Journal of Visualized Experiments

# High-frequency ultrasound echocardiography to assess zebrafish cardiac function

--Manuscript Draft--

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|---|---|
| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE60976R1 |
| **Full Title:** | High-frequency ultrasound echocardiography to assess zebrafish cardiac function |
| **Section/Category:** | JoVE Biology |
| **Keywords:** | Zebrafish, Echocardiography, Cardiac function, High-frequency ultrasound, Cardiac output, Ejection fraction, Doppler blood flow velocity |
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| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US$2,400) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Palo Alto, CA, USA |

**TITLE:**

# High-Frequency Ultrasound Echocardiography to Assess Zebrafish Cardiac Function

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**KEYWORDS:**

Zebrafish, Echocardiography, Cardiac function, High-frequency ultrasound, Cardiac output, Ejection fraction, Doppler blood flow velocity

**SUMMARY:**

We describe a protocol to assess heart morphology and function in adult zebrafish using high-frequency echocardiography. The method allows visualization of the heart and subsequent quantification of functional parameters, such as heart rate (HR), cardiac output (CO), fractional area change (FAC), ejection fraction (EF), and blood inflow and outflow velocities.

**ABSTRACT:**

The zebrafish (*Danio rerio*) has become a very popular model organism in cardiovascular research, including human cardiac diseases, largely due to its embryonic transparency, genetic tractability, and amenity to rapid, high-throughput studies. However, the loss of transparency limits heart function analysis at the adult stage, which complicates modeling of age-related heart conditions. To overcome such limitations, high-frequency ultrasound echocardiography in zebrafish is emerging as a viable option. Here, we present a detailed protocol to assess cardiac function in adult zebrafish by non-invasive echocardiography using high-frequency ultrasound. The method allows visualization and analysis of zebrafish heart dimension and quantification of important functional parameters, including heart rate, stroke volume, cardiac output, and ejection fraction. In this method, the fish are anesthetized and kept underwater and can be recovered after the procedure. Although high-frequency ultrasound is an expensive technology,

the same imaging platform can be used for different species (e.g., murine and zebrafish) by adapting different transducers. Zebrafish echocardiography is a robust method for cardiac phenotyping, useful in the validation and characterization of disease models, particularly late-onset diseases; drug screens; and studies of heart injury, recovery, and regenerative capacity.

## INTRODUCTION:

The zebrafish (*Danio rerio*) is a well-established vertebrate model for studies of developmental processes and human diseases[1]. Zebrafish have high genetic similarity to humans (70%), genetic tractability, high fecundity, and optical transparency during embryonic development, which allows direct visual analysis of organs and tissues, including the heart. Despite having just one atrium and one ventricle, the zebrafish heart (**Figure 1**) is physiologically similar to mammalian four-chambered hearts. Importantly, the zebrafish heart rate, electrocardiogram morphology, and action potential shape resemble those of humans more than murine species[2]. These features have made zebrafish an excellent model for cardiovascular research and have provided major insights into cardiac development[3,4], regeneration[5], and pathologic conditions[1,3,4], including arteriosclerosis, cardiomyopathies, arrhythmias, congenital heart diseases, and amyloid light chain cardiotoxicity [1,4,6]. Assessment of cardiac function has been possible during the embryonic stage (1–days post fertilization) through direct video analysis using high-speed video microscopy [7,8]. However, zebrafish lose their transparency beyond the embryonic stage, limiting functional evaluations of normal mature hearts and late-onset heart conditions. To overcome this limitation, echocardiography has been successfully employed as a high-resolution, real-time, noninvasive imaging alternative to evaluate adult zebrafish heart function[9-15].

In zebrafish, the heart is located ventrally in the thoracic cavity immediately posterior to the gills with the atrium located dorsal to the ventricle. The atrium collects venous blood from the sinus venosus and transfers it to the ventricle where it is further pumped to the bulbus arteriosus (**Figure 1**). Here, we describe a physiological, underwater, protocol to assess cardiac function in adult zebrafish by non-invasive echocardiography using a linear array ultrasound probe with a center frequency of 50 MHz for B-mode imaging at a resolution of 30 µm. Since ultrasound waves can easily travel through water, keeping close proximity between the fish and the scanning probe underwater provides enough contact surface for heart detection with no need for ultrasound gel and is overall less stressful for the fish. Although alternative zebrafish echocardiography systems were reported by several authors[9,12,13], here we present the general and most commonly used setup that applies to high-frequency ultrasound in animals.

The method allows high resolution imaging of the adult zebrafish heart, tracing of cardiac structures, and quantification of peak-velocities from Doppler blood flow measurements. We show reliable in vivo quantification of important systolic and diastolic parameters, such as ejection fraction (EF), fractional area change (FAC), ventricular blood inflow and outflow velocities, heart rate (HR), and cardiac output (CO). We contribute to establishing a reliable range of normal healthy adult zebrafish cardiac functional and dimensional parameters to allow a more precise evaluation of pathologic states. Overall, we provide a robust method to assess cardiac function in zebrafish, which has proven extremely useful in establishing and validating zebrafish

heart disease models[6,16], heart injury and recovery[10,13], and regeneration[11,12], and can be further used to evaluate potential drugs.

**PROTOCOL:**

All procedures involving zebrafish were approved by our Institutional Animal Care and Use Committee and are in compliance with the USDA Animal Welfare Act.

## 1. Experimental set-up

### 1.1. Setting up the platform for image acquisition

1.1.1. Using small scissors or a scalpel make an incision on a sponge at the 12 o'clock position to hold the fish during scanning. Place the sponge in a glass container (**Figure 2A**).

NOTE: The position of the incision should allow enough room to move transducer and also to keep the fish bellow the water line when the platform is tilted for scanning (**Figure 2**). The incision can vary depending on the size of the fish; however, for a standard size and weight, the incision should be approximately 2.5 cm x 0.7 cm x 0.5 cm (length, width, and depth, respectively). The glass container should be at least 6 cm deep to avoid water leakage while imaging the fish.

1.1.2. Affix the glass box containing the sponge on the ultrasound platform, for instance using double-sided tape. Ensure the glass box is at the center of the platform and firmly attached (**Figure 2B**).

1.1.3. Tilt the platform forward about 30° using the knob on the left side of the platform holder (**Figure 2B**). Fill the glass square with 200–250 mL of fish system water containing 0.2 mg/mL tricaine methanesulfonate (MS222).

NOTE: Tricaine can be prepared as a 4 mg/mL stock solution in Tris 40 mM pH 7 and further diluted to the desired concentration in fish system water; 0.2 mg/mL was found to be the best concentration[16]. The 4 mg/mL tricaine stock solution can be stored for a long period of time at -20 °C or at 4 °C for one month.

1.1.4. Insert the transducer within the micromanipulator holder on the working rail station, turning the notch of the transducer towards the operator. Keep the array parallel to the ground with the working side longitudinal with respect to the stage (see **Figure 2B**). Leave enough room (10 cm on both sides) for the now connected transducer-rail system to move along the x- and y-axes.

1.1.5. Log in to the control software and choose **Mouse (Small) Vascular**. Create a new study as well as a new series for each animal included in the study. Find the **new study** button located on the bottom left side of the screen on the browser page (the view starts in B-mode).

## 2. Handling the Fish

NOTE: Zebrafish used in this study were adult, 11-month-old males of the wild-type strain AB/Tuebingen (AB/TU). Zebrafish were maintained in a stand-alone flow-through aquarium system at 28 °C in a constant light cycle set as 14 h light/10 h dark. Zebrafish were fed twice daily with brine shrimp (*Artemia nauplii*) and dry food flakes.

2.1. Using a fish net, transfer the fish into a small tank containing system water with 0.2 mg/mL tricaine. Wait until the fish is fully anesthetized (no movement and no response to touch).

2.2. Using a plastic teaspoon, gently and quickly transfer the fish into the glass box containing the sponge into the previously made incision with ventral side of the fish facing up.

NOTE: Make sure the head of the fish is positioned towards the operator (same direction as the notch of the transducer) and at a slightly higher level compared to the rest of the body to achieve better heart visualization.

2.3. Gently lower the transducer (keeping its original position) using the handle on the rail system, placing it longitudinally and close to the ventral side of the fish with the notch of the transducer facing the operator. Leave 2-3 mm (no more than 1 cm) clearance from the fish. Adjust the platform in respect to the transducer using the micromanipulator in all 3 axes until the fish heart is visualized and then start image acquisition. The angle of the transducer should not be changed during the entire image acquisition (**Figure 2C**).

NOTE: As long as there is enough proximity (up to 1 cm), the water on top of the fish will provide a contact surface via liquid surface tension that allows transmission of the ultrasound waves between the probe and the fish. Therefore, there is no need to push the transducer against the fish. Try to complete this step and finish the scan in less than 3 minutes to prevent fish death or a decrease of the heart rate during image acquisition. If needed, use a timer. The heart can be found on the upper side of the screen towards the left side of the eye, which can be easily visualized if moving the x-axis all the way to the right. If there is continued difficulty in finding the heart while in B-Mode, switch to color Doppler mode, which will allow for tracking blood flow (red indicates blood flowing towards the operator) and locating the heart.

## 3. Image acquisition

NOTE: See **Table of Materials** for imaging system and image analysis software.

### 3.1. Longitudinal View B-Mode

3.1.1. After localizing the heart, select or stay in B-Mode (found at the bottom left side of the touchscreen after having initiated a new series) and reduce the field in order to zoom in and have a closer look at the heart for easier tracing during analysis.

3.1.2. In order to have a closer and clearer view of the heart in B-Mode image acquisition, reduce the field by zooming in. Use the touchscreen to manually narrow the field on both the x- and y-axes.

3.1.3. If needed, enhance the quality/contrast of the image by setting the dynamic range to 45–50 dB. Go to the B-mode controls in the **More Controls** option and subsequently save the change to **Mode Presets**. Tap **Mode Presets** to select the optimized image acquisition setting every time before starting to image a new series.

3.1.4. Take as many images as desired in the long axis plane by selecting **Save Image**.

NOTE: More detailed information and training resources on image acquisition can be found at <https://www.visualsonics.com/product/software/vevo-lab> and <https://www.visualsonics.com/Learning-hub-online-video-training-our-users>.

3.2. **Longitudinal View Pulse Wave**

3.2.1. Switch to Color Doppler for blood flow detection (select **Color** button) and acquisition (found at the bottom left side of the touchscreen after having initiated a new series).

3.2.2. Using the touch screen position the quadrant on top of the atrioventricular valve and localize the inflow, which will be distinguished by the red color signal (**Figure 3A**). Reduce the quadrant area as much as possible to increase the frame rate.

NOTE: Lower the Color pulse-repetition-frequency (Color PRF) (velocity range) to ensure yellow color can be seen in the velocity profile of the Color Doppler image. This will increase the range of velocities that can be seen and will help to create a mosaic of color that will allow to visualize more clearly the peak velocities.

3.2.3. Activate pulse wave (select **PW**) Doppler Mode to sample ventricular blood inflow velocity. Position the sample volume gate at the center of the atrioventricular valve (where the red color signal becomes more yellowish) to detect the maximum flow velocity. Adjust the PW angle on the screen using your fingers so it aligns with the direction of the blood inflow. Press **start** or **update** to start sampling the velocity of blood flowing into the ventricle.

NOTE: Make sure the angle correct line is parallel to the blood flow in order to provide consistent and reproducible results. Placing the angle correct line so it matches the direction of blood flow will ensure that velocities are accurately captured.

3.2.4. Repeat step 3.2.3 to determine the outflow velocity by placing the Color Doppler quadrant at the junction between the ventricle and the bulbus (bulbuventricular valve) and localize the flow, which will be distinguished by a blue color signal (**Figure 3B**). Position the sample volume gate right before the ventricle-bulbus junction and adjust the angle correction line to match the direction of the blood flow.

NOTE: As mentioned before, to achieve accurate velocity values, make sure the PW angle is aligned with the blood flow.

3.2.5. Adjust the baseline (bar), lowering or raising it in the flow velocity panel, in order to detect and trace completely the signal peaks (**Figure 3C,D**). Identify the inflow peaks in the upper/positive quadrant (signal going towards the probe) and the outflow peaks in the lower/negative quadrant (signal going away from the probe).

4. **Fish recovery**

4.1. As soon as image acquisition is complete, using a teaspoon, transfer the fish into regular system aerated water free of tricaine and let the fish recover (usually takes 30 s to 2 min to resume gill movement and swimming);

4.2. To help recovery, squirt water repeatedly over the gills using a transfer pipette to promote aeration of the water and oxygen transfer.

**5. Image analysis**

5.1. Open the image analysis software.

5.2. Select an image and click on the image processing icon (**Figure 4**). Using the available scale (**Figure 4**), adjust brightness and contrast of the image to allow clear visualization of ventricular walls or blood flow pattern.

5.3. Using the B-mode image, open the drop-down list from the PSLAX (parasternal long axis) option on the cardiac package/measurements (**Figure 4**). Select **LV trace** and trace the ventricular inner wall at systole and diastole to obtain the ventricular area (VA) in systole (VAs) and diastole (VAd), end diastolic volume (EDV), and end systolic volume (ESV) (**Figure 5A,B**).

NOTE: Volume values are extrapolated from 2D image tracings and might deviate from the 3D entity. For all measurements, average at least 3 representative cardiac cycles per animal.

5.4. Note the stroke volume and ejection fraction that will be automatically calculated and displayed by the software.

NOTE: Stroke volume, and ejection fraction can also be manually calculated using the formulas
SV = EDV-ESV
EF = (EDV-ESV)/EDV
where SV is stroke volume, EDV is end diastolic volume, ESV is end systolic volume, and EF is ejection fraction

5.5. Calculate fractional area change using the formula

FAC = (VAd - VAs)/ VAd
where FAC is fractional area change, VAd is ventricular area in diastole, and VAs is ventricular area in systole.

5.6. Calculate the cardiac output using the formula
CO = HR x SV
where CO is cardiac output, HR is heart rate, and SV is stroke volume

5.7. Using the Pulsed Wave Doppler Mode image, measure the inflow blood velocity by selecting the **MV Flow** option under the cardiac package (**Figure 4**). Select **E** or **A** for early diastole and late diastole, respectively, and determine the peak-velocities on the graph (**Figure 3C**).

5.8. Measure the outflow blood velocity by selecting **AoV Flow** and determine the peaks on the tracing (**Figure 3D**).

5.9. Measure the heart rate using 2 different methodologies for a more reliable assessment:

5.9.1. When the heart is visualized on the screen during image acquisition, count the beats within 10 s and multiply it by 6.

5.9.2. Using the Pulse Wave Doppler image on the Vevo LAB software, choose the **heart rate** button and trace intervals between 3 consecutive aortic flow peaks (**Figure 4** and **Figure 6**).

5.9.3. To export data to a spreadsheet after having traced the LV and the peaks of the blood flow, click on **report | export | save as | excel**.

**REPRESENTATIVE RESULTS:**

The described protocol allows for measurement of important cardiac dimensional and functional parameters, analogous to the technique used in human and animal echocardiography. The B-Mode images allow for tracing of ventricular inner wall in systole and diastole (**Figure 5**) and obtaining of dimensional data, such as chamber and wall dimensions, and functional data, such as heart rate, stroke volume, and cardiac output as well as parameters of ventricular systolic function, such as fractional area change and ejection fraction (**Table 1**). Measurements at the level of the atrioventricular valve using color Doppler Mode images also provide ventricular inflow and outflow blood velocities (velocity at which blood fills and exits the ventricle, respectively) (**Figure 3** and **Table 1**).

The parameters obtained in this study were comparable with the ones reported in previous studies using similar experimental conditions[6,16,17] (**Table 1**), further demonstrating the reproducibility of the method. Overall, we show that using this detailed protocol one can effectively and consistently assess zebrafish cardiac function, which is critical when comparing different cardiac phenotypes during a study.

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Illustration of adult zebrafish heart.** Blood flow circulation is represented by arrows: the blood flows from the sinus venosus to the atrium and is further transferred to the ventricle, where it is pumped to the bulbus arteriosus.

**Figure 2. Fish-imaging chamber.** (**A**) To prepare a fish-imaging "chamber", a sponge with an incision towards one end in a vertical orientation is placed in a glass container. (**B**) The glass container is then firmly taped on the inclined imaging platform. (**C**) The transducer is mounted on the manipulator and placed parallel to the incision for correct imaging positioning (the transducer notch is pointing towards the operator).

**Figure 3. Atrioventricular inflow (A) and outflow (B) in Color Doppler mode and corresponding Pulsed Wave Doppler to assess velocities of the respective ventricular diastolic wave peaks (C) and ventricular outflow (D).**

**Figure 4. Image analysis.** After image processing (to achieve desired contrast and brightness of the image), measurements can be performed in the PW Doppler mode (left) and B-mode (right) images. To trace the LV wall in the B-mode image, select **Cardiac Package** from the drop-down menu, go to **PSLAX**, and select **LV Trace**. To measure peak velocities in the PW Doppler mode image, select **Cardiac Package** from the drop-down menu. To measure the ventricular blood inflow velocity, select the **MV Flow** option and select **E** or **A** for early diastole and late diastole, respectively. For determination of the outflow blood velocity, select **AoV Flow** and **AV peak velocity**.

**Figure 5. B-mode images.** (**A**) B-Mode image of the ventricle (V) in diastole, filled with blood coming from the atrium (A). (**B**) B-Mode image of the ventricle in systole, ejecting blood through the bulbus arteriosus (B, green tracing).

**Figure 6. Pulse Wave Doppler image.** A heart rate value can be generated by tracing 3 consecutive aortic flow peaks. The aortic flow peaks can be displayed by selecting the **heart rate** button in measurements tab in the analysis software.

**Table 1. Echocardiographic parameters in adult zebrafish.** Values obtained for the cardiac function parameters evaluated in the current study for adult male or female zebrafish between 3 and 12 months anesthetized in a 0.2 mg/mL tricaine solution. A range of the values obtained for the same parameters in previous studies[6,16,17] performed in similar conditions is presented for validation and to help standardize the method.

**DISCUSSION:**

We describe a systematic method for echocardiographic imaging and assessment of cardiac function in adult zebrafish. Echocardiography is the only available non-invasive and most robust method for live adult fish cardiac imaging and functional analysis, and it is becoming increasingly popular in zebrafish cardiovascular research. The amount of time needed is short and allows for high-throughput and longitudinal studies. However, there is considerable variation in the

methodology employed and data analysis. Standardization of zebrafish echocardiography is very difficult when so many variables can influence the outcoming parameters. When conducting experimental studies, one should consider conditions that can produce variability, including anesthesia, body weight, age, sex, and background strain. Wang, L et al.[16] assessed the variability introduced by these factors and compiled the available data on zebrafish cardiac function in order to help standardize the method. Their study is a very useful resource to design experimental studies involving zebrafish echocardiographic assessment. Based on the information provided by Wang, L et al.[16] and references within and our own observations[6], we provide an outline of critical steps and conditions we considered important for protocol optimization and reproducibility:

**Choice of specimen**: Previous studies suggest that while systolic function parameters (EF, FAC) are not significantly affected by sex differences, diastolic function (namely peak wave E/A ratio) can be considerably lower in females older than 6 months. It was also observed that ventricular areas and volumes significantly increase with fish age (3 months and older) and are considerably higher in females due to their higher body weight and size. Indexing diastolic volumes to body-mass index (BMI) and body surface area (BSA) can help abolish differences between age-matched females and males, and indexing to BSA and weight can help overcome age related diastolic volume differences[16]. There were also reports of different diastolic functions between fish with different background strains[16]. Overall, when choosing experimental design, it is advisable to use age- and strain-matched controls and avoid mixing different sexes. Using males is recommended, as image quality was lower in gravid females.

**Scanning position**: In this setup two scanning positions are possible: longitudinal axis and short axis. We found that in short axis mode it was very hard to identify the cardiac chambers. Therefore, we used only longitudinal axis and recommend the latter for delineation of the cardiac chambers in B-mode and derivation of ventricular size and function.

**Anesthesia**: Adequate sedation is critical to avoid significant bradycardia during measurement. Heart rate will affect cardiac functional measurement, compromising the accuracy of the study. Tricaine is the most common anesthetic agent and a dose of 0.2 mg/mL was found to provide adequate sedation. However, measurement time is critical since heart rate starts to decrease after 3–4 min under sedation[16]. To avoid introducing variability, it is critical to keep measurements under 3 min.

**Critical parameters**: Heart rate can be considered as a critical parameter when aiming for consistency and accuracy. Heart rate should be comparable between experimental groups tested and within the range of values reported for the conditions used. We found that a range of 118 ± 14 to 162 ± 32 bpm can represent the normal values for wild type zebrafish 3–12 months old adults anesthetized with 0.2mg/mL of tricaine for less than 3 min.

**Result accuracy**: To ensure accuracy, measurements should be taken over a minimum of 3 cardiac cycles. To obtain more accurate manual image tracings, the analysis should be done in a blinded manner.

Besides choosing the most appropriate conditions, several aspects are critical to ensure accurate measurement. Ideally, conditions should be kept as close to the normal fish physiologic state as possible. Performing the scan under water has the advantage of keeping the fish in their natural environment and close to normal conditions for gas exchange, water composition, hydrostatic pressure, and temperature. These are clear advantages over previous studies, where during the scanning fish are placed in a wet sponge exposed to room air and conductivity is enabled by ultrasound gel instead of water[9,10]. Underwater scanning also allows for recovery of the fish after the procedure, provided that the time between anesthesia and recovery is kept under 3 min and the fish is returned to recovery water immediately after measurement. To ensure the procedure is performed as quickly and effectively as possible, a considerable amount of time spent on training is advisable before performing experiments.

Echocardiography is a very well established method to evaluate cardiac function in clinical practice as well as in murine (or other mammalians) animal models. However, unlike murine or human echocardiography, performing fish ultrasound underwater does not allow connection of the specimen to the electrodes. Therefore, direct measurement of heart and respiratory rates is not possible. In that case, heart rate can be measured by counting the beats per min in a 10 or 15 min interval or by manually tracing 3 consecutive aortic flow peaks (**Figure 6**). Heart rate also affects determination of other parameters, such as cardiac output, that have to be calculated manually once parameters such as stroke volume have been obtained through ventricular inner wall tracing. Another aspect to consider is that fish heart morphology is quite different from mammals. In the two-chambered zebrafish heart, ventricular filling is mostly determined by atrial contraction, and fish typically present a much lower early to late ventricular filling ratio when compared to mammals[18]. This explains the different profile obtained by pulse wave Doppler in A and E peaks between zebrafish and healthy mammalian hearts.

Echocardiography enables a thorough characterization of the fish cardiac profile and quantification of multiple functional parameters. The values obtained for ejection fraction, fractional area change, blood inflow and outflow velocities, heart rate, and cardiac output are in the range reported by previous studies (**Table 1**), highlighting the reproducibility of the method. Taken together, our data shows that high-frequency ultrasound echocardiography is a robust and reproducible method to measure zebrafish cardiac morphology and function when evaluating disease models or drug testing.

**ACKNOWLEDGMENTS:**
We thank Fred Roberts technical support and revision of the manuscript

**DISCLOSURES:**
The authors have nothing to disclose

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Figure 1



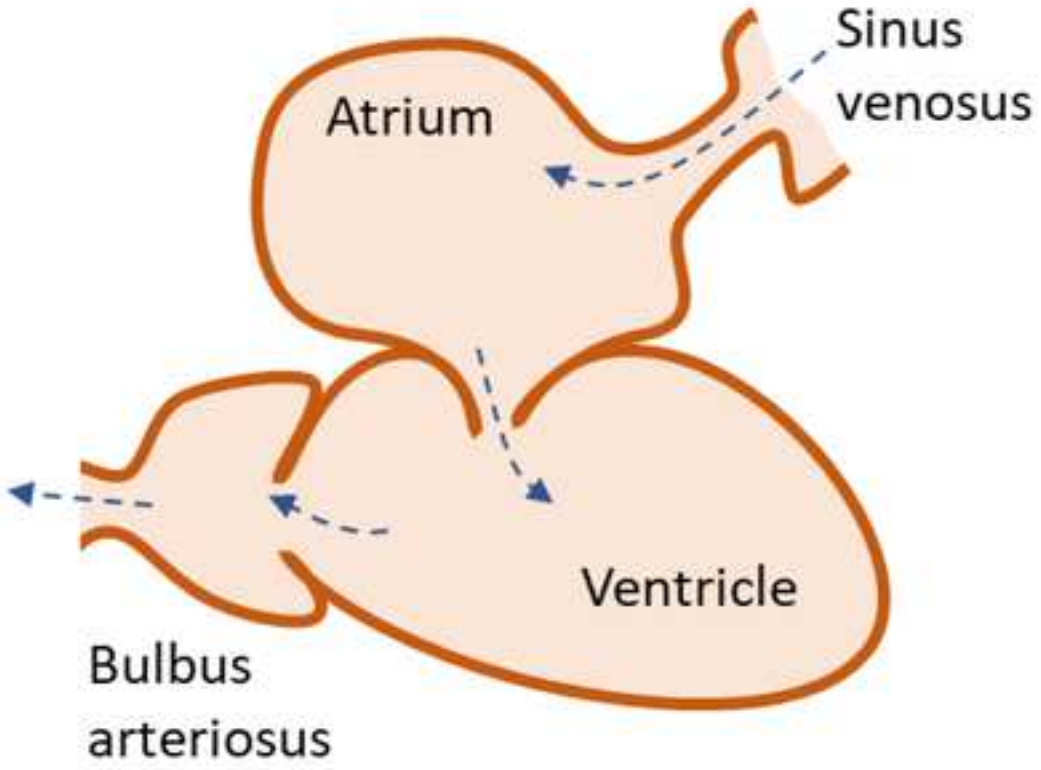

Figure 2

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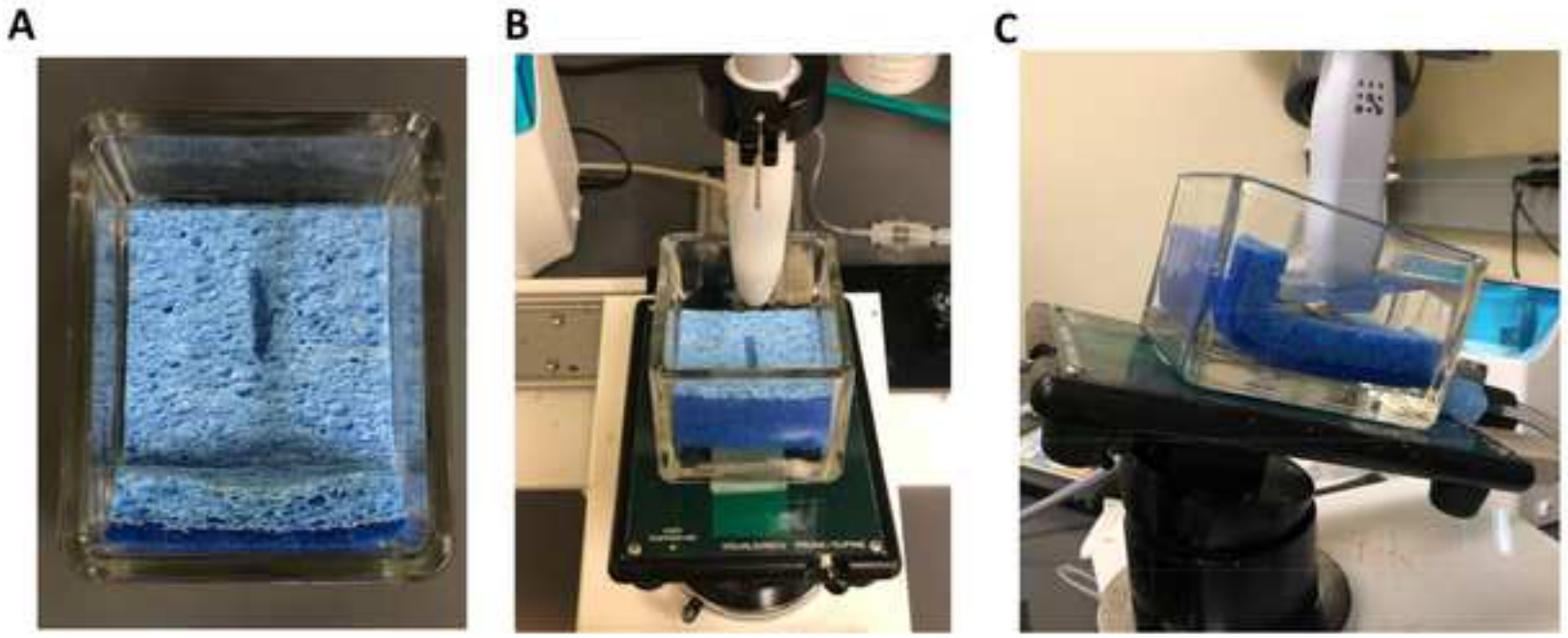

Figure 3

Click here to access/download;Figure;Figure 3.psd

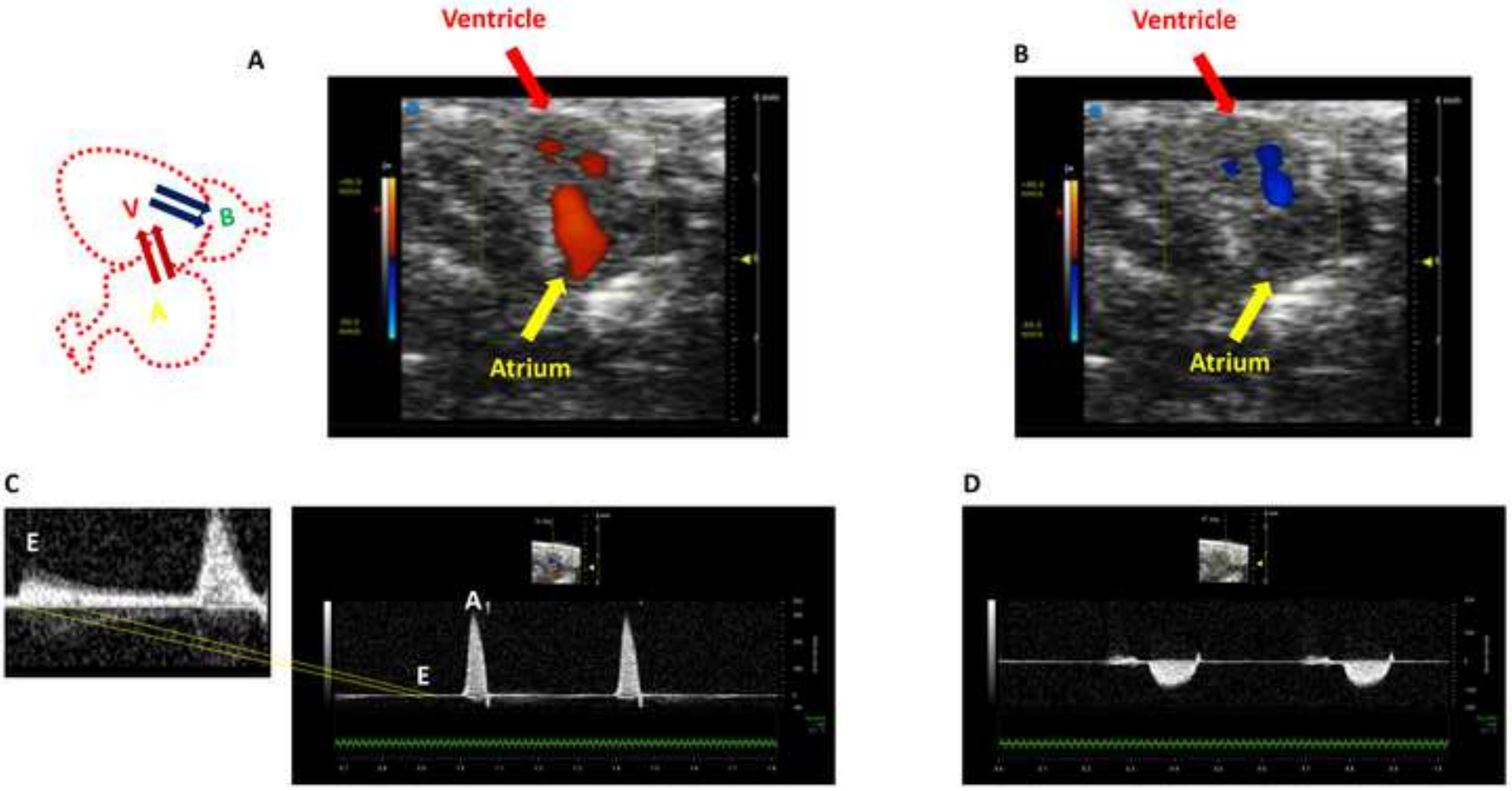

Figure 4



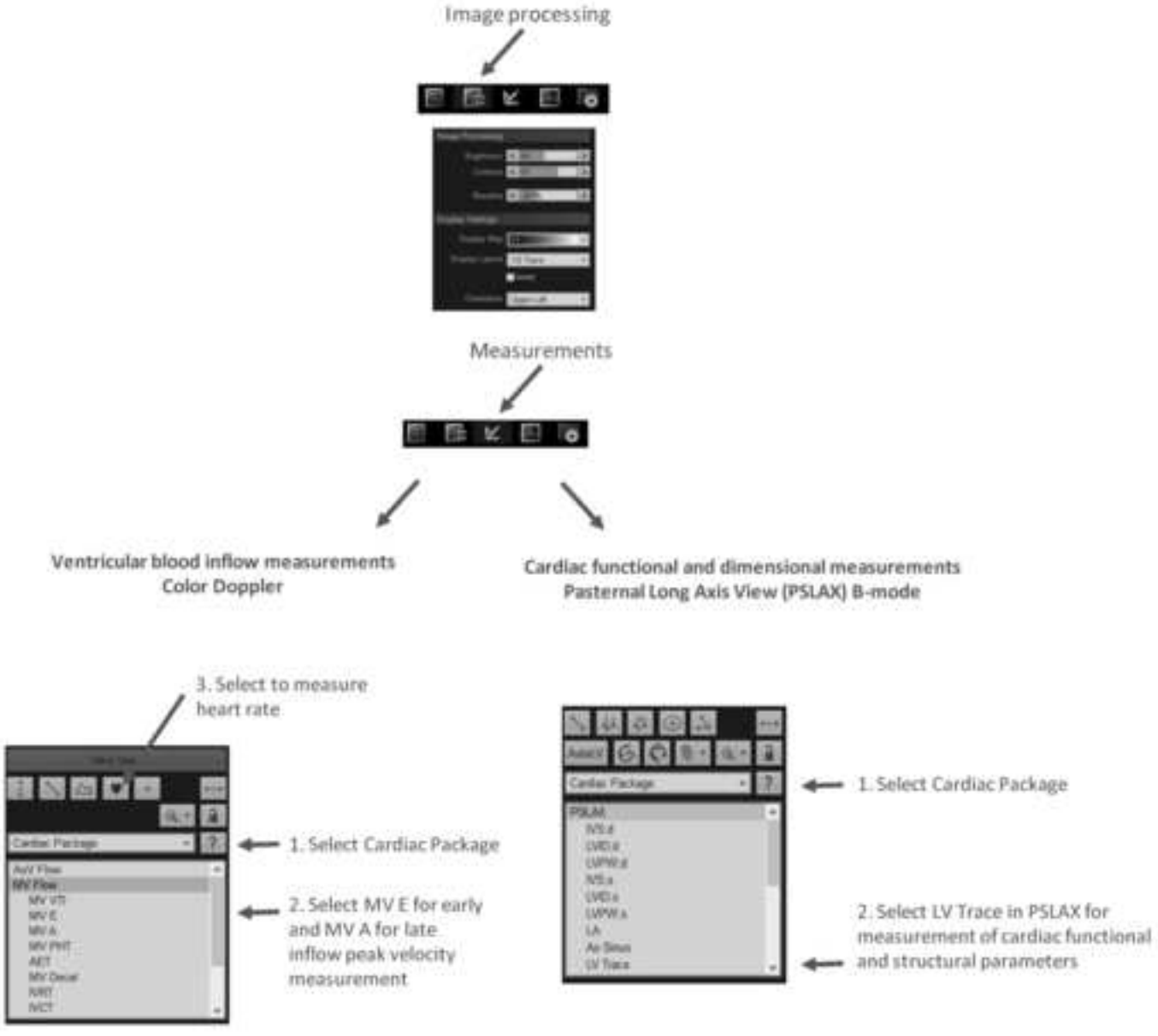

Figure 5

Click here to access/download;Figure;figure 5.psd

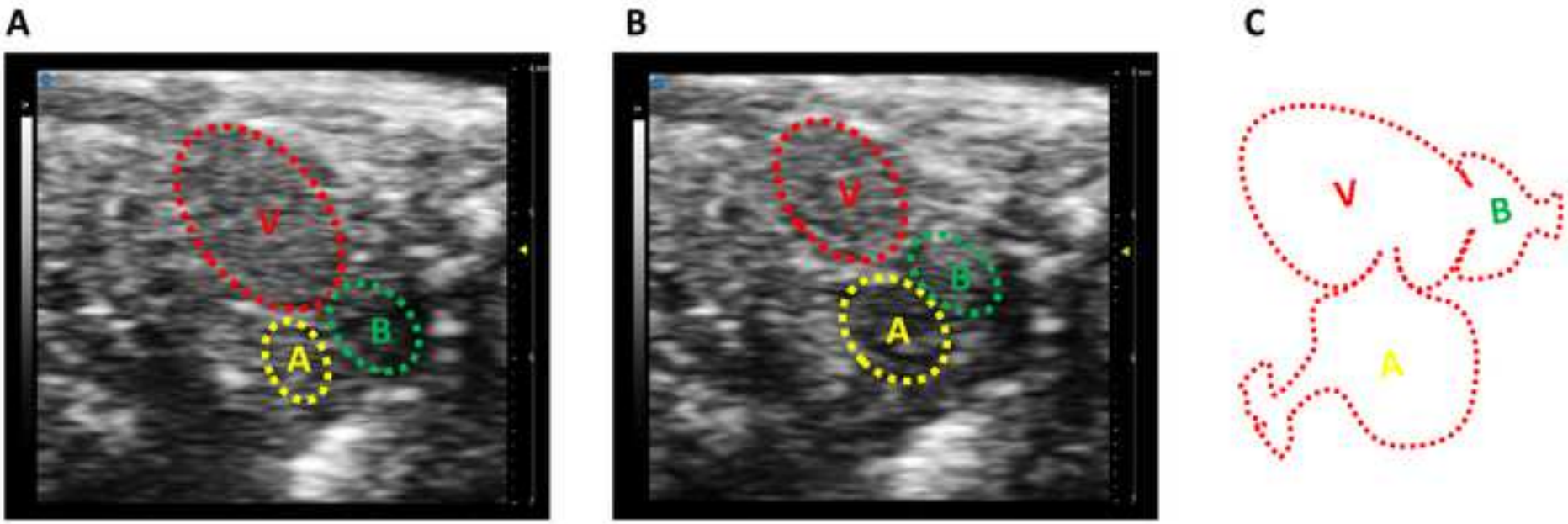

Figure 6

Click here to access/download;Figure;Figure 6.psd

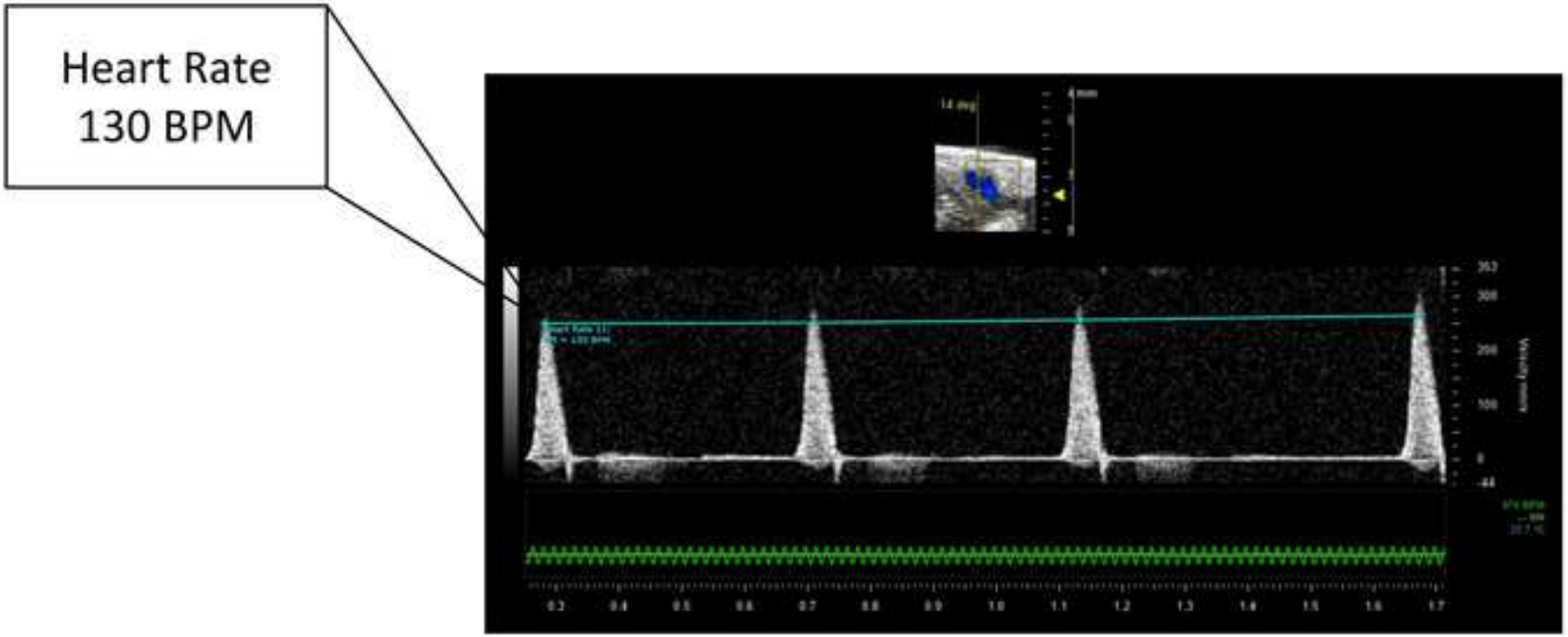

Table 1



| Parameters, units ± sd | This study | Wang, L. *et al*, 2017; Lee, L. *et al*, 2016 & Mishra, S. *et al* , 2019 | Comments/Description |
|---|---|---|---|
| Heart rate (HR), bpm | 133 ± 7 | 118 ± 14 - 162 ± 32 | Wild-types AB/ABTU males and females between 3-12 months anesthetized in tricaine 0.2 mg/mL |
| Fractional area change (FAC) | 0.38 ± 0.03 | 0.29 ± 0.07 - 0.39 ± 0.05 | |
| Ejection fraction (EF), [%] | 42 ± 7 | 34 ± 0.04 - 48 ± 0.03 | |
| Stroke volume (SV), µL | 0.21 ± 0.01 | 0.18 ± 0.06 - 0.28 ± 0.08 | |
| Cardiac output (CO), µL min-1 | 27.3 ± 1.69 | 19 ± 9.5 - 36.1 ± 7.8 | |
| E peak velocity (early ventricular inflow), mm/s | 30 ± 6.8 | 25 ± 7 - 51 ± 16 | |
| A peak velocity (late ventricular inflow), mm/s | 152 ± 32 | 144 ± 36 - 288 ± 54 | |
| Ventricular outflow, mm/s | 86.6 ± 19 | n/a | |

Table of Materials



| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| Double sided tape | | |
| Fish net | | |
| Glass container_ 100 inch high | | |
| High frequency transducer | Fujifilm/VisualSonics | MX700 |
| Plastic teaspoon | | |
| Scalpel or scissors | | |
| Small fish tanks | | |
| Sponge (kitchen sponge) | | |
| Transfer pipets (graduated 3 mL) | Samco Scientific | 212 |
| Tricaine (MS-222) | Sigma-Aldrich | A5040 |
| Vevo 3100 Imaging system and imaging station | Fujifilm/VisualSonics | |
| Vevo LAB sofware v 1.7.1 | Fujifilm/VisualSonics | |

| Comments/Description |
| --- |
| Band width 29-71 MHz, Centre transmit 50 MHz, Axial resolution 30 µm |

December 13th, 2019

Editor in Chief, Jove-Journal of Visualized Experiments

1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Editor,

We are submitting the revised version of our manuscript entitled “High-frequency ultrasound echocardiography to assess zebrafish cardiac function”. We have now addressed all the issues raised by the editor and reviewers. All the modifications in the main manuscript are underlined and all the filmable steps were highlighted as requested. All the figures (except Figure 1) were modified and a new figure (Figure 4) was added to address reviewers’ concerns. All figures were converted to vector image file (psd) to ensure high resolution and tables were upload as .xlsx files.

We greatly thank you for considering our manuscript and we hope the manuscript is now suitable for publication.

Sincerely,

Isabel Morgado, PhD
Instructor of Medicine
Stanford University School of Medicine
1651 Page Mill Road
Stanford, CA 94304

**Editorial comments:**

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

R: The authors read the final revised manuscript for proofreading and corrected any spelling or grammar issues encountered

2. Please include email addresses for all authors in the manuscript itself.

R: The email address of all authors has been added

3. Please include at least 6 key words or phrases.

R: There are now 7 keywords /phrases

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

R: The steps for the video have been highlighted in the protocol as indicated

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

R: The protocol has been revised to address this points

Figures:

1. Please cite Figure 5 outside of the Figure legend section.

R: The figures have been re-organized. Figure 5 is now figure 3 and it is cited in the text

2. Please include a legend for Table 1.

R: A legend for Table 1 has been added

References:

1. Please do not abbreviate journal titles.

R: The journal titles that were abbreviated have now been corrected to full length

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Re: We have added to the Table missing materials (scissors or scalpel and plastic spoon) and equipment used and respective company/catalogue number for the scientific equipment/material (see Table 2)

**Reviewers' comments:**
Reviewer #1:

Manuscript Summary:
This is a very good topic that is suitable for JOVE. I believe a lot of researchers will benefit from such a protocol. Even though, there are multiple research papers that have utilized echocardiography on zebrafish, most of these lacks providing sufficient details that would enable other researchers to adapt the approach. The protocol gives general steps. However, it would be better, if the authors prepare a better detailed protocol that would give more details about image acquisition and image analysis. Also, I think the authors should prepare the protocol suitable for all echo platforms and then explain the utilization based on visualsonics platform.

R: More details on image acquisition have now been added on sections 3.1. We appreciate the suggestion of the reviewer regarding a protocol suitable for all echo platforms. However, we believe this would be very difficult because most of the existent alternative echo platforms do not use high-frequency ultrasound, which is a very important feature of the method we are presenting. Therefore, presenting a general protocol for all platforms would not be feasible within the context and goals of the manuscript.

Major Concerns:

I believe, once the authors provide more details for below points, the manuscript will be strengthened significantly

- At the beginning of the manuscript, authors should state they are using Visualsonics 3100 platform to prevent confusion. At line 114,they state the transducer without brand information.
R: We have added a paragraph at the beginning of the section "Protocol" stating that we are using VisualSonics 3100 platform. The brand of the transducer has also been added accordingly.

- Authors mention about image acquisition underwater. This is confusing. Is the fish submerged underwater completely? In that case how is the ultrasound beam coupled with the fish body. I would expect ultrasound would dissipate in water and would not reach to body in that case. A schematic would help to explain that.
R: The ultrasound beam will reach the fish body as long as kept in close proximity. This has now been explained in the introduction and in the Methods section 2.3. The water around the fish establishes the contact between the ultrasound probe and the fish via liquid surface tension and transmission of the ultrasound signal. The following sentence has been added: "Since ultrasound waves can easily travel through water, keeping close proximity between the fish and the scanning probe underwater provides enough contact surface for heart detection with no need for ultrasound gel and it is overall less stressful for the fish."

- Orientations of the probe with respect to fish body is important for AV and OFT valve

measurements, for alignment with blood flow. I suggest the authors look at a recent relevant paper for this (doi: 10.3389/fbioe.2019.00096). If they can show orientations of the probe with the body and for such orientations what are seen on b-mode screen, that would be very helpful.

R: We thank the reviewer for pointing out the above paper; however, the therein described system is different to the one we use as in it utilizes a single element probe. In this case, aligning the probe with the blood flow is necessary. We here describe a platform that employs 265 elements to capture flow and therefore, the position of the probe does not need to be changed during acquisition.

- For image acquisition section, authors mention things specific to the setup, without explaining what they really mean. I think it should be reverse. They should explain what these mean and then tell how these are managed in that system. For example, in line 164 they mention "Setting the dynamic range to 45 - 50 dB will notably increase the contrast of the image and result in better image acquisition". Instead, it should be something like this: "The contrast should be enhanced for better image acqusition. In this system, it can be managed by Setting the dynamic range to 45 - 50 dB". Same for aligning the ultrasound with the flow.
R: We thank the reviewer for the useful suggestion. We have now rephrased several notes on sections 3.1.3, 3.2.2, 3.2.3. and 3.2.5 accordingly

- Why is Figure 4 is mentioned before Figure 3 in the text
R: Thank you for pointing out this mistake. The figures have been re-organized for clarity and they are mentioned in the text following a logical sequence

- I know it is difficult to present still b-mode ultrasound images. However, Figure 4 can be improved with small schematic drawings to accompany the images, to show chambers
R: Figure 4 (now Figure 5) includes now a panel C with a schematic to illustrate the heart chambers detected in b-mode
- There is no Figure 4 C and D, as stated in the figure legend. It should be Figure 5 C and D (reply)
R: The figures have been re-organized for clarity and this mistake has been corrected

- Again, color doppler flow images in Figure 5 A and B, is hard to follow. Schematics would help
R: Figure 5 is now Figure 3 and a schematic to illustrate the heart chambers has been added

- Figure 5 is not referred in the text.
R: The figures have been re-organized and all figures are now mentioned in the text

- Regarding the image analysis part, I believe few screenshots from the analysis window will be helpful.
R: We have now added Fig. 4 to illustrate the analysis window

- Regarding Doppler snapshots of Figure 5C and D, It will be better if time scale at the bottom is

seen which will give an idea about the heartbeat.
R: Figure 5 is now Figure 3 and it has been modified accordingly to show the time scale

- Regarding figure 5C, I think this is not a good representative profile, since there is no E peak but only A peak present
R: Figure 5 is now Figure 3. The E peak is usually very small and difficult to detect within the same scale as the A peak. We have now included an insert figure with modified scale showing the E peak

- Regarding Figure 5D, the flow on the snapshot is negative because of the orientation of the probe (velocity signal is getting away from the probe). This might be explained in the text. Also, it looks like there is an initial regurgitation for this one. Do the authors see this for all cases? This may also be interference with the inflow signal. This should be mentioned. Please see relevant references
R: The negative flow has been explained in the text on section 3.2.5 " The inflow peaks will appear in the upper/positive quadrant (signal going towards the probe), while the outflow peaks in the lower/negative quadrant (signal going away from the probe)". The signal of regurgitation is minor and considered neglectable.

- Table should be better prepared with border lines. Also, values for the outflow signal can be added. Some part of the table is in following page
R: The border lines have now been added as well as the outflow signal

- Does the visualsonic software enable to trace the flow profile and export to a software like excel for further analysis? If so, it will be very helpful to explain this in the text.
(export peak velocities)
R: Exporting data to excel is possible and this has now been described in section 5.7.1: "NOTE: Data can be exported to an excel sheet. For that, after having traced the peaks, click on "report" > "export"> "save as" > excel."

Minor Concerns:

- Some typos are present. Please make a through check. For example, in abstract line 31, it should be heart rate, not heart hate. We all love heart research :).
R: This typo and several other have been corrected as indicated throughout the manuscript

Reviewer #2:

Manuscript Summary:
The authors describe to a great extent a protocol for ultrasound echocardiography of adult zebrafish. This is an interesting protocol and timely, since an increasing amount of adult models of zebrafish cardiovascular disease and regeneration are being published.

Major Concerns:

This a protocol describing a specific set up and a specific transducer model, although one of the most popular ones. They provide a detailed enough protocol for taking measurements but the most challenging aspect of this experiments is the analysis and quality control of the data obtained by the system, which they do not discuss enough.

For example they could suggest some milestones for proper measurements that should be established as wild-type values also when referring to discrepancies with previous publications in Table 1. (Should one standardize based on heart rate? or velocity values? of wild-type fish in order to compare with mutant lines). They could use pharmacological treatments or mutant lines to provide these data.

Re: A detailed description of critical steps and conditions to for quality control of the data have been added in the discussion (second paragraph). Discrepancies reported with previous publications are likely to result from the variability of the parameters used (e.g. fish age, sex, weigh, anesthesia, background strain, ultrasound system). Therefore, we chose to compare only with the studies that used a similar setup and specimen type as we present in our protocol. A previous study by our group (Mishra, S., *et al* 2019, Ref 6 on the manuscript) showing differences detected between wild types and a mutant line of light chain amyloidosis (using a light chain sequence associated with cardiomyopathy in humans) demonstrated the feasibility of the method and is cited on the manuscript. We used that and other publication (Wang, L. et al 2016 (Ref 16 on the manuscript) and references within) to provide some standardization rather than conducting a whole study for that here. In this manuscript it was not our goal to provide a standardization study but rather a detailed protocol to perform the method in a way that in our view is robust and reproducible.

Also they should further discuss how much is the angle of measurement affecting the measurements and how this should be standardized

Re: We have now rephrased the importance of using a pulse wave (PW) angle parallel to the flow in point 3.2.3: "Make sure the angle correct line is parallel to the blood flow in order to provide consistent and reproducible results. Placing the angle correct line so it matches the direction of blood flow will ensure that velocities are accurately captured."

Minor Concerns:

Figure 5 is not discussed in the main text and Figure 4 and Figure 5 legends are too brief.
It is now very clearly shown in Figure 3 (or the figure legend) what is described in 5.3 (line 219-222)

Re: We apologize for the mistake. All figures are now discussed in the text. The legend of Figure 4 (now Figure 5) and Figure 5 (now Figure 3) have been edited to introduce more detail. The description of line 5.3 has now been improved by adding Figure 4 which illustrates the image analysis commands.