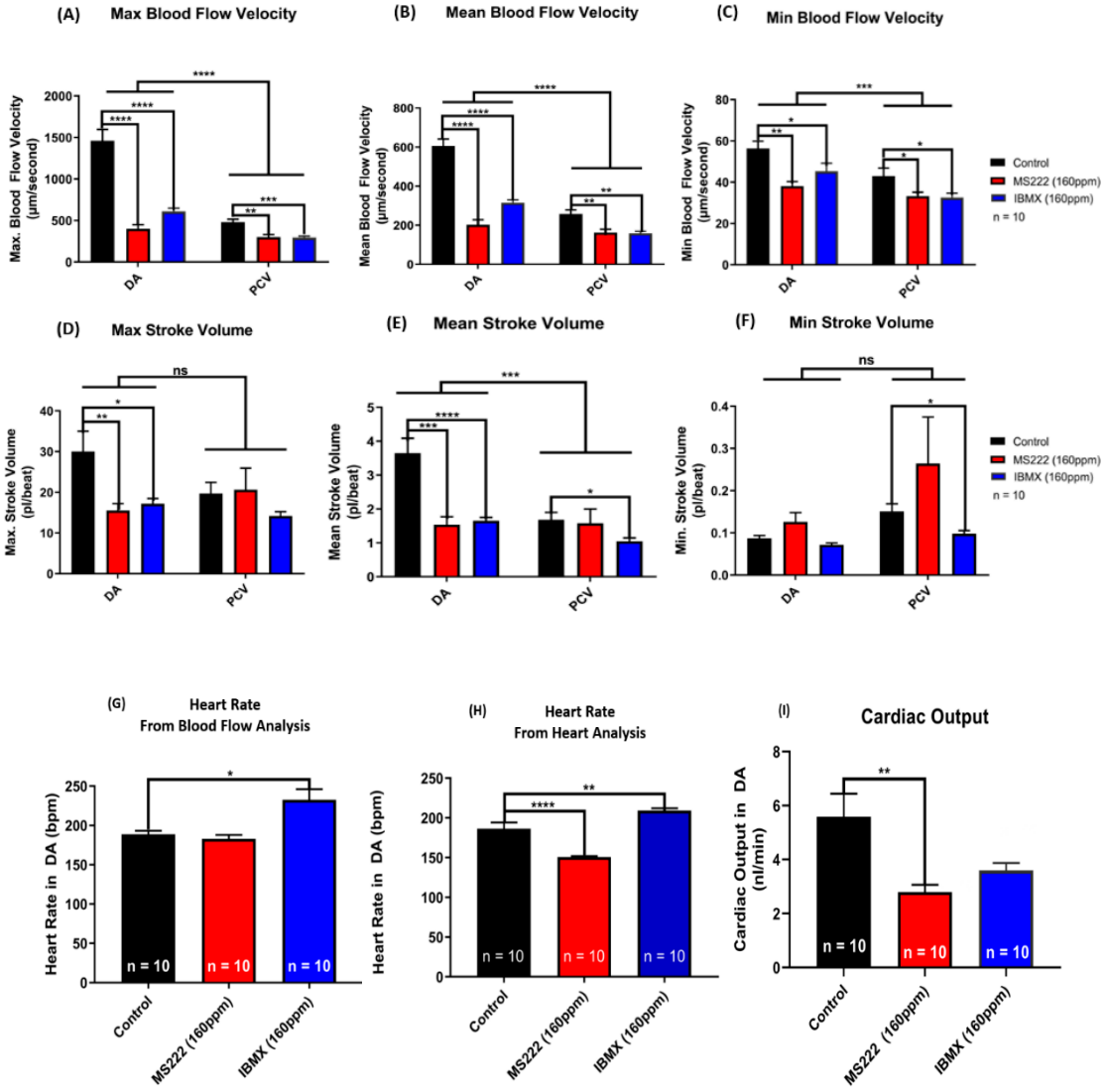

Figure 5. Functional assessment of cardiovascular performance in zebrafish embryos after treatment with MS222 and IBMX based on images captured from the artery or vein. MS222 and IBMX were applied to zebrafish embryos at 72 hpf at 160 ppm and the cardiovascular performance was recorded and analyzed by the ImageJ-based method established in this study. (**A**–**C**) Maximum, mean, and minimum blood flow velocity in dorsal aorta (DA) and posterior cardinal vein (PCV), (**D**–**F**) Maximum, mean, and minimum stroke volume in the DA and PCV, (**G**) Heart rate in the DA calculated from blood flow, (**H**) Heart rate calculated directly from heart, and (**I**) Cardiac Output of the DA. Data are expressed as mean ± SEM values and were analyzed by two-way ANOVA in (**A**–**F**) and one-way ANOVA in (**G**–**I**) (n = 10 for each chemical treatment; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$, ns = non-significant).

To validate the designed methods, the potential effects of two different compounds on cardiovascular function were evaluated. MS222, one of the anesthetics extensively used to reduce body movement and avoid discomfort, stress, and pain in aquatic animals during experimental procedures This drug consists of benzocaine that blocks the activity of both sensory and motor neurons [8,19–25]. However, previous studies found that MS222 can reduce heart rate by 37.7% and 27.1% in zebrafish when administered at a concentration of 40 ppm and 30 ppm, respectively [8]. The effect of MS222 and other compounds on other cardiovascular function parameters, such as stroke volume and cardiac output, remains elusive. In this study, zebrafish embryos which were incubated in 160 ppm MS222 elicited a 72% blood flow velocity reduction. The maximum, mean, and minimum blood flow of the DA

were 398 ± 51, 202 ± 25, 38 ± 2 µm/s, respectively. While the maximum, mean, and minimum velocity of the PCV were 301 ± 28, 162 ± 17, 33 ± 2 µm/s, respectively (Figure 5A–C, Video S3). These results are consistent with a previous study that demonstrated blood flow velocity reduction in zebrafish after MS222 treatment [14]. The second compound used is IBMX, one of the methylxanthines having coupled heterocyclic organic compounds consisting of pyrimidinedione and imidazole rings [10]. This compound reportedly has two major toxicities, cardiac toxicity and hemodynamic perturbations [9]. In this study, IBMX (160 ppm) caused 58% blood flow velocity reduction. The maximum, mean, and minimum blood flow velocity of the DA were 608 ± 40, 314 ± 15, 45 ± 4 µm/s, respectively. By contrast, the maximum, mean, and minimum blood flow velocity of the PCV were 294 ± 18, 158 ± 9, 32 ± 2 µm/s, respectively (Figure 5A–C, Video S3). Overall, the reduction of blood flow velocity in treated fish compared to the control group was observed.

Stroke volume (SV) can be determined by multiplying the area of the aorta/vein (A) by velocity-time integral (VTI). The maximum, mean, and minimum stroke volume of the DA was 30 ± 5, 3 ± 0.4, 0.09 ± 0 pl/beat, respectively. On the contrary, the maximum, mean and minimum stroke volume of the PCV was around 19 ± 2, 1.6 ± 0.2, 0.15 ± 0 pl/beat, respectively. The stroke volume of the DA was approximately 1.6-fold higher than the PCV in zebrafish. In treated fish, MS222 and IBMX significantly decreased the maximum stroke volume of the DA, but no significant difference in maximum stroke volume of the PCV was found. Furthermore, this study also showed that both compounds produced a significant reduction in the mean stroke volume of the DA, and only IBMX could significantly reduce the mean and minimum stroke volume of the PCV (Figure 5D–F). Stroke volume is dependent on the diameter of the blood vessel. A higher diameter leads to a higher stroke volume. Therefore, there was no significant difference in the maximum stroke volume of the DA and the PCV.

Heart rate measurement was also performed indirectly based on the recorded blood flow video. It was determined by multiplying the time interval (in seconds) between two peaks by a factor of 60. This study showed that only IBMX produced a significant difference in heart rate (Figure 5G). However, based on a previous study, incubation of zebrafish in 40 ppm and 30 ppm of MS222 caused a 37.7% and 27.1% decrease in heart rate respectively, and incubation in 160 ppm led to a heart rate reduction to 97 bpm [8]. IBMX also induced an increase in heart rate [26]. To validate this result, we also measured the heart rate of treated fish directly from the heart. Interestingly, the results are comparable to other studies [8,26] showing that fish treated with MS222 had a significantly reduced heart rate, whereas treatment with IBMX had significantly increased the heart rate (Figure 5H). These results suggest that heart rate measurement calculated from blood flow pattern may lead to bias. In connection with the results, it was reported in another study that no significant difference in the heart rate in different concentrations of MS222 was reported when it was calculated from the blood flow [14]. Therefore, it can be concluded that heart rate measurement must not be done based on blood flow pattern because it may lead to biased results.

The side effects of anesthetic drugs also can be seen from changes in cardiac output. The cardiac output can be determined by multiplying the stroke volume with heart rate (CO = SV × HR). The anesthetic MS222 significantly reduced cardiac output. On the other hand, treatment with IBMX did not reduce the cardiac output (Figure 5I). In this study, analysis of both the blood flow and heart rate is suggested to obtain accurate measurements of the cardiac functions and better assessment of cardiovascular performance.

3. Discussion

Heartbeat and blood flow are highly correlated, thus assessment of cardiotoxic drugs could be done with either approach to support one another. Many previous studies have established several methods to analyze the cardiovascular performances in zebrafish, however most of them require expensive instruments, a fluorescent probe or transgenic fish, and complicated software analysis [1,11–14]. This study designed a method that utilizes a simple and low-cost instrument with a high-speed CCD camera mounted on an inverted microscope. In this method, fluorescent and/or transgenic fish is not required

however, a pigment-blocking reagent such as phenylthiourea (PTU) must be used as pigmentation in zebrafish can hinder the blood flow velocity measurement. In addition, the current ImageJ-based method for blood flow calculation involves multiple steps before obtaining cardiac function data. Macro language implantation is required to reduce the operational difficulties and boost the calculation performance in the future.

The DA vessels are directly connected to the heart chamber, making the DA regional blood flow pattern in accordance with the pumping of the ventricle. The PCV is connected into the atrium region, collecting oxygen-deficient blood from the body. Tracking single blood cells over the frames is required to access the precise impact caused by a drug in the heart and blood flow alteration. High contrast difference between foreground and background facilitates the TrackMate plugin application in detecting subtle changes in intensity. The algorithm is based on the LoG operation [27]. TrackMate, a plugin of ImageJ could provide single-particle tracking via segmenting and following the object over time. It has several modules that are designated for different functionalities. The first is a spot detector to detect the blood cell based on its size. This detector was based on the LoG algorithm. The second is a spot analyzer that can filter the object. The third functionality is selecting a view that opens a new 3D window and displays the spots as 3D spheres and 3D lines. The fourth functionality is a spot tracker that filter the spots and link them together. These trackers are based on Linear Assignment Problem (LAP). Finally, the functionality of the miscellaneous actions functionality completes the whole tracking process and export all statistic data for further analysis. Red blood cells show lower pixel intensity than the background; therefore, it is very difficult to identify using TrackMate. Other than the size, the TrackMate plugin also recognizes the object based on the threshold. If the background intensity value is greater than the object, it is recognized as a false positive result. Therefore, to minimize the false-positive results affected by background noise, the pixel values were inverted using ImageJ resulting in object pixel intensity greater than the background.

Furthermore, to analyze the blood flow pattern chronically, the Time Series Analyzer plugin was used. Stack Difference plugin was used beforehand to distinguish the foreground from the background. Only the blood cell movement was calculated, with the background subtracted. The calculation is based on pixel intensity change. The principle of pixel intensity change is based on the cell numbers and differences in the degree of an object that could be affected by speed. In the DA, the highest peak of blood flow pattern represents the systolic phase and the red blood cell velocity. This study found the time interval of blood flow pattern to be in agreement with heart rate. The dorsal aorta blood flow pattern corresponded to the heart beating rate and there was almost no flow in between pulses. Vasculogenesis in zebrafish resembles that in other higher vertebrates. The peak in the DA was 2100 μm/s and seldom ceased during the oscillatory cycle. The blood flow in the PCV is relatively less pulsatile and steadier [1]. After angioblasts differentiated from the ventral-lateral mesoderm, they migrated to the DA and the PCV. In the first circulation, the blood cell exits the heart through the bulbous arteriosus and ventral aorta and branches into mandibular aortic arches. The blood exits arches directly and forms into a single medial DA. Many factors contribute to the differences in blood flow pattern between the DA and the PCV. The ends of the DA anterior vessels are connected directly into the developing heart via the outflow tract. Blood flow continues into the tail and turns 180° entering the caudal vein. The size of the PCV vessel diameter is greater than the diameter of the DA. Therefore, more red blood cells could be accommodated in the PCV [3,28].

General anesthetics are commonly used in fish research for immobilization and reduction of pain, discomfort and handling stress. Tricaine methanesulfonate or MS222 is the most frequently used anesthetic in fish; however, depending on the dose and exposure time, it can have adverse effects, such as aversion, epidermal and corneal lesions, hypoxemia, decreased heart rate, and death. Moreover, MS222 has been reported to be toxic to humans [7]. Previous findings on the normal sedating dosage of MS222 has shown that it could lead to bradycardia in zebrafish [29]. In this study, the result is in corroboration that MS222 affects heart rhythm. The average heart rate was reduced when the fish was incubated in 160 ppm MS222. Blood flow velocity was also reduced to 72% after 160 ppm of

MS222 treatment. On the other hand, IBMX, a methylxanthine increased the heart rate of zebrafish by 12%. Methylxanthines are adenosine receptor antagonists, which cause an increase in the heart rate in the treated embryos [9]. Although IBMX increases the heart rate, it concurrently decreased the blood flow velocity. This result is in agreement that heartbeat and blood flow velocity could have different outcomes [6]. IBMX can cause defective heart formation, irregular heartbeat, decreased blood circulation, mild pericardial and yolk sac edema in zebrafish embryo which explains why it is not approved as a drug. [9].

Stroke volume is the amount of residual blood in the ventricle chamber after each heartbeat. It is an indication of how much blood could be pumped out and the power of the heart pushing blood through blood vessels. It is also defined as the difference between systolic and end-diastolic volume [30]. MS222 and IBMX lowered the stroke volume compared to the control. The amount of blood pumped out from the heart could be measured by cardiac output. It can be determined by the stroke volume and heart rate produce cardiac output. Cardiac output is an important indicator of how efficient the heart can fulfill the demands of the body for blood perfusion [31]. It was found that the cardiac output was also reduced after MS222 treatment; however, no significant difference in cardiac output was shown after IBMX treatment. This suggests that both blood flow and heart rate analysis should be done side by side to measure cardiac function precisely.

To magnify and enhance blood cell contrast, 40× Hoffman lens was used. This may be the first study that performed cardiovascular measurements in zebrafish by using a Hoffman modulation lens. The Hoffman contrast modulation increases visibility and contrast of an unstained and living material by detecting optical gradients (or slopes) and converting them into variations of light intensity. Thus, Hoffmann lens can obtain a clear blood cell image which comparable to the result obtained by using expensive Nomarski differential interference-contrast microscopy. However, in this study, we found the blood flow velocity obtained from non-Hoffman and Hoffman lens are consistent and comparable. These results strong indicates the ImageJ-based method reported here can tolerant low image contrast to extract precise blood flow velocity.

The blood flow velocity was also measured in zebrafish larvae ontogenically from 1 to 5 dpf. Blood flow velocity continued to increase and reached the highest velocity on day 3, with a subsequent significant decrease on the fourth day. The increase in size of the ventricular-bulbar valve and the increased hematocrit due to ongoing production of blood cells may possibly account for the reduction of velocity. Moreover, on 4 dpf, a motion occurred due to the closure of the valve pushing red blood cells back into the ventricle and this phenomenon was not present in the 3 dpf fish [16].

In conclusion, the designed method and instrument setting provides an easy and low-cost methodology to study the blood flow velocity and pattern in zebrafish embryo for drug/compound screening. It can also provide precise measurement of cardiac functions, including heart rate, stroke volume, and cardiac output. Most importantly, the simple setting result corresponds with other studies which measured cardiovascular performance in zebrafish larvae using an expensive setting like laser-scanning velocimetry [14]. We believe this simple method will do great contribution for scientists on doing cardiovascular function assessment in fish embryo/larvae.

4. Methods

4.1. Zebrafish Maintenance

Zebrafish care and maintenance system, breeding, and larvae raising were in accordance with standard protocols [32]. Wild type AB strain zebrafish were used for breeding and observation in this study. Blood flow velocity was determined in 72 hpf zebrafish larvae whereas cardiovascular function was evaluated in 1 to 5 dpf of zebrafish larvae. Phenylthiourea (PTU, Sigma, St Louis, MO, USA) treatment was applied to prevent pigment formation on masking the blood flow tracking according to the previous method [33]. Ethyl 3-aminobenzoate methanesulfonate (MS222, cat. A5040, Sigma) and 3-isobutyl-1-methylxanthine (IBMX, cat. A1914048, Aladdin, Shanghai, China) were purchased

from commercial companies. All experimental protocols involving zebrafish were approved by the Institutional Animal Care and Use Committee of Chung Yuan Christian University with the approval number 106025 (25 May 2017).

4.2. Video Recording

Healthy larva was transferred into 3 cm-Petri dish. Excess water was removed from the plate and 3% methylcellulose (Sigma) was applied to fix the fish position. The Petri dish was mounted on an inverted microscope (ICX41, Sunny Optical Technology, Zhejiang, China) equipped with a high-speed digital charged coupling device (CCD) (AZ Instrument, Taichung City, Taiwan). Hoffman objective lens 40× was used to magnify and enhance image contrast. In this study, the dorsal aorta (DA) and posterior cardinal vein (PCV) were selected as the regions of interest (ROI). HiBestViewer (AZ Instrument, Taichung City, Taiwan) software was used to record the video at 200 fps for 10 s and saved at a rate of 30 fps.

4.3. Video Processing

The captured videos in mp4 format were first converted to avi format by using Wondershare Video Converter Ultimate software (available online: https://videoconverter.wondershare.net/). The video was then rendered with VirtualDub software (available online: http://virtualdub.sourceforge.net/) to make it compatible with ImageJ. X264vfw (available online: https://sourceforge.net/projects/x264vfw/) software was installed to run the VirtualDub.

4.4. Blood Cell Tracking

The Fiji distribution of ImageJ (Available online: https://imagej.net/Fiji/Downloads) was used to track the blood cell velocity. Before blood flow velocity analysis, the distance of each blood cell was measured. This was done by using the MTrackJ plug-in (available online: https://imagescience.org/meijering/software/mtrackj/). The ROI was selected by the freehand selection tool and the outside area was cleared to facilitate the analysis. Trackmate plug-in (available online: https://github.com/fiji/TrackMate/releases/tag/TrackMate_-3.5.3) is one of the ImageJ Plugin to track the particle/cell movement. Several filters and parameters are implemented in the TrackMate to screen the object such as threshold, color, contrast, estimated diameter, etc. The object detection algorithm of TrackMate is based on the Laplacian of Gaussian (LoG) segmentation. There are three different detectors based on the size of the object and for this study, downsample of LoG detector was used. The tracking method is based on the Linear Assignment Problem (LAP) that is most suitable most for particles undergoing Brownian motion.

4.5. Blood Flow Pattern and Heart Rate Calculation

Stack Difference plugin was used to discriminate the foreground and the background. DA and PCV regions were selected as ROI to track the blood flow pattern. Then, Time Series Analyzer plug-in (Available online: https://imagej.nih.gov/ij/plugins/time-series.html) was used to analyze the dynamic pixel change pattern over time [1,18]. Next, the heart rate was measured from the dynamic pixel change data. The heartbeat rhythm was determined using Origin 9.1 software (Originlab Corporation, Northampton, MA, USA). Peak Analyzer function in Origin 9.1 was used to calculate the time of the peak. The time of peak was then processed by using Microsoft Excel (Microsoft 2016 version, Microsoft Corp. Seattle, WA, USA) to calculate time intervals and beats per minute. Heart rate is defined as beats per minute (bpm) and can be obtained by multiplying the time interval (in seconds) between two peaks by a factor of 60. Furthermore, to validate the result of the heartbeat, the heart rate was measured directly from the heart by following a previously established ImageJ-based method [34].

4.6. Stroke Volume, and Cardiac Output Calculation

Stroke volume (SV) was determined by multiplying the area of the DA/PCV (A) by the distance blood pumping around the aorta/vein in one beat (dx) (Equation (1)):

$$\mathrm{SV} = \mathrm{A} \times \mathrm{dx} \tag{1}$$

Since a blood vessel is cylindrical, the area (A) was measured using Equation (2), where blood vessel diameter (d) was measured with line selection in ImageJ function:

$$A = \pi \left(\frac{d}{2}^2\right) \tag{2}$$

Because velocity is defined as the distance traveled per unit time, the distance traveled (dx) was determined by integrating cell velocities as a function of time. The velocity-time integral (VTI) was measured by multiplying average speed with Δt. Therefore, the final equation of stroke volume was obtained by Equation (3):

$$SV = \pi \left(\frac{d}{2}^2\right) \times VTI \tag{3}$$

Cardiac output (CO) was determined by multiplying the stroke volume (SV) by the heart rate (HR) (Equation (4)):

$$\mathrm{CO} = \mathrm{SV} \times \mathrm{HR} \tag{4}$$

4.7. Statistics

All statistical data were compiled and plotted using GraphPad Prism (GraphPad Software version 7 Inc., La Jolla, CA, USA). One-way or two-way ANOVA test was given by following the post hoc test of Tukey, depending upon the data normality for significant data. Level of Significance was set at a p value < 0.05.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2411-5134/4/4/65/s1.

Author Contributions: Conceptualization, F.S., B.P.S. and C.-D.H.; Data curation, F.S. and B.P.S.; Formal analysis, F.S., B.P.S. and C.-D.H.; Funding acquisition, C.-D.H.; Investigation, E.H., J.-R.C. and C.-D.H.; Methodology, C.-D.H.; Resources, E.H.; Validation, Y.-H.L., S.-T.L. and E.H.; Writing—original draft, F.S., B.P.S., J.-R.C. and C.-D.H.

Funding: This work was funded by Ministry of Science and Technology, Taiwan (grant no. MOST105-2313-B-033-001-MY3; MOST107-2622-B-033-001-CC2 and MOST108-2622-B-033-001-CC2) to Chung-Der Hsiao; Guangxi Innovation Driven Development Project, NO.: GUIKEAA18242040 to Erwei Hao.

Acknowledgments: The authors wish to thank Roni J. Bollag, Medical College of Georgia, Augusta University, Kuan-Cheng Chiu of Department of Physics, Chung Yuan Christian University and Rhenz Alfred D. Liman from University of Santo Tomas for helpful criticism and proof reading of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Anton, H.; Harlepp, S.; Ramspacher, C.; Wu, D.; Monduc, F.; Bhat, S.; Liebling, M.; Paoletti, C.; Charvin, G.; Freund, J.B. Pulse propagation by a capacitive mechanism drives embryonic blood flow. *Development* **2013**, *140*, 4426–4434. [CrossRef] [PubMed]
2. Boselli, F.; Freund, J.B.; Vermot, J. Blood flow mechanics in cardiovascular development. *Cell. Mol. Life Sci.* **2015**, *72*, 2545–2559. [CrossRef] [PubMed]
3. Isogai, S.; Horiguchi, M.; Weinstein, B.M. The vascular anatomy of the developing zebrafish: An atlas of embryonic and early larval development. *Dev. Biol.* **2001**, *230*, 278–301. [CrossRef] [PubMed]
4. McGrath, P.; Li, C.-Q. Zebrafish: A predictive model for assessing drug-induced toxicity. *Drug Discov. Today* **2008**, *13*, 394–401. [CrossRef] [PubMed]

5. Zon, L.I.; Peterson, R.T. In vivo drug discovery in the zebrafish. *Nat. Rev. Drug Discov.* **2005**, *4*, 35. [CrossRef] [PubMed]
6. Dong, W.; Teraoka, H.; Yamazaki, K.; Tsukiyama, S.; Imani, S.; Imagawa, T.; Stegeman, J.J.; Peterson, R.E.; Hiraga, T. 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: Local circulation failure in the dorsal midbrain is associated with increased apoptosis. *Toxicol. Sci.* **2002**, *69*, 191–201. [CrossRef]
7. Martins, T.; Diniz, E.; Felix, L.M.; Antunes, L. Evaluation of anaesthetic protocols for laboratory adult zebrafish (danio rerio). *PLoS ONE* **2018**, *13*, e0197846. [CrossRef]
8. Huang, W.-C.; Hsieh, Y.-S.; Chen, I.-H.; Wang, C.-H.; Chang, H.-W.; Yang, C.-C.; Ku, T.-H.; Yeh, S.-R.; Chuang, Y.-J. Combined use of ms-222 (tricaine) and isoflurane extends anesthesia time and minimizes cardiac rhythm side effects in adult zebrafish. *Zebrafish* **2010**, *7*, 297–304. [CrossRef]
9. Basnet, R.M.; Zizioli, D.; Guarienti, M.; Finazzi, D.; Memo, M. Methylxanthines induce structural and functional alterations of the cardiac system in zebrafish embryos. *BMC Pharmacol. Toxicol.* **2017**, *18*, 72. [CrossRef]
10. Basnet, R.; Guarienti, M.; Memo, M. Zebrafish embryo as an in vivo model for behavioral and pharmacological characterization of methylxanthine drugs. *Int. J. Mol. Sci.* **2017**, *18*, 596. [CrossRef]
11. Pan, X.; Yu, H.; Shi, X.; Korzh, V.; Wohland, T. Characterization of flow direction in microchannels and zebrafish blood vessels by scanning fluorescence correlation spectroscopy. *J. Biomed. Opt.* **2007**, *12*, 014034. [CrossRef] [PubMed]
12. Iftimia, N.V.; Hammer, D.X.; Ferguson, R.D.; Mujat, M.; Vu, D.; Ferrante, A.A. Dual-beam fourier domain optical doppler tomography of zebrafish. *Opt. Express* **2008**, *16*, 13624–13636. [CrossRef] [PubMed]
13. Zeng, Y.; Xu, J.; Li, D.; Li, L.; Wen, Z.; Qu, J.Y. Label-free in vivo flow cytometry in zebrafish using two-photon autofluorescence imaging. *Opt. Lett.* **2012**, *37*, 2490–2492. [CrossRef] [PubMed]
14. Malone, M.H.; Sciaky, N.; Stalheim, L.; Hahn, K.M.; Linney, E.; Johnson, G.L. Laser-scanning velocimetry: A confocal microscopy method for quantitative measurement of cardiovascular performance in zebrafish embryos and larvae. *BMC Biotechnol.* **2007**, *7*, 40. [CrossRef] [PubMed]
15. Marr, D.; Hildreth, E. Theory of edge detection. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **1980**, *207*, 187–217.
16. Jamison, R.A.; Fouras, A.; Bryson-Richardson, R.J. Cardiac-phase filtering in intracardiac particle image velocimetry. *J. Biomed. Opt.* **2012**, *17*, 036007. [CrossRef]
17. Sato, Y. Dorsal aorta formation: Separate origins, lateral-to-medial migration, and remodeling. *Dev. Growth Differ.* **2013**, *55*, 113–129. [CrossRef]
18. Watkins, S.C.; Maniar, S.; Mosher, M.; Roman, B.L.; Tsang, M.; St Croix, C.M. High resolution imaging of vascular function in zebrafish. *PLoS ONE* **2012**, *7*, e44018. [CrossRef]
19. Collymore, C.; Tolwani, A.; Lieggi, C.; Rasmussen, S. Efficacy and safety of 5 anesthetics in adult zebrafish (danio rerio). *J. Am. Assoc. Lab. Anim. Sci.* **2014**, *53*, 198–203.
20. Matthews, M.; Varga, Z.M. Anesthesia and euthanasia in zebrafish. *ILAR J.* **2012**, *53*, 192–204. [CrossRef]
21. Lockwood, N.; Parker, J.; Wilson, C.; Frankel, P. Optimal anesthetic regime for motionless three-dimensional image acquisition during longitudinal studies of adult nonpigmented zebrafish. *Zebrafish* **2017**, *14*, 133–139. [CrossRef] [PubMed]
22. Ramlochansingh, C.; Branoner, F.; Chagnaud, B.P.; Straka, H. Efficacy of tricaine methanesulfonate (ms-222) as an anesthetic agent for blocking sensory-motor responses in xenopus laevis tadpoles. *PLoS ONE* **2014**, *9*, e101606. [CrossRef] [PubMed]
23. Cho, G.; Heath, D. Comparison of tricaine methanesulphonate (ms222) and clove oil anaesthesia effects on the physiology of juvenile chinook salmon oncorhynchus tshawytscha (walbaum). *Aquac. Res.* **2000**, *31*, 537–546. [CrossRef]
24. Schwartz, F.J. Use of ms 222 in anesthetizing and transporting the sand shrimp. *Prog. Fish Cultur.* **1966**, *28*, 232–234. [CrossRef]
25. Polese, G.; Winlow, W.; Di Cosmo, A. Dose-dependent effects of the clinical anesthetic isoflurane on octopus vulgaris: A contribution to cephalopod welfare. *J. Aquat. Anim. Health* **2014**, *26*, 285–294. [CrossRef] [PubMed]
26. De Luca, E.; Zaccaria, G.M.; Hadhoud, M.; Rizzo, G.; Ponzini, R.; Morbiducci, U.; Santoro, M.M. Zebrabeat: A flexible platform for the analysis of the cardiac rate in zebrafish embryos. *Sci. Rep.* **2014**, *4*, 4898. [CrossRef]
27. Huertas, A.; Medioni, G. Detection of intensity changes with subpixel accuracy using laplacian-gaussian masks. *IEEE Trans. Pattern Anal. Mach. Intell.* **1986**, *8*, 651–664. [CrossRef]

28. Gore, A.V.; Monzo, K.; Cha, Y.R.; Pan, W.; Weinstein, B.M. Vascular development in the zebrafish. *Cold Spring Harb. Perspect. Med.* **2012**, *2*, a006684. [CrossRef]
29. Sun, P.; Zhang, Y.; Yu, F.; Parks, E.; Lyman, A.; Wu, Q.; Ai, L.; Hu, C.-H.; Zhou, Q.; Shung, K. Micro-electrocardiograms to study post-ventricular amputation of zebrafish heart. *Ann. Biomed. Eng.* **2009**, *37*, 890–901. [CrossRef]
30. Moore, F.B.; Hosey, M.; Bagatto, B. Cardiovascular system in larval zebrafish responds to developmental hypoxia in a family specific manner. *Front. Zool.* **2006**, *3*, 4. [CrossRef]
31. Jacob, E.; Drexel, M.; Schwerte, T.; Pelster, B. The influence of hypoxia and of hypoxemia on the development of cardiac activity in zebrafish larvae. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2002**, *283*, R911–R917. [CrossRef] [PubMed]
32. Avdesh, A.; Chen, M.; Martin-Iverson, M.T.; Mondal, A.; Ong, D.; Rainey-Smith, S.; Taddei, K.; Lardelli, M.; Groth, D.M.; Verdile, G. Regular care and maintenance of a zebrafish (Danio rerio) laboratory: An introduction. *J. Vis. Exp.* **2012**, *18*, e4196. [CrossRef] [PubMed]
33. Karlsson, J.; Von Hofsten, J.; Olsson, P.-E. Generating transparent zebrafish: A refined method to improve detection of gene expression during embryonic development. *Mar. Biotechnol.* **2001**, *3*, 522–527. [CrossRef] [PubMed]
34. Sampurna, B.P.; Audira, G.; Juniardi, S.; Lai, Y.-H.; Hsiao, C.-D. A simple imagej-based method to measure cardiac rhythm in zebrafish embryos. *Inventions* **2018**, *3*, 21. [CrossRef]