High-Resolution Tissue Doppler Imaging of the Zebrafish Heart During Its Regeneration

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Abstract

The human heart cannot regenerate after injury, whereas the adult zebrafish can fully regenerate its heart even after 20% of the ventricle is amputated. Many studies have begun to reveal the cellular and molecular mechanisms underlying this regenerative process, which have exciting implications for human cardiac diseases. However, the dynamic functions of the zebrafish heart during regeneration are not yet understood. This study established a high-resolution echocardiography for tissue Doppler imaging (TDI) of the zebrafish heart to explore the cardiac functions during different regeneration phases. Experiments were performed on AB-line adult zebrafish ($n=40$) in which 15% of the ventricle was surgically removed. An 80-MHz ultrasound TDI based on color M-mode imaging technology was employed. The cardiac flow velocities and patterns from both the ventricular chamber and myocardium were measured at different regeneration phases relative to the day of amputation. The peak velocities of early diastolic inflow, early diastolic myocardial motion, late diastolic myocardial motion, early diastolic deceleration slope, and heart rate were increased at 3 days after the myocardium amputation, but these parameters gradually returned to close to their baseline values for the normal heart at 7 days after amputation. The peak velocities of late diastolic inflow, ventricular systolic outflow, and systolic myocardial motion did not significantly differ during the heart regeneration.

Introduction

THE ADULT ZEBRAFISH (*Danio rerio*) has recently become an important small-animal model for studying human pathologies in many fields of preclinical research and translational medicine, particularly those related to cardiovascular diseases.^{1–3} In 2002, Poss *et al.* found that an adult zebrafish can fully regenerate its heart even after 20% of its ventricular myocardium has been surgically removed.⁴ This was an exhilarating finding, since it opened the possibility of identifying factors that could stimulate a regenerative response in the human heart. Researchers have studied how the zebrafish regenerates its heart from cellular, molecular, and genetic viewpoints.^{5–10} Figure 1 illustrates the time course of zebrafish heart regeneration.⁷ Two of the interesting questions that have emerged about zebrafish heart regeneration are (1) how does the zebrafish heart functions during different regeneration phases and (2) do dynamic cardiac functions play any role in heart regeneration?

Studies that have evaluated the regeneration phases of the adult zebrafish heart have mainly observed the cardiac morphology using optical or fluorescence microscopy in histological specimens. Although histological slides can provide

very-high-resolution images of tissue structure, they are not suited to elucidate the dynamic cardiac functions of the zebrafish, since they require the animal to be sacrificed. Therefore, a noninvasive imaging modality is needed for observing the heart structure and evaluating the cardiac functions of adult zebrafish *in vivo*. Micro magnetic resonance imaging (micro-MRI)¹¹ and fluorescence microscopy¹² have been proposed for imaging the adult zebrafish heart noninvasively. However, the temporal resolution of micro-MRI is inadequate for monitoring cardiac function, and a genetically modified transparent zebrafish is needed for fluorescence microscopy imaging—the drawbacks of this optical modality include that it is not easy to produce genetically modified transparent zebrafish and the low optical penetration makes it difficult to estimate the cardiac function of adult zebrafish.

High-frequency (>50 MHz) ultrasound imaging has recently been proposed as a useful tool for adult zebrafish imaging due to its high image resolution, noninvasiveness, and real-time capabilities.¹³ A commercial array imaging system with a center ultrasound frequency of 50 MHz is currently available for small-animal imaging applications (Vevo 2100; Visualsonics, Toronto, Canada). Although such

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FIG. 1. Illustration of the different phases of zebrafish heart regeneration. The zebrafish heart consists of an atrium and a ventricle, with the outlet of the ventricle connected to a bulbus arteriosus. Amputating the apex of the ventricle resulted in a blood clot forming that filled up the missing tissue below the plane of amputation within 3 days. A fibrin layer subsequently formed along the inner side of the wound margin at 7 days after amputation. By 14 days the fibrin layer was reduced, with it being replaced by new myocytes. The fibrin layer was eventually replaced by new myocardium at 30 days after amputation.⁷

an array system can provide a high imaging frame rate for the cardiac imaging of small animals, the use of higher frequency ultrasound would improve the image resolution.¹⁴ The heart of the adult zebrafish is smaller than $1.5 \times 1.5 \times 1.5$ mm^{3,15} and so the ultrasound frequency needs to be higher than 75 MHz to provide sufficient resolution for imaging the detailed heart structures according to the suggestions from previous studies.^{14,16} Array transducers operating at frequencies higher than 50 MHz are not available, but a single-element transducer system based on mechanical scanning is still an option for zebrafish heart imaging. An 80-MHz duplex echocardiography system based on mechanical scanning has been built in our laboratory for obtaining high-frame-rate color Doppler images of the zebrafish heart.¹⁶ There is now a considerable amount of evidence that high-frequency ultrasound imaging is a suitable modality for obtaining images with both high temporal and spatial resolutions for monitoring the cardiac functions of the zebrafish heart. Therefore, we believe that this acoustic modality may make it possible to answer some of the remaining questions about the cardiac functions of the zebrafish heart during regeneration phases, without requiring the use of genetic modifications.

Duplex echocardiography including color M-mode imaging (CMI) and pulsed-wave Doppler sonography has been extensively used for diagnosing cardiovascular diseases in human^{17,18} and in studies of small animals such as rats and mice.^{19,20} Tissue Doppler imaging (TDI) is a relatively new echocardiography technique proposed for the clinical evaluation of the cardiac functions of the human heart. Duplex echocardiography uses the Doppler technique to assess the blood flow velocity by measuring high-frequency and low-amplitude signals from fast-moving blood cells. In TDI, the same Doppler principle is used to quantify the higher-amplitude and lower-frequency signals of myocardial tissue motion, particularly from the mitral annulus and basal left ventricle.^{21,22} Several cardiovascular diseases and ventricular functions can be detected or estimated by measuring the velocity of myocardial motion using TDI, such as left ventricular systolic and diastolic functions, heart failure, hypertrophic cardiomyopathy, and cardiac dyssynchrony.^{23,24} TDI might be suitable for assessing the ventricular systolic and diastolic functions when attempting to understand how the zebrafish heart functions during its regeneration.

This study established a high-resolution echocardiography and TDI system based on high-frequency ultrasound technology for assessing the functions of the adult zebrafish heart during its regeneration. The construction of the imaging system is described, including the hardware and Doppler signal processing algorithm. Animal experiments were performed with adult AB-line zebrafish in which 15% of the ventricular myocardium was surgically removed from the apex. The blood flow velocity within the ventricular chamber and the motion velocity of the ventricular myocardium were measured by CMI and TDI, respectively, during different heart regeneration phases. The echocardiography and TDI parameters were also calculated according to these velocity patterns. The data reported here can provide a framework for cardiovascular function analysis of the zebrafish heart and provide another viewpoint for studying zebrafish heart regeneration.

Materials and Methods

Zebrafish experimental arrangement

Experiments were carried out using healthy AB-line adult zebrafish (GENDANIO Ocean Technology, New Taipei City, Taiwan) aged 4–5 months. The zebrafish were reared in separate tanks at a density of 10 fish per 10 L of unchlorinated water, with the temperature maintained within the range 27.5–28.5°C. The zebrafish was anesthetized with ethyl 3-aminobenzoate methanesulfonate salt (MS-222; Sigma-Aldrich, St. Louis, MO) dissolved in water at pH 7.2 to a concentration of 0.16% as a stock, which was kept at 4°C. For echocardiography examinations, the zebrafish were anesthetized to stage-4 anesthesia, defined as the zebrafish turning abdominal side up for more than 5 s but with the operculum still moving. After the zebrafish was anesthetized and fixed in the sponge, high-resolution echocardiography was used to measure the blood flow velocity within the ventricular chamber and the tissue motion velocity of the ventricular myocardium using CMI and TDI, respectively. The weight and length of the experimental fish used in this study ($n=40$) were 0.32 ± 0.04 g and 3.82 ± 0.20 cm (mean $\pm$ standard error of the mean), respectively. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of Fu-Jen Catholic

FIG. 2. Photographs of the zebrafish surgery (a) and the setup for ultrasound examinations (b). All of the surgical operations were performed under microscopy assistance. The zebrafish was anesthetized by immersion in 4.2% MS-222 (tricaine methanesulfonate) solution and then transferred onto a moist sponge that had been shaped so as to stably hold the fish upside down. The posterior medial margin of the heart was located visually, and straight operating scissors were used to puncture the skin. The scissors were used to make a small incision above the heart through the skin and muscles. The silvery epithelial layer of the hypodermis was torn gently using the tip of the scissors so as to provide direct access to the ventricle. The ventricle was then exposed using gentle pressure, and the scissors were used to remove 15% of the ventricle from the apex.

University (Taiwan). Figure 2 shows photographs of the zebrafish surgery and the setup for ultrasound examinations. The animal survival rate is 92% in this study.

High-resolution echocardiography system

Figure 3 shows a block diagram of the high-resolution echocardiography system. The core of this imaging system is an 80-MHz single-element high-frequency ultrasonic transducer (NIH Ultrasonic Transducer Resource Center). The axial and lateral resolutions of this imaging system were ~ 21 and $56 \mu\text{m}$, respectively. The transducer was driven by a customized high-frequency bipolar-pulse signal generator. The center frequency of the bipolar excitation pulses was 80 MHz, and their peak-to-peak voltage was 200 V. Backscattering signals were amplified 45 dB using a preamplifier (AU-1313; MITEQ, Hauppauge, NY), filtered by a band-pass

filter, and then digitized by an 8-bit analog-to-digital converter (ADC; PXI 5152; National Instruments, Austin, TX) at a sampling rate of 500 MHz. The transducer was mounted on a one-axis piezoceramic motor (HR8; Nanomotion, Yokneam, Israel) to allow mechanical scanning. The transducer could also be moved in the other two orthogonal directions using two axial microstep motors (CM1-C-17L30A; Cool Muscle, Tokyo, Japan). The three-axes motor stage was controlled by a motor controller (DMC-1842; Galil Motion Control, Rocklin, CA). A software program was developed for motor control and backscattering signal acquisition using LabVIEW software (National Instruments). The pulser-receiver and ADC were synchronized using a trigger signal from a function generator (AFG3252; Tektronix, Beaverton, OR). High-resolution two-dimensional (2D) B-mode images of the zebrafish heart were reconstructed by processing the backscattering signals using demodulation, logarithmic compression, and gray-level transformation. The lateral step distance of each scan A-line was $4 \mu\text{m}$. The echocardiography frame rates were adjusted to 10 frames per second within a scanning width of 2 mm for obtaining real-time B-mode images.

Blood flow velocity and tissue velocity estimation

Before the CMI and TDI measurements, the position of the zebrafish heart was determined using real-time echocardiography system. The measurement locations for the blood flow velocity and ventricular myocardium were then selected via a user interface of the echocardiography system, and the transducer was automatically moved to that location. Consecutive backscattering signals were then acquired from the moving tissues at a certain scan location at a pulse repetition frequency (PRF) of 40 kHz by the ADC. These measurement locations for the blood flow velocity and ventricular myocardium velocity were located within the ventricle and atrioventricular valve annulus, respectively. The ultrasound M-mode image was obtained first using the same signal processing procedure as used for the B-mode image, except that the motion information of the tissue relative to the transducer along the beam direction was displayed as a function of time. CMI and TDI images were subsequently reconstructed according to the Doppler signal processing

FIG. 4. Flow chart of Doppler signal processing for tissue Doppler imaging (TDI) and determining the blood flow velocity.

procedure, as shown in Figure 4. After the demodulation processing of backscattering signals, the tissue velocities (including of the blood and ventricular myocardium) were estimated by a 2D autocorrelation velocity estimation algorithm given by

$$V = \frac{c}{2} \frac{\frac{\text{PRF}}{2\pi} \tan^{-1} \left\{ \frac{\sum_{m=0}^{M-1} \sum_{n=0}^{N-1} [Q(m, n)I(m, n+1) - I(m, n)Q(m, n+1)]}{\sum_{m=0}^{M-1} \sum_{n=0}^{N-1} [Q(m, n)Q(m, n+1) + I(m, n)I(m, n+1)]} \right\}}{\frac{f_s}{2\pi} \left\{ 2\pi f_{\text{dem}} + \tan^{-1} \left\{ \frac{\sum_{m=0}^{M-2} \sum_{n=0}^{N-1} [Q(m, n)I(m+1, n) - I(m, n)Q(m, n+1)]}{\sum_{m=0}^{M-2} \sum_{n=0}^{N-1} [Q(m, n)Q(m+1, n) + I(m, n)I(m+1, n)]} \right\} \right\}}, \quad (1)$$

where V is the estimated velocity, c is speed of sound in the tissue, f_s is the sampling frequency, f_{dem} is the ratio of the center frequency to the sampling frequency, I and Q are the in-phase and quadrature sampled signals, respectively, M is the window depth (quantified as the number of samples), N is the total number of A-line backscattering signals, and indexes m and n correspond to the fast-time (depth direction) and the slow-time (pulse number) directions, respectively, of I and Q data. The slow-time window was 100 lines for continuous backscattering signals and the fast-time window was $40 \mu\text{m}$ when calculating the 2D autocorrelation algorithm in the zebrafish experiments.

After the Doppler information of blood and tissue were obtained along the depth direction, the motion velocities were encoded in color on the grayscale M-mode image to reconstruct the CMI image. The motion of the target toward and away from the transducer was displayed in red and blue,

respectively. The gating locations of the ventricle and ventricular myocardium were subsequently selected to obtain the blood flow velocity and TDI image, respectively. The Doppler data were recorded for 20 s in each measurement.

Statistical analysis

The echocardiography and TDI parameters were measured for the normal zebrafish heart (uninjured) and the injured zebrafish heart (on days 1, 3, 7, 14, and 30 after amputation). Differences between the normal and injured groups were assessed by one-way analysis of variance. A probability value of $p < 0.05$ was considered as indicative of a statistically significant difference (relative to the normal zebrafish group). The statistical analysis was performed using commercial software (SigmaPlot 12.0; Systat Software, San Jose, CA).

Results

Echocardiography of the zebrafish heart

Figure 5a shows a typical B-mode image of a normal zebrafish heart as obtained by high-resolution echocardiography. Blood drains into the triangular single-chamber atrium from the sinus venosus, and then the single-chamber ventricle pumps blood through the bulboventricular orifice into the pear-shaped bulbus arteriosus, which is interposed between the ventricle and the ventral aorta. The diameter of the adult zebrafish bulboventricular annulus is typically $100\text{--}150 \mu\text{m}$, as measured by scanning electron microscopy.¹⁵ A bulboventricular annulus of similar size was observed by echocardiography, and even better dynamic blood flow information was obtained using this modality. Figure 5b shows a typical CMI image of a normal zebrafish heart and its corresponding anatomical measurement locations.

The measurement location for the ventricular myocardium was selected as the atrioventricular valve annulus (the dorsal side of the ventricle); this is where the ventricle motion is greatest, and this position also corresponds to the mitral annulus of the human heart, whose velocity is often measured in TDI-based assessments of human cardiac functions.

Blood flow velocity and tissue velocity estimation

Figure 6 shows examples of the quantitative measurements of blood flow and myocardium motion velocity during the cardiac cycle of a zebrafish heart on uninjured and day 3. Several parameters for characterizing the blood flow velocity are clearly displayed in the figure, including the early diastolic inflow (E wave), late diastolic inflow (A wave), and ventricular systolic outflow (S wave). The measured waveforms for characterizing the myocardium

FIG. 5. Typical B-mode image of a normal zebrafish heart obtained using high-resolution echocardiography (a). The two major chambers—atrium and ventricle—are clearly evident in the figure, without requiring the fish to be modified genetically so as to make it transparent. The locations of the blood flow and tissue could be easily identified using the features of the speckle pattern. Typical color M-mode imaging image of a normal zebrafish heart and its corresponding anatomical locations of measurement (b). The motion velocities of both the blood and myocardium have been encoded in color, showing that the peak blood flow velocity was faster than the tissue motion velocity. The boundaries of the ventricular wall are clearly evident. Color images available online at www.liebertpub.com/zeb

motion velocity are also presented for different phases of the cardiac cycle: the early diastolic myocardial motion (Em wave), late diastolic myocardial motion (Am wave), and systolic myocardial motion (Sm wave). The peak velocities and patterns of various blood flow and myocardium motion waveforms were clearly altered by the amputation of part of the myocardium.

FIG. 6. Typical examples of quantitative measurements of the blood flow and myocardium motion velocity during the cardiac cycle of a zebrafish on uninjured (left) and day 3 (right). E, early diastolic inflow; A, late diastolic inflow; S, ventricular systolic outflow; Em, early diastolic myocardial motion; Am, late diastolic myocardial motion; Sm, systolic myocardial motion.

Echocardiography and TDI parameters measurements

A schematic diagram of the blood flow velocity and tissue motion velocity waveforms for a normal zebrafish during the cardiac cycle is shown in Figure 7. The slope of early diastolic deceleration and the heart rate were measured from these velocity patterns. Figure 8 shows the blood flow and TDI parameters obtained from different fish during the heart regeneration that occurred during 30 days following the myocardium amputation. It is obvious that the peak velocities of the E wave, Em wave, Am wave, early diastolic deceleration slope, and heart rate were increased at 3 days after the myocardium amputation. However, those parameters gradually returned to their baseline values for the normal heart at 7 days after myocardium amputation (with no statistically significant differences between the values on uninjured and day 7). The peak velocities of the A wave, S wave, and Sm wave did not significantly differ during the various heart regeneration phases. The quantitative data of these parameters are listed in Table 1. Two other parameters are also presented in the Table 1: the early diastolic velocity ratio (E/Em) and the early-to-late ventricular filling velocity ratio (E/A), defined as the E-wave velocity divided by the A-wave velocity.

Discussion

A conventional TDI system detects tissue velocity based on the tissue motion being slower than the blood flow and the echo signal being stronger for solid tissue than for blood cells. This allows the myocardium motion velocity of a patient to be easily extracted using an adaptive frequency filter or using an amplitude threshold to separate the echoes from blood and

FIG. 7. Schematic diagram of the blood flow velocity and tissue motion velocity waveforms for a normal zebrafish during the cardiac cycle. This figure combines the information on the blood flow pattern in the ventricle and the tissue motion pattern in the atrioventricular valve annulus, which facilitates the understanding of the relationship between blood flow and tissue motion of the zebrafish heart for evaluating the cardiac functions during different heart regeneration phases. SV, sample volume; BA, bulbus arteriosus; E, early diastolic inflow; A, late diastolic inflow; S, ventricular systolic outflow; Em, early diastolic myocardial motion; Am, late diastolic myocardial motion; Sm, systolic myocardial motion. The mean values of peak velocity for each flow are present.

other tissue. However, this principle cannot be fully applied for TDI of the zebrafish. The peak velocities of the E wave and A wave for healthy young adults are about 57 and 44 cm/s, respectively, and the peak velocity is ~ 15 cm/s for both Em and Am.²⁵ The peak velocity of the E wave is typically 15 times greater than the Em peak for patients with heart disease.²³ A frequency filter can therefore be used to easily separate the flow blood and tissue motion velocity in human TDI examinations. However, for the normal zebrafish heart the blood flow velocity approximately ranges from 5 to 29 mm/s (in different flow directions) and the myocardium motion velocity approximately ranges from 2 to 7 mm/s, as listed in Table 1. The presence of some overlap in these velocities within the zebrafish heart makes it difficult to separate the blood and tissue motions using the frequency-filter principle.

It is known from Rayleigh scattering theory that the magnitude of scattering from blood cells is proportional to the fourth power of frequency up to 90 MHz,²⁶ which means that the intensity of the signal backscattered from flowing blood markedly increases with the ultrasound frequency. The chambers of the human heart therefore appear as dark regions in echocardiography (the echo from the myocardium is typically 32 dB stronger than that from blood at 5 MHz).²⁷ However, Figure 5a shows that we can still obtain a strong echo signal from blood within the ventricle of the zebrafish at an ultrasound frequency of 80 MHz. Therefore, separating blood and tissue signals using an amplitude threshold is also not suitable in TDI of the zebrafish heart (as is also the case for the frequency-filter principle). However, the stronger echoes from blood provide another way to identify the region of the ventricular chamber, where the speckle pattern is more dense than that from the myocardium in the B-mode image, as shown in Figure 5a.

Two kinds of TDI (pulsed-wave TDI and color-coded TDI) have frequently been used for assessing the myocardium motion velocity in patients. Pulsed-wave TDI provides a high temporal resolution of typically 250–300 samples/s in a conventional system; however, a velocity filter needs to be set

at ~ 24 cm/s to prevent aliasing of the velocity signals.²⁴ Again, a velocity filter will work only because there is a huge difference in the peak velocity between blood flow and tissue motion. Since mechanical scanning cannot yield a 2D color-coded Doppler image, CMI was the best option for obtaining the TDI images of the zebrafish heart in this study. The sampling locations can be selected according to the speckle pattern in CMI, and a higher PRF of the ultrasound pulse provides a high temporal resolution (40,000 samples/s) for velocity estimation. Another advantage of CMI is that it allows synchronous measurements of both the tissue and blood flow velocities as a function of time.

The E/A ratio was smaller than 1 for the normal zebrafish, which is consistent with observations made in previous studies.^{14,28} Under normal conditions most of the blood will fill the ventricle during early diastole in the human heart. Thus, the E-wave peak velocity is larger than the A-wave velocity, which means that the E/A ratio is larger than 1 for the normal human heart. This difference in E/A ratio between humans and zebrafish may be attributed to differences in the heart structure. The human heart comprises separate discrete chambers, whereas the ventricle of the zebrafish heart is filled with muscular trabeculae to form a spongy network and the atrium was mainly composed of pectinate muscle.¹⁵ The long and slender trabecular network creates many tiny intertrabecular lacunae in the ventricle, which increases the contractile efficiency in pumping the blood into the bulbus arteriosus. However, this spongy network of the ventricle may increase the resistance to blood inflow or decrease the pressure gradient between the two chambers during early diastole. In contrast, a rapid contraction of the atrium by pectinate muscle pumps more blood into the ventricle during late diastole. Therefore, velocity orders of A-wave velocity > E-wave velocity and Am-wave velocity > Em-wave velocity were observed for the normal zebrafish, whereas the opposite orders of E-wave velocity > A-wave velocity and Em-wave velocity > Am-wave velocity were found for the normal human heart.²⁹

FIG. 8. Blood flow and TDI parameters obtained from different zebrafish during heart regeneration during 30 days following myocardium amputation: E wave (a), A wave (b), S wave (c), early diastolic deceleration slope (d), Em wave (e), Am wave (f), Sm wave (g), and heart rate (h). Data are mean and SEM values. Day 0 presents uninjured heart. * $P < 0.05$.

The E-wave velocity was clearly increased within the first 3 days after amputation, and it had returned to the baseline value at 7 days after amputation. However, the peak velocities of the A and S waves seemed invariable during all phases of heart regeneration. The ventricular diastolic filling pattern (E and A waves) is frequently used to represent the ventricular relaxation, which is affected by several factors such as internal loading forces, wall stress, and myocardial contraction.¹⁷ There are many clinical reports of the E-wave velocity and E/A ratio being decreased in patients with ischemic myocardial injury,¹⁸ and of the E-wave velocity being faster for young adults than for older adults (as well as E/A ratio

usually also decreasing with age), which may be attributed to reductions of the ventricular compliance and contraction.²⁵ However, the E-wave velocity and E/A ratio of the zebrafish were increased after partial amputation of the ventricle, which means that myocardial injury appears to promote ventricular diastolic filling. The Em-wave velocity, E/Em ratio, and early diastolic deceleration slope were increased within the first 3 days after amputation, which means that the myocardial motion is greater during the early stage of myocardial injury and results in a faster change of the pressure gradient between the atrium and ventricle. These results are opposite to those observed in patients with heart disease, in

TABLE 1. THE CARDIAC PARAMETERS OF THE ADULT ZEBRAFISH DURING DIFFERENT HEART REGENERATION PHASES

	<i>Normal</i>	<i>Day 1</i>	<i>Day 3</i>	<i>Day 7</i>	<i>Day 14</i>	<i>Day 30</i>
Peak E-wave velocity (mm/s)	5.4±0.8	10.0±1.6*	7.7±1.8*	5.2±1.3	4.8±1.3	4.6±0.9
Peak A-wave velocity (mm/s)	28.7±6.6	32.2±13.6	36.2±10.5	31.1±7.7	28.6±8.7	28.6±8.8
Peak S-wave velocity (mm/s)	-11.6±2.4	-10.8±2.5	-11.1±3.7	-13.4±3.4	-12.6±4.0	-12.3±3.0
Peak Em-wave velocity (mm/s)	-2.6±0.5	-3.7±1.2*	-3.1±1.3	-2.6±1.0	-2.3±0.5	-2.3±0.5
Peak Am-wave velocity (mm/s)	-7.0±1.4	-11.3±5.0*	-10.4±4.8*	-9.6±2.6	-6.9±3.8	-6.2±1.3
Peak Sm-wave velocity (mm/s)	4.8±1.4	4.7±2.2	5.0±1.3	4.1±2.5	4.8±1.3	4.1±0.8
Early diastolic deceleration slope	-0.06±0.01	-0.13±0.07*	-0.12±0.05*	-0.09±0.03	-0.08±0.03	-0.07±0.04
Heart rate (beats/min)	124.4±17.6	198.3±45.3*	193.9±33.5*	151.2±23.4	132.9±33.1	125.3±28.3
Mean E/Em ratio	2.07	2.70	2.48	2.00	2.08	2.00
Mean E/A ratio	0.19	0.31	0.21	0.17	0.17	0.16

* $P < 0.05$.

E, early diastolic inflow; A, late diastolic inflow; S, ventricular systolic outflow; Em, early diastolic myocardial motion; Am, late diastolic myocardial motion; Sm, systolic myocardial motion; E/Em, early diastolic velocity ratio (only represent the absolute mean value), E/A, early to late ventricular filling velocity ratio (only represent the absolute mean value).

whom reduced Em velocities have been documented in a variety of disease settings, including postinferior myocardial infarction, chronic pulmonary hypertension, and chronic heart failure.^{23,30} All of the measured parameters indicated that myocardial injury does not debilitate the ventricular relaxation but promotes ventricular filling. However, myocardial injury does not appear to affect the ventricular systolic function, since the peak velocities of the S and Sm waves remained the same after injury. Furthermore, the heart rate substantially increased by 3 days after amputation, which means that the cardiac output was increased to maintain the circulation. To confirm that the skin incision and heart exposure did not affect the measured results, the Em and Am wave were measured from sham-operated zebrafish ($N=5$) at uninjured, 1, and 3 days after skin incision and heart exposure. The result showed that the Em and Am wave velocities have no statistically significant difference between uninjured fish and sham-operated fish. In other words, the major cause for parameters changes can be attributed to heart amputation in this study.

The results of this study differ from previous studies,^{5,10} which means that the regenerating heart is still functionally impaired even at 10–14 days after trauma. The 15% of the ventricular myocardium was surgically removed from the apex in this study. However, the heart was removed/injured about 20% in these studies.^{5,10} It is possible to prolong the period of cardiac function recovery if 20% of the heart was removed. The adult zebrafish heart regeneration was studied from the cellular and molecular viewpoints, which indicated that electrically coupled cardiac muscle regenerates after resection injury, primarily through activation and expansion of cardiomyocyte populations.¹⁰ An optical voltage mapping method was used to measure the conduction velocity on the surface myocardium near the apex of injured heart. The results showed that electrical conduction is reestablished between existing and regenerated cardiomyocytes between 7

and 28 days postinjury. However, our finding shows that many physiological parameters of the injured heart were gradually returned to their baseline values for the normal heart at 7 days after myocardium amputation, as shown in Figure 8. The discrepancy between these two studies may be attributed to two possible reasons: (1) the TDI provides an overall effect on myocardial motions and blood flows, however, the electrical conduction was only measured near the injured tissue; (2) the optical voltage mapping method was performed from the explanted heart, however, the TDI was carried out *in vivo* from living zebrafish. The zebrafish heart regeneration was studied after cryoinjury.⁵ They found that cardiomyocyte proliferation was enhanced during heart regeneration (from 4 to 30 days after cryoinjury). However, they also showed that the electrocardiogram parameter, QTc-interval, was only increased at 7 days after cryoinjury due to the formation of scar, and no significant difference between the QTc values before surgery and at 30 days after cryoinjury. In other words, the regenerating heart is still impaired even at 7 days after trauma in the cellular and molecular viewpoints, however, the cardiac functions (physiological parameters) of impaired heart were gradually recovered by 3 days after myocardium amputation. This is reasonable because the cardiac functions should be first recovered to maintain the circulation, and then the heart can be regenerated gradually via various cellular and molecular mechanisms.

The ejection fraction (EF) is another parameter used for assessing the cardiac function. The EF of human heart can be measured by echocardiography. Since the ventricle of human is an empty chamber, the left ventricular volume in diastole and systole can be calculated from a single ultrasonic dimension (M-mode imaging). The end-diastolic volume (EDV) and end-systolic volume (ESV) were estimated by the cube of the echo diastolic dimension and systolic dimension, respectively. Stroke rate (SV) was derived from the difference of EDV and ESV, and $EF = SV/EDV$. This estimation

can be applied to zebrafish heart by using this high resolution echocardiography theoretically. Since the high resolution echocardiography is sufficient to define the border of the ventricle by B-mode imaging, it is possible to measure the variations of ventricular volume during diastolic and systolic phases. However, a high frame rate imaging technology is needed for this purpose. The frame rate of current system is 10 frames per second, which is not fast enough. We will increase the imaging frame rate by gating technology to achieve this idea in our future works.

In summary, a novel method of high-resolution TDI based on CMI was applied to adult zebrafish in this study. The cardiac parameters of the zebrafish were measured to explore the dynamic cardiac functions during different heart regeneration phases. The ventricular inflow pattern of the zebrafish differs from the filling pattern of the human left ventricle, with $E/A < 1$ for zebrafish and $E/A > 1$ for humans with normal diastolic function. The E-wave velocity, Em-wave velocity, Am-wave velocity, E/Em ratio, E/A ratio, and early diastolic deceleration slope were increased by 3 days after heart amputation, whereas the S-wave and Sm-wave velocities remained the same after injury. Some of these results differ from those observed in patients suffering from various types of myocardial injury. In other words, this unique cardiac function of the zebrafish may be another factor that influences the heart regeneration. Finally, we have shown how high-frequency ultrasound imaging can be particularly useful for investigating cardiac functions and for analyzing the zebrafish heart during its regeneration. The new technique is simple, rapid, widely available, and relatively inexpensive.

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Disclosure Statement

No competing financial interests exist.

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