

Video Article

Echocardiographic Approaches and Protocols for Comprehensive Phenotypic Characterization of Valvular Heart Disease in Mice

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Abstract

The aim of this manuscript and accompanying video is to provide an overview of the methods and approaches used for imaging heart valve function in rodents, with detailed descriptions of the appropriate methods for anesthesia, the echocardiographic windows used, the imaging planes and probe orientations for image acquisition, the methods for data analysis, and the limitations of emerging technologies for the evaluation of cardiac and valvular function. Importantly, we also highlight several future areas of research in cardiac and heart valve imaging that may be leveraged to gain insights into the pathogenesis of valve disease in preclinical animal models. We propose that using a systematic approach to evaluating cardiac and heart valve function in mice can result in more robust and reproducible data, as well as facilitate the discovery of previously underappreciated phenotypes in genetically-altered and/or physiologically-stressed mice.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54110/>

Introduction

Aging is associated with progressive increases in cardiovascular calcification¹. Hemodynamically significant aortic valve stenosis affects 3% of the population over the age of 65², and patients with even moderate aortic valve stenosis (peak velocity of 3–4 m/s) have a 5 year event-free survival of less than 40%³. Presently, there are no effective treatments to slow the progression of aortic valve calcification, and surgical aortic valve replacement is the only available treatment for advanced aortic valve stenosis⁴.

Studies aimed at gaining a deeper understanding of the mechanisms that contribute to the initiation and progression of aortic valve calcification are a key first step in moving towards pharmacological and non-surgical methods to manage aortic valve stenosis^{5,6}. Genetically-altered mice have played a major role in developing our understanding of the mechanisms that contribute to a variety of diseases and are now coming to the forefront of mechanistic studies aimed at understanding the biology of aortic valve stenosis^{6,7,8}. Unlike other cardiovascular diseases such as atherosclerosis and heart failure—where standard protocols for evaluating vascular and ventricular function are for the most part well-established—there are unique challenges associated with *in vivo* phenotyping of heart valve function in mice. While recent reviews have provided thorough discussions regarding the advantages and disadvantages to numerous imaging and invasive modalities used to assess valve function in rodents^{9,10,11}, to date, we are not aware of a publication that provides a comprehensive, step-by-step protocol for phenotyping heart valve function in mice.

The purpose of this manuscript is to describe the methods and protocols to phenotype heart valve function in mice. All methods and procedures have been approved by the Mayo Clinic Institutional Animal Care and Use Committee. Key components of this protocol include the depth of anesthesia, the evaluation of cardiac function, and the evaluation of heart valve function. We hope this report will not only serve to guide investigators interested in pursuing research in the field of heart valve disease, but will also start a national and international dialogue related to protocol standardization to ensure data reproducibility and validity in this rapidly-growing field. Importantly, successful imaging using high-resolution ultrasound systems requires a working knowledge of the principles of sonography (and terminology commonly used in sonography), an understanding of the fundamental principles of cardiac physiology, and significant experience with sonography to allow for accurate and time-efficient assessment of cardiac function in rodents.

Protocol

1. Prepare the Materials and Equipment (Table 1 and Figure 1)

1. Turn on the ultrasound machine. Enter the animal ID, date, and time (for serial imaging experiments) and other relevant information.
2. Use a high-frequency ultrasound transducer, 40 MHz for imaging mice less than ~20 g or 30 MHz for mice greater than ~20 g.
3. Connect the platform to the electrocardiogram (ECG) monitor for ECG gating of imaging for certain modalities.
NOTE: Critically, this also allows for the instantaneous calculation of the heart rate (HR), which can be used as one of several indices of an appropriate depth of anesthesia.
4. Pre-heat the platform to 37 °C.
NOTE: All commercially-available ultrasound machines have a control panel that provides image acquisition controls and study management controls for B-mode, M-mode, and Doppler echocardiography. A cardiac measurement tool is embedded in the machine for the automatic measurement and computation of common echocardiographic parameters of cardiac and valvular functions.

2. Prepare the Mouse for Imaging and Induction of Anesthesia

1. Gently pick up the mouse by its tail and firmly hold the animal at the nape of its neck.
2. Guide the nose of the animal into the nose cone. Begin anesthesia flow at 1% isoflurane. Ensure that the animal is sedated within 3-5 s of exposure to the gas.
3. Quickly and accurately lay the animal on the platform in a supine position, making sure that the forefeet and hind feet lie on the ECG sensors of the platform.
4. Gently secure the animal with adhesive tape on all four limbs, lightly apply adhesive tape to stabilize the head in the nose cone apparatus, and apply adhesive tape to stabilize the tail. Both hind feet and forefeet should lie flat to ensure stable and clear ECG signal acquisition by the physiological imaging system.
5. Check the HR. Do this using an imaging platform with ECG capabilities or with external ECG devices. Ensure that the baseline HR is between 600 to 700 bpm. Ensure that the HR does not fall below 450 bpm under any circumstances.
NOTE: During the procedure, the HR may decrease slightly due to anesthesia, but it should be above 500 bpm in most instances.
6. Adjust the anesthesia flow by small increments accordingly (~0.1% increments every 15 s until a stable state of anesthesia is reached).
NOTE: A stable state of anesthesia is a condition in which the above-mentioned cardiac parameters are maintained (see step 2.5) and the animal does not overtly respond to stimuli from the placement of the probe on various imaging windows. Importantly, this is not a surgical plane of anesthesia, which results in marked cardiodepression in mice. For prolonged imaging sessions, the application of vet ointment to the eyes to prevent dryness is recommended.
7. Check the body temperature using a rectal thermometer. Keep the temperature between 36.5 °C and 38 °C.
NOTE: In an appropriately environmentally-controlled room and on a heated platform, the body temperature (measured rectally) remains constant during the entire procedure and, consequently, is not a confounding factor influencing cardiovascular hemodynamics over time.
8. Shave off the hair from the chest using an electric clipper designed for use with fine hair. Wipe clean the chest with a damp paper towel. The animal is ready for imaging.
NOTE: While chemical removal of hair can also be performed, avoid the use of such compounds, as they can cause significant skin irritation over time in long-term experiments. Furthermore, the appropriate application and removal of such chemically-based hair removal products can prolong the duration of anesthesia exposure by 2-3 min (~10-20%). The total time from induction of anesthesia to completion of skin preparation should take less than 3 min.

3. Follow Basic Principles and Guidelines in Acquiring Cardiac Ultrasound Images

NOTE: There are three ultrasound modalities used in acquiring the images: B-mode/2-D, M-mode, and Doppler (spectral pulsed-wave Doppler and color flow Doppler imaging). There are two basic transducer positions used to acquire images of the heart and heart valves: the parasternal and apical windows (**Figure 2**).

1. From each transducer position, obtain multiple tomographic images of the heart relative to its long and short axes by manually rotating and angulating the transducer.
NOTE: Rotation refers to pivoting or twisting the transducer from a fixed position on the chest wall, while angulation refers to the side-to-side movement of the transducer from a fixed point on the chest wall. All ultrasound transducers have an image index marker in the form of a groove (notch), external ribbing, or button.
2. Ensure that the ultrasound signal is perpendicular to the target structure by adjusting the transducer position accordingly.
3. Optimize the color flow and peak velocity signals by aligning the transmitted ultrasound beam parallel to the flow. The angle between the ultrasound beam and flow should be less than 60°.
4. Optimize the image quality using the control panel controls. Only the area of interrogation should fill up the image display.
NOTE: Fine adjustments in transducer and platform positions are almost always necessary to obtain clear images. Even during optimal conditions, respiratory movements, chest wall anatomy (e.g., small rib spacing), and variations in internal anatomy (both inherent and disease-induced) can limit the acoustic window and make image acquisition very challenging.
5. When measuring the left ventricular dimensions in M-mode and 2-D/B-mode, place the measurement caliper in the most continuous echo line.
6. Adjust the color Doppler sector and sample volume to the area of interrogation by adjusting the sector control, which is found on the panel.
NOTE: The color-coding scheme in Doppler studies indicates the speed and directionality of blood flow. Doppler signals that are red indicate laminar blood flow towards the transducer. Doppler signals that are blue indicate laminar flow away from the transducer. A "mosaic" color pattern indicates regions of turbulent or non-laminar blood flow (which commonly occurs in valvular stenosis or valvular regurgitation).

7. Record a minimum of two 5 s strips (or 100 frames) of real-time B-mode/2D echo from each imaging window for offline analysis.
NOTE: Commercially-available echo machines have image acquisition settings that capture a pre-set number of frames or cine-loop sizes. The image acquisition settings can be modified so that longer cine loops can be acquired. Acquisition of high-quality images requires extensive experience and experimentation. Investigators must find the right combination of transducer placement and platform angle to obtain images from many views and acoustic windows.

4. Evaluation of Aortic Valve (AV) Function

NOTE: Assessments of aortic valve function include qualitative evaluations of the valve (e.g., perceived cusp thickness, increased echogenicity due to valvular calcification, and the presence or absence of regurgitant jets using color Doppler) and quantitative measures of valve function (e.g., peak transvalvular velocity and cusp separation distance).

1. Begin to image the aortic valve by selecting B-mode image acquisition.
2. With the animal securely fastened on the platform and the head facing away from the investigator, tilt the table 15-20° to the left. This will bring the heart forward and leftward, closer to the chest wall. Apply a generous amount of ultrasound gel on the transducer or directly on the animal's chest.
3. Position the transducer parasternally, about 90° perpendicular with the long axis of the heart, with the image index marker of the transducer pointing posteriorly (**Figure 2**). While in 2D/B-mode, slide the transducer cephalad until the AV comes into view. This is the "short axis" view of the aortic valve.
NOTE: A normal aortic valve has three thin cusps that open widely during systole and close adequately during diastole so that there is no regurgitation of blood back into the left ventricle. The cusps are very thin, move very rapidly, and can often be challenging to visualize.
4. Rotate the transducer clockwise until the image index marker points caudad. Observe the aortic root, aortic valve, left ventricular outflow tract, mitral valve, left atrium, and part of the right ventricular outflow tract on the image display.
NOTE: This is the "parasternal long axis" view of the AV. The sonographer should ascertain that there are two aortic valve cusps visible throughout the cardiac cycle in the B-mode images, which will allow for subsequent M-Mode imaging and analysis (see below).
5. Evaluate the aortic root in this view. Carefully sweep back and forth so that the aortic root images contain the largest dimensions of the aortic root. Measure the largest antero-posterior dimension of the aorta using the electronic caliper associated with the measurement tool embedded in the machine.
6. Locate the aortic valve in the long axis. Reduce the image width so that only the aortic valve is on the image display by adjusting image width button in the control panel. Position the M-mode line of interrogation where it intersects the tips of the aortic valve to accurately assess aortic valve cusp separation.
7. In the M-mode display of the aortic valve, measure the cusp separation distance (box-like appearance in the systole) using the electronic caliper associated with the measurement tool embedded in the machine.
NOTE: The greatest advantage of M-mode imaging is the very high temporal resolution, which is essential for the evaluation of aortic valve function. While M-mode images of the AV can be acquired in both the short- and long-axis views, the parasternal long-axis view is generally preferred because the imaging plane allows the sonographer to readily identify the orientation and location of the tips of the cusps during systole.
8. While still in the parasternal long-axis view of the aortic valve, press the color Doppler control key in the control panel. Apply color Doppler to the region of the aortic valve.
NOTE: Normal flow from the left ventricle through the aortic valve during systole is toward the transducer and thus is encoded red.
9. Document the presence or absence of aortic valve regurgitation.
NOTE: Aortic valve regurgitation is an abnormal flow that occurs during diastole and is directed away from the transducer; thus, it is encoded blue.
10. Press the pulsed-wave Doppler control key. Using the track ball located in the control panel, place the pulsed-wave sample volume in the proximal ascending aorta, just above the aortic valve, making sure that the angle between the ultrasound beam and the blood flow is less than 60° by tilting the platform and/or the transducer. If possible, obtain the peak velocity across the aortic valve from the suprasternal notch window.
11. Measure the peak velocity from the spectral display using the electronic calipers associated with the measurement tool embedded in the machine (**Figure 3C** and **3F**).
NOTE: A mosaic color denotes high flow velocity that is likely to contain non-laminar flow patterns.

5. Evaluation of Mitral Valve (MV) Function

NOTE: Assessment of mitral valve function includes qualitative evaluations of the valve (e.g., perceived cusp thickness, increased echogenicity due to valvular calcification, presence or absence of regurgitant jets using color Doppler) and quantitative measures of valve function.

1. Place the transducer in the apical position in B-mode. Position the transducer so that it is angled towards the head of the mouse (**Figure 2C**). Observe the right ventricle (RV), left ventricle (LV), right atrium (RA), and left atrium (LA) on the image display. Manually tilt the platform slightly so that the animal is in a "head-down" position to visualize the mitral valve as it opens into the LV.
NOTE: The apical 4-chamber view is the optimal view for examining blood velocity across the mitral and tricuspid valves, as well as the tissue velocity of the mitral annulus. This is also a good view to assess the motion and size of the RV and interventricular septum.
2. From the apical 4-chamber view, bring the mitral valve in focus by reducing the image width. Observe that the mitral valve leaflets appear as two thin, mobile filaments opening and closing during each cardiac cycle.
NOTE: Mitral leaflets of a "normal" mouse can be difficult to visualize if imaging is done at physiological HR (i.e., >450 bpm).
3. Place the M-mode cursor across the mitral valve to assess the thickness of the leaflets.
NOTE: The anterior leaflet is best visualized in systole when it is perpendicular to the ultrasound beam (**Figure 4**).
4. Using the apical 4-chamber view, apply color Doppler to image the flow from the left atrium through the mitral valve during diastole. Observe for mitral valve regurgitation.

NOTE: Flow is directed toward the transducer and is therefore encoded red. Regurgitant flow will be encoded blue and occurs during systole (**Figure 5**).

- Using the apical long-axis view, switch to pulsed-wave mode. Move the Doppler sample volume to the tips of the mitral valve leaflet. Note the two peaks of the mitral inflow spectral display. If the leaflets are not well-visualized, use color Doppler to identify regions with bright red or mosaic color patterns and place the sample volume at that point.

NOTE: The spectral display of mitral flow has two peaks in slow HRs (<450 bpm). In normal HRs (>450 bpm), the early- (E) and late-filling (A) flows are fused. The spectral Doppler display of flow across the mitral valve is used in the assessment of left ventricular diastolic function (see step 7.5).

6. Evaluation of Right-sided Heart Valve Function

NOTE: The tricuspid and pulmonic valves comprise the right-sided heart valves. The tricuspid valve can be readily visualized in the apical long-axis view, while the pulmonic valve can be visualized in both the parasternal long- and short-axis views.

- From the apical long-axis view, tilt or point the transducer tip using a rocking motion so that the right ventricle is at the center of the image display. Reduce the image width so that only the right ventricle is visible in the image display.
- In the same image plane, visualize the tricuspid valve leaflets, which appear as thin, mobile filaments between the right atrium and right ventricle and that open and close over the course of each cardiac cycle.
- Apply color Doppler in the region of the tricuspid valve. Note for tricuspid valve regurgitation.
NOTE: Normal flow occurs during diastole, is directed toward the transducer, and therefore is encoded red. Abnormal regurgitant flow occurs during systole, is directed away from the transducer, and therefore is encoded blue. The peak velocity of the regurgitant jet is used to estimate right ventricular systolic pressure.
- Move the transducer to the parasternal short-axis position at the level of the aortic valve. Above the aortic valve are the right ventricular outflow tract, the pulmonic valve, the proximal main pulmonary artery, and the right and left pulmonary arteries (**Figure 6**).
- Rotate the transducer clockwise to a modified parasternal long-axis position. Then, tilt the transducer slightly upward to obtain a short-axis view of the pulmonic valve.
- In this view, apply M-mode imaging to evaluate the separation distance of the pulmonic valve cusps (**Figure 7**).
- Apply color Doppler in the region of the pulmonic valve to assess for valvular regurgitation (a mosaic-patterned, high-velocity jet during diastole) and stenosis (a mosaic-patterned, high-velocity jet during systole).
- Press the pulsed-wave control key and place the sample volume just after the pulmonic valve.

NOTE: Analysis of the spectral Doppler display of flow is used to estimate pulmonary artery pressure (**Figure 8**).

7. Evaluation of Cardiac Function

NOTE: The assessment of cardiac function includes qualitative evaluations of left ventricular contractility (e.g., visual estimation of the ejection fraction, the regional wall motion abnormality, and the perceived thickness of the walls) and quantitative measures of left ventricular function (e.g., the ejection fraction, left ventricular mass, left ventricular diastolic function, and indices of myocardial performance).

- Obtain a short-axis view of the LV in 2D/B-mode, with the transducer in the parasternal short-axis position at the level of the papillary muscles. Move the transducer up and down to scan the LV from the base to the apex. Observe for wall motion abnormalities.
- From a parasternal short-axis view of the left ventricle, press the M-mode button, located in the control panel. Using the track ball, position the M-mode cursor at the center of the left ventricular cavity at the level of the papillary muscles and obtain M-mode images.
- Measure the left ventricular cavity dimension at end-diastole, where the distance between the anterior wall and posterior wall is the largest, and in end-systole, where the inward motion of both anterior and posterior walls is maximal (**Figure 9**).
- Measure the anterior and posterior wall thickness at end-diastole and end-systole.
NOTE: While the papillary muscles are an essential landmark to ensure the correct imaging plane, be careful not to include them in any measurements.
- Move the transducer to the apical window. See step 5.1. Assess the left ventricular diastolic function using pulsed-wave Doppler of blood flow across the mitral valve in the apical long-axis view.
- Place the sample volume at the tips of the mitral valve leaflets. Measure the peak mitral inflow velocity from the spectral display of pulsed-wave Doppler velocities across the mitral valve.
- Position the sample volume between LV inflow and outflow. Note the mitral and aortic valve closing and opening signals. Measure the isovolumic relaxation time, isovolumic contraction time, and left ventricular ejection time (**Figure 10**).
- Perform tissue Doppler imaging (TDI) of the mitral annulus in the apical long-axis view. Press the TDI control key and place the sample volume at the medial aspect of the mitral annulus. Make sure that the sample volume does not encroach on the mitral leaflets. Keep the Doppler sample volume size between 0.21 mm and 0.27 mm. Measure the early diastolic velocity (e') of the mitral annulus (**Figure 11**).

8. Final Steps

- Review the acquired images. Ascertain that all required images were obtained.
- Remove any excess ultrasound gel from the chest of the mouse and gently remove the tape securing the animal in place. Turn off the anesthesia.
- Place the animal on an absorbent paper towel (not bedding, which can be aspirated or can block airways during recovery). Observe the animal until sternal recumbency is attained. If the anesthesia is administered appropriately, recovery should occur within 30 to 60 s.

Representative Results

Examples of images that are routinely obtained from animal cardiac ultrasound imaging are included in this manuscript. An illustration of transducer placement on the animal's chest is provided to give the reader a clear understanding of where the transducer is positioned to obtain the images as described. A photograph of the ultrasound laboratory set-up is also included to emphasize the importance of the proper equipment, particularly the ultrasound transducer to be used and the method of anesthesia. The 2D/B-mode, M-mode, and color and Doppler displays of the normal and abnormal valves, right and left ventricles, and aortic root are properly labeled. Although strain-rate imaging is not routinely performed, an example is also included.

Mitral regurgitation is characterized by a high, usually non-laminar blood flow velocity (mosaic coloring) across the valve during systole (**Figure 5**). The presence of such a mosaic-color Doppler flow pattern from the left ventricle to the left atrium across the MV, occurring after the QRS complex in the ECG tracing, allows for an unequivocal diagnosis of MR. When this occurs in the absence of aortic valve regurgitation and/or left ventricular dysfunction, this can be characterized as isolated mitral valve prolapse. If there is significant dilation of the left ventricle (due to experimentally-induced heart failure or excessive depth of anesthesia), this can be characterized as ischemic mitral regurgitation (or regurgitation secondary to cardiac dysfunction). A pulsed-wave spectral Doppler display can be used to confirm the presence and timing of a regurgitant jet of blood flow.

A normal aortic valve has three thin, pliable cusps that open and close adequately during each cardiac cycle. Aortic valve cusp separation is measured in 2D-guided M-mode of the aortic valve in the long-axis view. Electronic calipers are used to measure from the leading edge of the right aortic cusp to the leading edge of the left aortic cusp (**Figure 3**). Aortic valve cusp-separation distance in normal mice is 0.9 to 1.3 mm. Color Doppler shows a laminar flow across the valve and into the aortic root during systole. Turbulent flow can be appreciated in conditions of increased flow, such as in aortic valve regurgitation, or increased pressure, as in aortic valve stenosis. This is demonstrated as mosaic coloring in the outflow tract. Even small amounts of aortic valve regurgitation can result in significant increases in peak transvalvular velocity due to hyperdynamic cardiac function and increased left ventricular preload. Peak aortic velocity in normal mice ranges from 0.90 m/s to 1.50 m/s. Peak aortic valve velocity of > 5 m/s has been recorded in mice with severe aortic valve stenosis.

The pulsed-wave spectral Doppler tracings can also be used to provide an index of pulmonary artery hemodynamics¹² (**Figure 8**). Pulmonary artery acceleration time is the time interval from the onset of systolic pulmonary arterial flow to the peak flow velocity. Right ventricular ejection time is the interval between the onset of right ventricular ejection to the point at which there is cessation of systolic pulmonary artery systolic flow. The combination of a shortened pulmonary artery acceleration time with a decrease in the ratio of pulmonary artery acceleration time to right ventricular ejection time suggests the presence of pulmonary arterial hypertension (which can be confirmed using invasive or direct measures of pulmonary arterial or right ventricular pressure).



Figure 1: Animal Cardiac Ultrasound Laboratory. The laboratory is equipped with the small-animal-dedicated ultrasound machine with high frequency (30 MHz and 40 MHz) transducers (MS 400 and MS 550D), isoflurane diffuser, animal platform, temperature and heart rate monitor, 1% to 1.5% isoflurane mixed with 1 L/min 100% O₂, nose cone and tubing connected to isoflurane diffuser and 100% O₂, hair razor, ultrasound gel, electrode gel, adhesive tapes, and paper towels. [Please click here to view a larger version of this figure.](#)

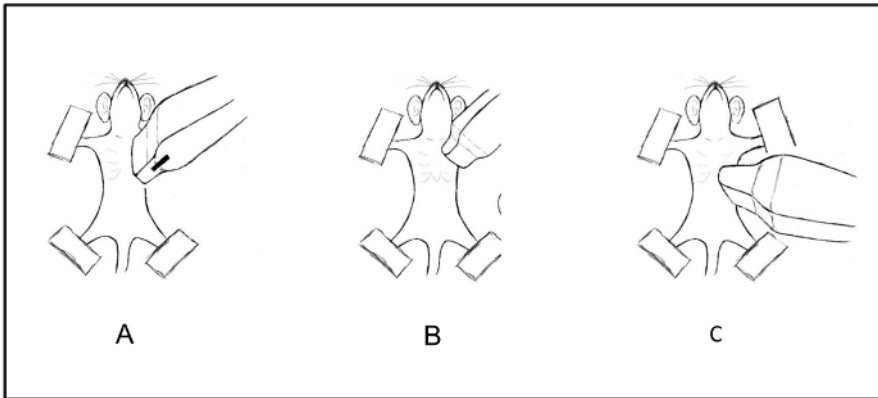


Figure 2: Basic Transducer Positions. (A) Parasternal window. The transducer head is positioned in the left parasternal border, with the image index marker of the transducer directed caudally. From this position, the long-axis view of the left ventricle, aortic valve, and aortic root and the short-axis view of the pulmonic valve can be obtained. (B) From the parasternal window, the transducer head is rotated counterclockwise, with the notch directed posteriorly. From this position, the short-axis view of the left ventricle and aortic valve and the long-axis view of the pulmonic valve can be obtained. (C) Apical window. The transducer head is positioned at the apex of the heart. From this position, the long-axis view of the right and left ventricles and mitral and tricuspid valves can be obtained. [Please click here to view a larger version of this figure.](#)

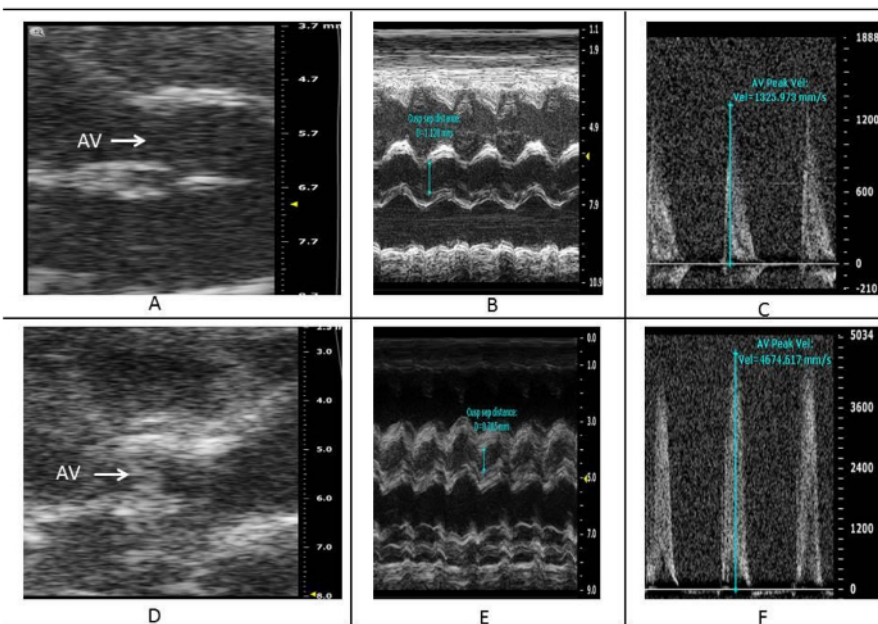


Figure 3: Assessment of Aortic Valve Function in a Normal Mouse versus Aortic Valve Function in a Mouse with Calcific Aortic Valve Disease. (A) 2D image of a normal aortic valve in the long-axis view. Note that the aortic valve opens well during systole. (B) M-mode image depicting normal aortic valve function (box-like appearance). Note that the cusp-separation distance is measured at 1.12 mm. (C) Spectral Doppler display of peak velocity across the normal aortic valve was measured at 1.3 m/s. (D) 2D image of a calcified aortic valve in the long-axis view from a low-density lipoprotein receptor deficient ($ldlr^{-/-}$) and apolipoprotein B100-only ($apoB^{100/100}$) mouse fed with western diet. The cusps are thickened and have increased echogenicity, which results in restricted opening during systole. (E) An M-mode image depicting the same stenotic aortic valve shows a cusp-separation distance measurement of 0.7 mm. (F) The spectral Doppler display of peak velocity across the stenotic aortic valve was measured at 4.6 m/s. [Please click here to view a larger version of this figure.](#)

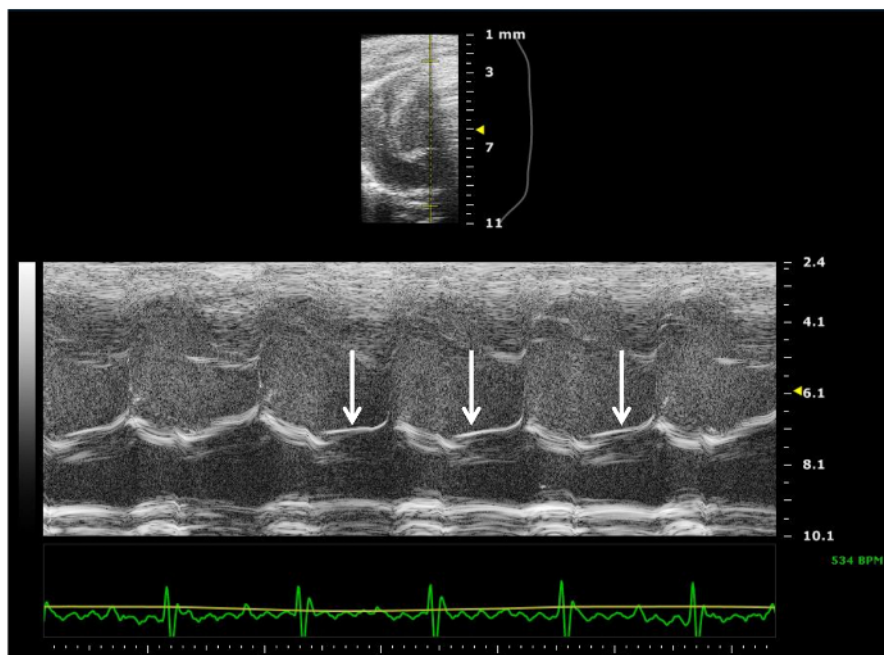


Figure 4: M-mode of a Normal Mitral Valve. From the apical window, a long-axis view of the mitral valve is obtained. The M-mode line of interrogation is applied across the mitral valve leaflet. While mitral leaflet thickness can theoretically be measured using electronic calipers, this can be extremely challenging given the thin, poorly echogenic, and rapidly moving leaflets of the normal mitral valve. The arrows point to the M-mode of the mitral valve leaflet in systole. [Please click here to view a larger version of this figure.](#)

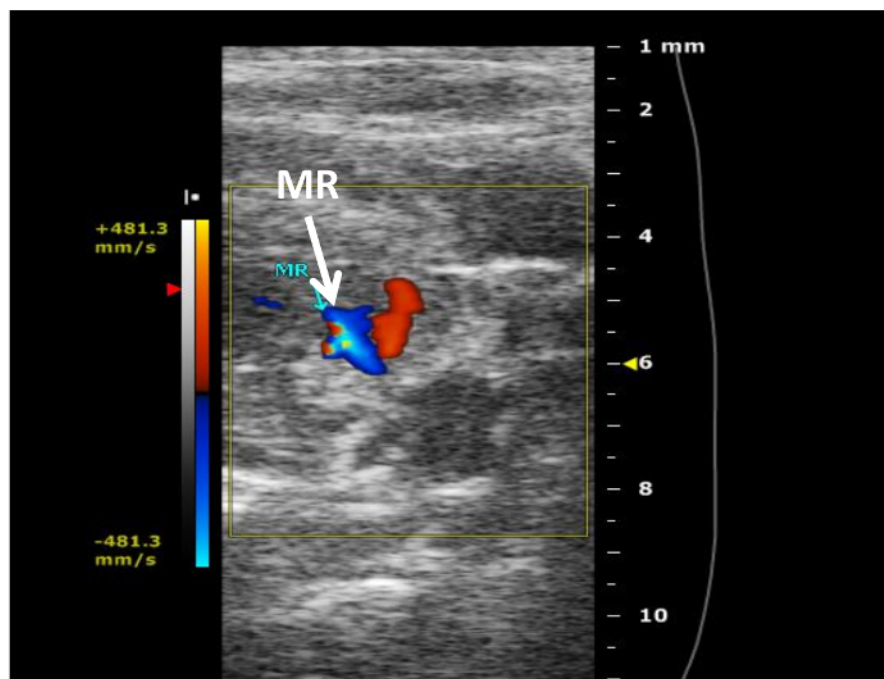


Figure 5: Evidence of a Mitral Valve Regurgitant Jet using Color Doppler Imaging. From the parasternal window, a modified long-axis view of the mitral valve is obtained. Color Doppler interrogation shows a mosaic-color jet at the mitral valve during systole (highlighted by an arrow). [Please click here to view a larger version of this figure.](#)

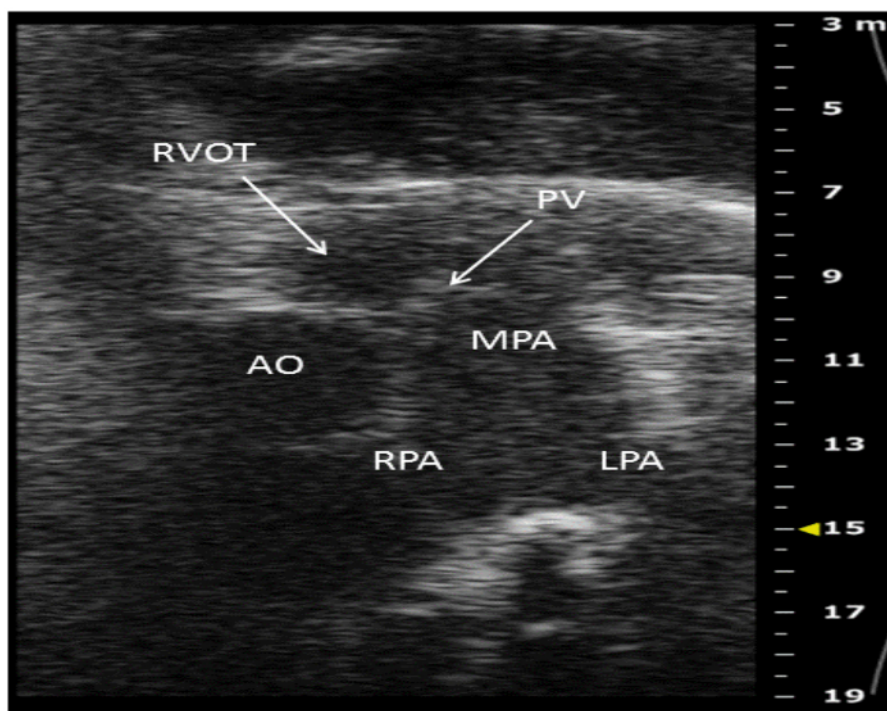


Figure 6: Long-axis View of the Main Pulmonary Artery and its Major Branches. The long-axis view of the main pulmonary artery (MPA) and right (RPA) and left (LPA) branches can be obtained from the parasternal window. The right ventricular outflow tract (RVOT), pulmonic valve (PV), and aorta (AO) are partly seen. [Please click here to view a larger version of this figure.](#)

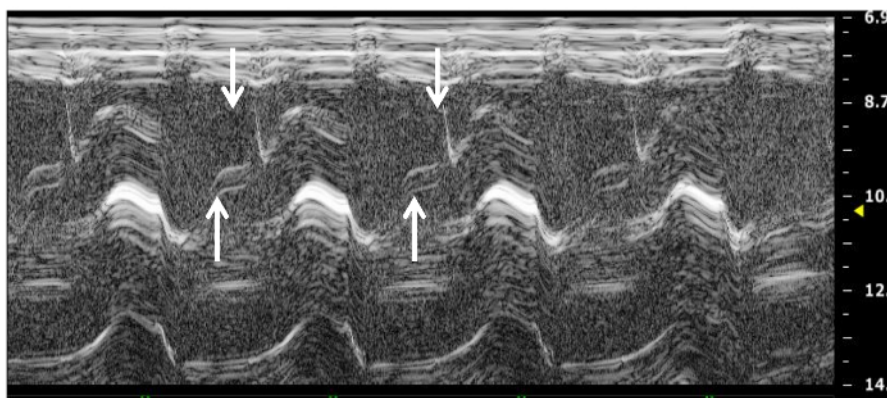


Figure 7: M-mode Image Depicting a Normal Pulmonic Valve. From the parasternal window, both short- and long-axis views of the pulmonic valve can be obtained. The M-mode line of interrogation is applied across the pulmonic valve. The pulmonic valve cusp-separation (arrows) distance can be measured from this view. [Please click here to view a larger version of this figure.](#)

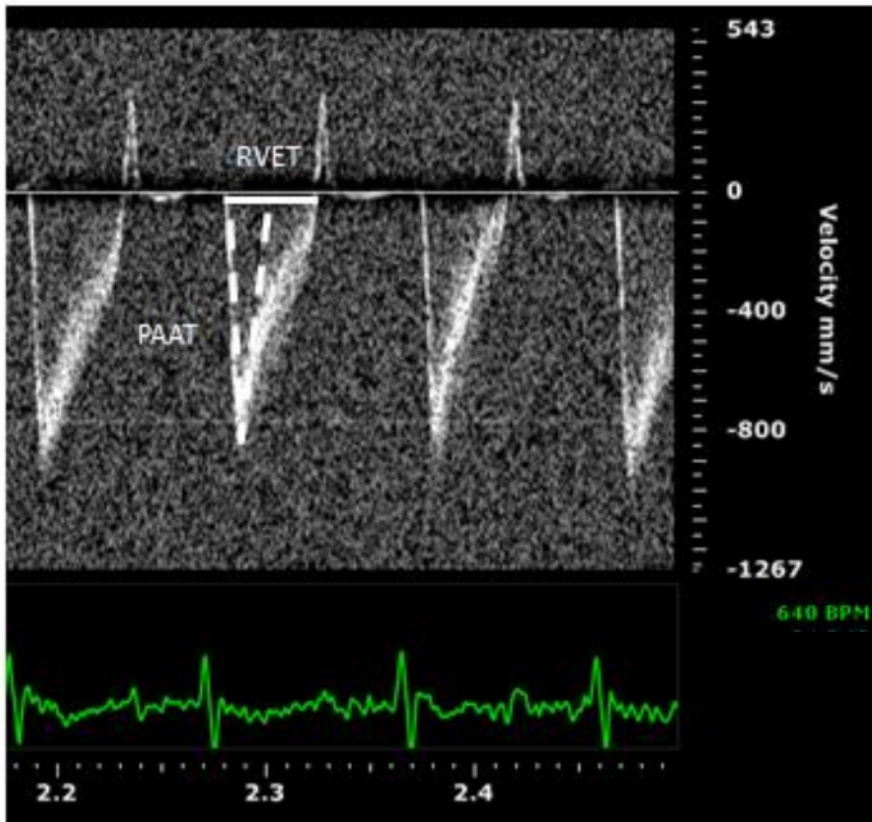


Figure 8: Pulsed-wave Doppler Interrogation of Flow Across the Pulmonic Valve. The pulmonary artery acceleration time (PAAT) is the time interval from the onset of systolic pulmonary arterial flow to the peak flow velocity. Right ventricular ejection time (RVET) is the interval between the onset of right ventricular ejection to the point at which there is cessation of flow. [Please click here to view a larger version of this figure.](#)

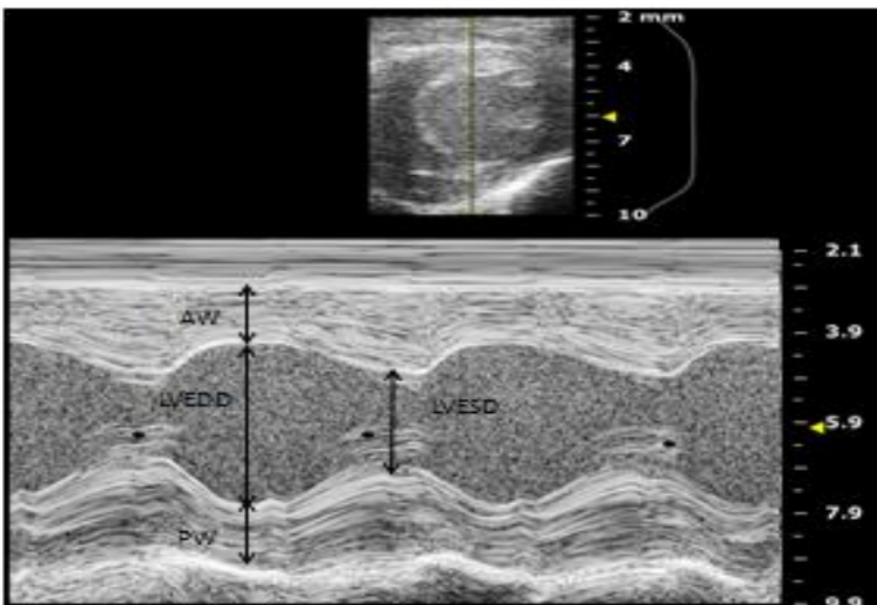


Figure 9: M-mode Image Depicting a Short-axis View of the Left Ventricle. From the parasternal window, the short-axis view of the left ventricle is obtained by rotating the transducer head counterclockwise so that the image index marker points posteriorly or dorsally. The M-mode line of interrogation is applied across the left ventricle at the level of the papillary muscles. Left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), and anterior wall (AW) and posterior wall (PW) thicknesses can be readily measured. Be careful not to include the papillary muscle (*) in any measurements. [Please click here to view a larger version of this figure.](#)

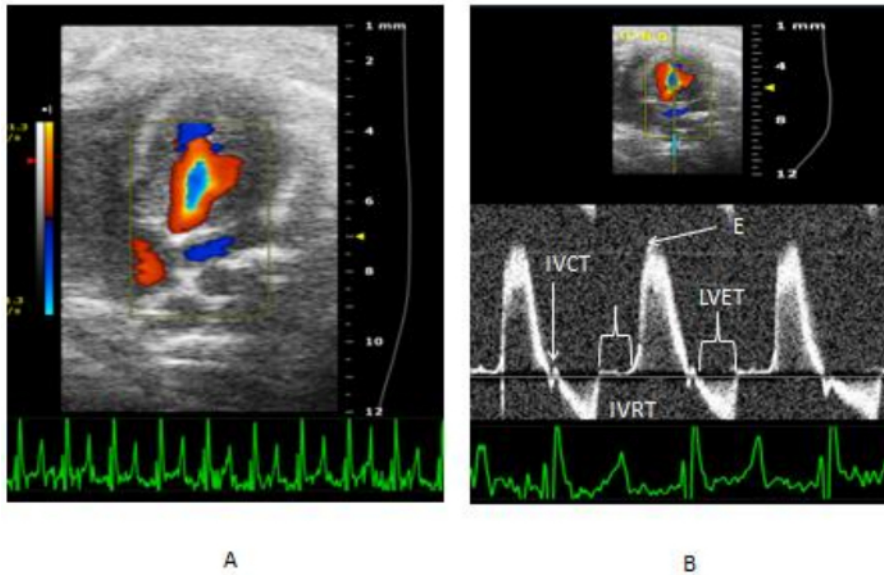


Figure 10: Color Doppler Evaluation and Pulsed-wave Doppler Spectral Display of Mitral Valve Inflow. (A) Image showing a color Doppler evaluation of mitral valve inflow in the apical long-axis view. Note that the 2D color Doppler image can be a critical tool for guiding the appropriate sample volume position for the acquisition of pulsed-wave Doppler tracings (depicted in panel B). (B) Spectral display of mitral valve inflow using pulsed-wave Doppler. The pulsed-wave Doppler assessment of blood flow across the mitral valve (in the apical long-axis view) is performed to assess left ventricular diastolic function. The sample volume is placed at the tips of the mitral valve leaflets. The isovolumic relaxation time (IVRT), isovolumic contraction time (IVCT), left ventricular ejection time (LVET), and peak mitral inflow velocity (E) can all be derived from the spectral display of pulsed-wave Doppler velocities across the mitral valve. [Please click here to view a larger version of this figure.](#)

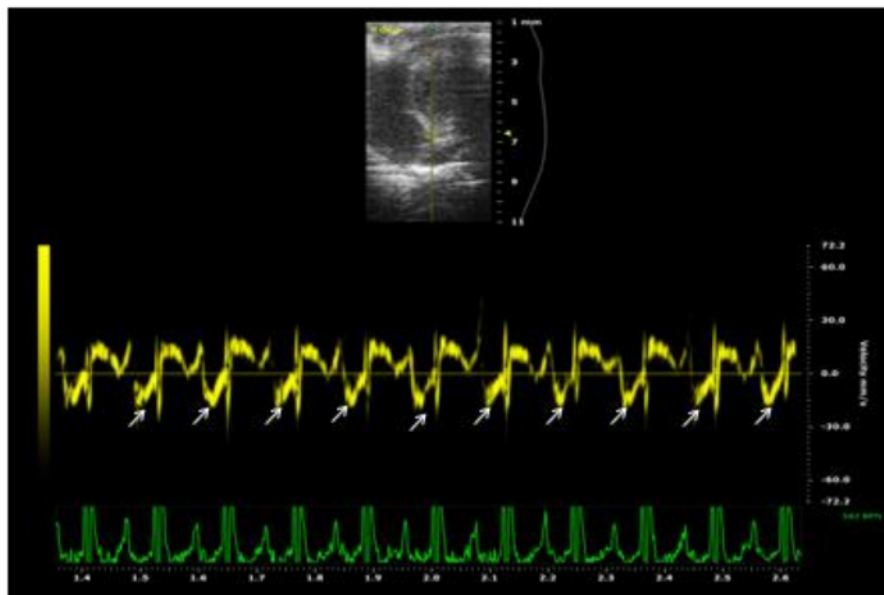


Figure 11: Tissue Doppler Imaging of the Septal Mitral Annulus. From the apical window, a long-axis view of the mitral valve is obtained. The tissue Doppler sample volume is positioned at the septal region of the mitral annulus. The ratio between the peak mitral inflow velocity (variable E in Figure 10B) and the peak mitral annulus tissue velocity (e' , denoted by white arrows) is used to assess left ventricular diastolic function (commonly referred to as E/e'). [Please click here to view a larger version of this figure.](#)

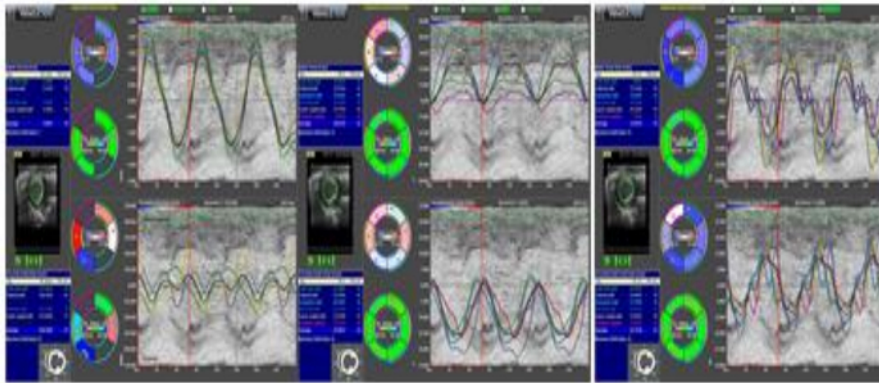


Figure 12: Assessment of the Strain and Strain Rate of the Left Ventricular Myocardium. There are specialized analysis software packages available commercially, and the strain and strain rate variables can be obtained as measures of early or sub-clinical changes in intrinsic myocardial contractile properties. The examples shown above depict radial strain and strain rate in commonly-acquired imaging planes in mice. Note that these imaging planes (and the subsequent shape of the strain tracings) can differ from images in humans, which are frequently acquired in the apical long-axis or 4-chamber view. [Please click here to view a larger version of this figure.](#)

Discussion

Induction of anesthesia

Proper induction and maintenance of anesthesia is critical for the accurate assessment of changes in heart valve and cardiac function in mice. Given the rapid induction of anesthesia elicited by isoflurane and the relatively long wash-out time of this anesthetic following deep anesthesia, we do not use a stand-alone anesthesia chamber for induction. Instead, as noted in detail above, animals are guided directly to the anesthesia cone, which allows for rapid and controlled induction of anesthesia at relatively low concentrations of the anesthetic.

Most strains of mice remain amply sedated at less than 1.5% isoflurane. The cumulative effects of isoflurane on cardiac function should be closely monitored, however, and small decreases in the concentration of anesthetic may be required over time. Reciprocally, small increments in the concentration of anesthetic may also be needed. Carefully monitor the animal for any movement (suggestive of inadequate depth of anesthesia) and for increases or decreases in HR; this allows for rapid and proactive management of the depth of anesthesia.

In contrast to humans, isoflurane elicits a decrease in HR in mice. While left ventricular function may initially be preserved during periods of excessive anesthetic administration, reductions in HR are nearly ubiquitously followed by left ventricular dilation secondary to the suppression of cardiac contractility. Consequently, the ejection fraction decreases, transvalvular (aortic valve and mitral valve) peak flow velocities fall, aortic valve closure occurs early, and tissue Doppler velocities decrease. It is therefore imperative to continuously monitor the physiological state of the animal to ensure that the HR remains well above 450 bpm. For individuals who are not experienced in imaging mice, an approach that includes a dedicated sonographer and a second investigator dedicated to monitoring the depth of anesthesia is recommended.

Analysis of AV function

Clinically, the American Society of Echocardiography guidelines¹³ recommend acquisition of the left ventricular outflow tract diameter and the left ventricular outflow tract velocity using pulsed-wave Doppler. The peak trans-aortic valve velocity should be measured using dedicated continuous-wave Doppler to calculate the aortic valve area using the continuity equation: $AVA = (CSA_{LVOT} \times VT_{LVOT}) / VT_{AV}$. In the absence of these Doppler data, the anatomic (geometric) cross-sectional area of the aortic valve orifice as measured by 2D or 3D is recommended. Although the transducer has high spatial and temporal resolution, the aortic valve cusps cannot be consistently delineated in the short-axis view. Thus, the AV orifice area cannot be accurately traced. Furthermore, and perhaps more importantly, currently-available high-frequency small-animal-dedicated ultrasound is not equipped with dedicated continuous wave Doppler capability. Thus, the identification of a "true" peak transvalvular velocity for use with the continuity equation is exceptionally challenging (and would not be accepted clinically). Likewise, other commercially-available ultrasound probes may not have the capability to record very high velocities and are thus limited to lower velocities. Given these major limitations, clinical imaging protocols using systems geared towards high-resolution imaging in small animals cannot be fully captured.

Analysis of MV function

Generally, mice are very resistant to the development of mitral valve prolapse. Visualization of a regurgitant jet across the mitral valve in the setting of a rapid HR can be very challenging. Moreover, in human echocardiography, the anterior and posterior mitral valve leaflets are clearly seen and the prolapsed or flail leaflet is easily appreciated. However, in mice, mitral valve leaflets cannot be well-delineated into anterior and posterior, and finding a flail or prolapsed leaflet is exceptionally challenging, given the low level of echogenicity of non-calcified, thin tissues. Thus, the use of color Doppler to show a regurgitant jet is the most useful means to assess mitral valve function in mice. A diagnosis of isolated mitral valve regurgitation should be made only after carefully assessing left ventricular function, aortic valve function, and mitral valve function.

To date, there are no robust mouse models of mitral valve stenosis. Increased echo density of the mitral valve can suggest calcification, but localization to either the anterior or posterior leaflet is difficult. Clinically, a diagnosis of mitral valve stenosis is made in the setting of thick, calcified leaflets with restricted motion. Measurement of leaflet thickness can be done by M-mode (**Figure 4**). Using Doppler, the peak E velocity is usually increased and is associated with prolongations in the pressure half-time. Thus, recapturing these features will be critical in the

evaluation of novel models of mitral valve stenosis. While the American Society of Echocardiography recommends that the estimation of mitral valve area is done using the pressure half-time ($MV \text{ area} = 220/\text{pressure half-time}$), such calculations have not been validated in mice¹³.

Analysis of tricuspid and pulmonic valvular function

The tricuspid valve is assessed for leaflet mobility, valvular stenosis, and valvular regurgitation. Typically, these data are expressed qualitatively and in a binary fashion (*i.e.*, presence or absence of dysfunction). The peak velocity of the tricuspid valve regurgitant jet is used to estimate right ventricular systolic pressure. Additionally, tricuspid regurgitation is not uncommon in normal, unstressed mice.

Pulmonic valve function can be assessed by 2D/B-mode, M-mode, and color-flow imaging (**Figures 6 and 7**). These modalities are used to assess pulmonic valve thickness (*e.g.*, visibility or echogenicity with 2D), measure pulmonic valve orifice opening (cusp-separation distance), and assess pulmonic valve mobility and coaptation (2D and color Doppler). Pulmonic valve regurgitation can be readily appreciated with color Doppler, as described above. The severity of pulmonic valve regurgitation can be assessed using the peak retrograde blood flow (measured with pulsed-wave Doppler) through the pulmonic valve during diastole.

Analysis of cardiac function

2D/B-mode imaging of the left ventricle in short- and long-axis views provides a visual assessment of cardiac function. While this imaging modality allows for coarse evaluations of left ventricular function, M-mode imaging offers significantly higher spatiotemporal resolution, making it a superior technique when compared to 2D/B-mode imaging. This is very important, considering the fact that normal mice can have HRs ranging from 450-700 bpm. We maintain the HR above 450 bpm so that the data is a close representative of non-anesthetized cardiac physiology and hemodynamics. If the HR is allowed to fall due to excessive anesthesia and/or over-sedation, left ventricular dilatation, reductions in estimates of cardiac contractility, and dramatic alterations in transvalvular blood velocities and other qualitative characterizations of valvular function (*e.g.*, changes in mitral regurgitation secondary to left ventricular dilatation, reductions in peak aortic valve flow velocity, and reductions in mitral blood inflow velocity) are often observed.

In the absence of segmental wall-motion abnormalities, ejection fraction (EF) and fractional shortening (FS) are highly reproducible measures of left ventricular systolic function. Using M-mode imaging, the maximal diastolic and systolic dimensions are obtained and used to calculate the EF, FS, and LV mass^{14,15}.

All of these measurements can be automatically computed in the software package associated with the ultrasound machine. While evaluation of cardiac and valvular function can be performed using "standard" clinical ultrasound systems, the relatively low levels of resolution (*e.g.*, 12-15 MHz probes) can make the accurate assessments of cardiac and valvular function in mice challenging.

Diastolic function is an integral part of assessing the function of the left ventricle. In clinical studies, diastolic heart failure has been found to be highly correlated with morbidity and mortality. Diastolic function is assessed by pulsed-wave Doppler echocardiography and tissue Doppler imaging. The E/A ratio (the ratio between the early rapid-filling wave, E, and the late-filling wave due to atrial contraction, A) and E deceleration time are not useful parameters of diastolic function in mice due to the fusion of the E and A waves secondary to the very high HRs present in appropriately-anesthetized mice.

To evaluate left ventricular diastolic function, peak mitral inflow velocity, isovolumic relaxation time (IVRT), isovolumic contraction time (IVCT), left ventricular ejection time, and mitral annulus tissue, the velocities (*e'*) are utilized. These Doppler parameters are readily obtainable, measurable, and reproducible. The early diastolic velocity (*e'*) of the mitral annulus measured with tissue Doppler imaging is a reliable indicator of left ventricular myocardial relaxation. The ratio between the peak mitral inflow velocity and the early mitral annulus tissue velocity have been shown in clinical studies to correlate well with pulmonary capillary wedge pressure¹⁶.

Global left ventricular function can be assessed using the myocardial performance index, also known as the Tei index. It incorporates both systolic and diastolic time intervals to allow for an integrated measure of both systolic and diastolic left ventricular function. Systolic dysfunction prolongs pre-ejection time (IVCT) and shortens the left ventricular ejection time (ET). Abnormalities in diastolic function or myocardial relaxation can result in significant prolongation of the IVRT. The left ventricular myocardial performance index (MPI) can be calculated as $MPI = IVCT + IVRT / LVET$ ¹⁷. In this context, reductions in the MPI are associated with improvements in cardiac function, whereas a higher MPI value is suggestive of cardiac dysfunction.

Emerging techniques to assess cardiac and valvular function in mice: future directions

Tissue Doppler

Tissue Doppler can be used to assess diastolic function using the E, *e'*, and E/*e'* variables, but this method is not currently widely used. As such, the variability and reproducibility of the measurements in a variety of rodent strains has not been rigorously tested by multiple research groups. Nevertheless, the use of E/*e'* and its correlation with left atrial pressure in clinical environments, potential for early detection of cardiac dysfunction in mice, and the application to disease mechanisms is likely to make this an integral component of assessing the cardiac consequences of valvular heart disease in translational research.

Strain rate imaging

Small animal models have proven to be an invaluable tool to understand the mechanisms underlying pathophysiological changes in cardiac function. While 2D and Doppler echocardiography provide comprehensive and non-invasive assessments of cardiac morphology, function, and hemodynamics *in vivo*, they lack the sensitivity to detect early changes in myocardial function in response to chronic pressure or volume overload (two of the most common stressors induced by valvular heart disease).

As a result of these limitations, there is growing interest in the application of clinically-used indices of cardiac function-such as myocardial strain and strain rate-that have the potential to more accurately detect early or sub-clinical changes in intrinsic myocardial contractile properties. Strain

and strain rate imaging have been used successfully in rodent studies on the progression of heart failure¹⁸ and hypertensive heart disease¹⁹, the reversal of cardiac dysynchrony and cardiac dysfunction²⁰, and the longitudinal function of the heart in juvenile mice²¹. It is recommended that strain-rate imaging be considered a supplemental imaging technique to thorough 2D and tissue Doppler-derived measures of cardiac function. To ensure that investigators have a basic understanding of the principles underlying the measurement of myocardial strain and strain rate, the subsequent sections aim to provide fundamental principles and limitations underlying strain calculation and strain rate imaging.

Strain and strain rate are derived from the change in length of the myocardial fiber with respect to the original length (In cardiology, the difference between end diastolic length and end systolic length is used for this calculation). The precise measurement of changes in myocardial fiber length is complicated by the spiral architecture of the myocardial fiber bundles, resulting in multidirectional strain deformation throughout systole (e.g., strain in the radial, longitudinal, and circumferential axes). Recent studies in mice suggest that tissue Doppler derived- and speckle tracking-derived strain and strain rate deformation parameters relate closely to intrinsic myocardial function²². Both techniques require the addition of specialized analysis software to research imaging systems, which allows for the relatively automated generation of the variables of interest (see examples in **Figure 12**)²³.

Although strain imaging holds promise, acquisition of high-quality 2D images for speckle-tracking analysis can be challenging. Further, manually tracing the endocardial and epicardial borders for strain measurement is difficult and cumbersome. A significant amount of practice and robust evaluation of the reproducibility and consistency of intra-investigator measurements (including image quality, consistent imaging planes, and off-line analysis) are critical when implementing the use of strain measurements to evaluate cardiac function. Thus, strain and strain-rate analyses should be conducted by completely blinded, trained investigators to ensure high-quality and reproducible data.

ECG-gated high-resolution ultrasound imaging

Tissue Doppler imaging and strain-rate imaging allow the measurement of the myocardial deformations over a complete cardiac cycle, but due to their temporal resolution (5 ms at best), they remain limited to the global motion of the heart²⁴. To achieve high frame rate ultrasound imaging, another approach based on the use of ECG-gated data acquisition has been recently proposed for cardiac and vascular applications. ECG-gated mechanical and electromechanical wave imaging of cardiovascular tissue is based on imaging the tissue using ultrasound at high frame rates, up to 8,000 frames per s (fps), by synchronizing the 2D image acquisition on the ECG signals²⁴. This clearly surpasses the 2D/B-mode frame rates of ~1,000 fps (providing greater resolution under physiological conditions where heart rate is ~500-650 bpm in a mouse), and *in vivo* feasibility of this imaging method for evaluation of ventricular function has been demonstrated in anesthetized animals (providing superior detection of cardiac wall motion abnormalities in small animal models²⁵).

Stress-induced cardiac function

While exercise testing is frequently used to evaluate cardiac responses to increased organismal stress in clinical settings, the need for conscious sedation and/or anesthesia in rodents makes the immediate post-exercise evaluation of cardiac function exceedingly challenging. Thus, pharmacological stress testing is likely to be a clinically-relevant parallel to assess the cardiac consequences of valvular heart disease (severe aortic stenosis, moderate-to-severe mitral stenosis, and severe primary mitral regurgitation). This will be a particularly important emerging area of research, given recent clinical guidelines that emphasize the role of stress testing to clarify symptom status, assess dynamic components of valvular abnormalities, and unmask subclinical myocardial dysfunction that is likely to be missed at rest²⁶.

As noted in previous sections, mice are exceedingly resistant to afterload-induced cardiac dysfunction. Thus, dobutamine stress echocardiography may be a very useful tool to detect early declines in the left ventricle that may not be apparent in mice with varying levels of valvular heart disease. Even mice with severe calcific aortic valve stenosis can have relatively well-preserved systolic function and are likely to provide a useful platform for the application of dobutamine stress echocardiography to predict the timing (and often very rapid) onset of heart failure in these animals. To date, we are not aware of any studies investigating the use of dobutamine stress echocardiography in mice with any degree of valvular heart disease.

3D echocardiography

Clinically, 3D cardiac imaging is a particularly powerful tool that enables precise measurements of diastolic and systolic volumes, stroke volume, and cardiac output. 3D echocardiography has become a new clinical standard in the assessment of the severity of valvular stenosis by means of accurate valve area measurement, and it allows for the accurate identification and quantification of prolapse of individual segments in mitral valve disease.

Research ultrasound systems with high-frequency transducers allow for the acquisition of cardiac-gated images and for the subsequent offline reconstruction of 3D images using custom software packages. While it is possible to acquire 3D images of the left ventricle using this hardware and software combination, this is often conducted under relatively deep levels of anesthesia (which lower the HR and minimize the respiratory artifact), making the extrapolation of the physiological significance of changes in cardiac function difficult.

With regards to the use of 3D imaging to assess heart valve function in mice, this is an exceptionally challenging proposition given the small size, relatively low echogenicity, and high velocity of the cardiac valves under normal physiological conditions. Until technological advances in image acquisition and processing allow for the clear discernment of cardiac valves under such conditions, our experience has been that 3D imaging is of limited utility in the accurate and thorough characterization of cardiac valve function in mice.

Collectively, technological advances in small animal imaging make this an exceptionally exciting time to gain insights into the pathophysiological mechanisms underlying valvular heart diseases and their cardiac consequences. We firmly assert that the thorough evaluation of both heart valve function and cardiac function is essential to understanding the effects of genetic, pharmacological, or mechanical manipulations of heart valve function in mice. We hope that this manuscript will not only serve as a useful resource for investigators pursuing research into the pathogenesis of cardiac valve disease, but will also spur discussion regarding the best methods to assess valvular and cardiac function in such studies within our research community.

Disclosures

The authors have nothing to disclose.

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