Video Article

In utero Measurement of Heart Rate in Mouse by Noninvasive M-mode Echocardiography

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Abstract

Congenital heart disease (CHD) is the most frequent noninfectious cause of death at birth. The incidence of CHD ranges from 4 to 50/1,000 births (Disease and injury regional estimates, World Health Organization, 2004). Surgeries that often compromise the quality of life are required to correct heart defects, reminding us of the importance of finding the causes of CHD. Mutant mouse models and live imaging technology have become essential tools to study the etiology of this disease. Although advanced methods allow live imaging of abnormal hearts in embryos, the physiological and hemodynamic states of the latter are often compromised due to surgical and/or lengthy procedures. Noninvasive ultrasound imaging, however, can be used without surgically exposing the embryos, thereby maintaining their physiology. Herein, we use simple M-mode ultrasound to assess heart rates of embryos at E18.5 *in utero*. The detection of abnormal heart rates is indeed a good indicator of dysfunction of the heart and thus constitutes a first step in the identification of developmental defects that may lead to heart failure.

Video Link

The video component of this article can be found at https://www.jove.com/video/50994/

Introduction

CHD is the most common noninfectious cause of death at birth[1]. Multiple surgeries are often needed to correct the structural defects in subjects whose quality of life may remain compromised[1]. Children with CHD frequently develop neurological disorders even if they have not undergone surgery, indicating important *in utero* consequences on development[2,3]. Both genetic and environmental factors, such as exposure to viruses or chemicals (alcohol) during pregnancy, cause CHD. Studying the genetic contributors is still at its early phase, but growing rapidly. To identify these contributors and understand their role in heart development, phenotyping mutant mice with a simple and powerful tool will be highly beneficial.

Mouse is indeed an animal model of choice to study CHD, and most of the human cases can be reproduced in mice[4,5]. Consequently, fetal mouse cardiac phenotyping has become increasingly important to investigate the etiology of human CHD and requires adequate tools. Although histological studies on fixed specimens are invaluable, real-time imaging of live animals is crucial to understand the physiology of the heart. Video microscopy offers live imaging. However, it requires laparotomy to expose embryos, thereby compromising their physiologic and hemodynamic state. Recently, echocardiography has become the standard imaging technique for cardiac assessments in the clinic as well as in mice.

Mouse fetal echocardiography is conducted using standard clinical ultrasound systems as well as ultra-high frequency ultrasound systems. The latter provide 30 MHz or higher frequency transducers that generate two-dimensional images and allow the assessment of early embryonic stages. These transducers have a relatively poor penetration depth (~13 mm), which is, however, sufficient to obtain adequate imaging planes and determine fundamental heart parameters, such as heart rate, left and right ventricular internal diameter at diastole and systole and septum and wall thickness, without performing laparotomy.

In our study, we have used an ultra-high frequency ultrasound system to assess heart rates of mouse embryos at embryonic day E18.5. We chose a 30 MHz transducer that provides a field view of 20 mm x 20 mm, which is ideal given the size of the fetuses, with a focal length of 12.7 mm. However, a higher frequency transducer may be chosen to analyze earlier developmental stages. The selected M-mode allows the visualization of tissues in motion thanks to a high temporal resolution of 1,000 frames/sec. The full procedure is simple and should be performed as quickly as possible to avoid any perturbation of the physiologic and hemodynamic states of the fetus. The analysis of about 8 embryos requires approximately 1 hr.

Protocol

All procedures shown in this protocol have been approved by the IRCM Animal Care Committee.

1. Ultrasound System and Station Preparation

1. Start the ultrasound imaging system and connect the scanhead as well as the physiology controller unit according to the manufacturer's instruction (**Figure 1**). Choose Cardiac Measurement Program and the scanhead that corresponds to the 30 MHz transducer.
2. Fill the nosepiece of the scanhead with deionized water. Avoid air bubbles as they interfere with imaging resolution. Place the scanhead with the handle side up on its holder near the imaging platform (**Figure 1A**).
3. Disinfect the imaging platform and working area.
4. Fill completely the bottle of ultrasound gel to avoid the formation of bubbles and place it upside down in its prewarming container set to 37 °C (**Figure 1B**).
5. Verify the levels of oxygen and isoflurane and the tubing system for anesthesia. One experiment with ~8 embryos would use approximately 15-20 L oxygen.
6. Place the bottles of ophthalmic balm, depilatory cream and electrode gel near the imaging platform (**Figure 1B**).
7. Verify that the infrared heat lamp functions. During training, set the heat level, the position of the lamp and the distance of the lamp to mice in order to maintain constant body temperature and heart rate.
   Note: Proper preparation of the system and material is critical. Lengthy procedures may affect the physiologic and hemodynamic characteristics of fetuses as long anesthesia depresses cardiac function by lowering heart rate, blood pressure and blood-oxygenation level. The processing time for ~8 fetuses (average litter size in C57BL/6) should be approximately 1 hr. Other mouse strains may provide a higher number of fetuses, possibly with a larger female size. Training is essential to optimize the processing time.

2. Mouse Preparation

1. Anesthetize the pregnant female in a chamber with continuous supply of 2% isoflurane in oxygen (200 ml/min) until it becomes nonresponsive.
2. Place the mouse on the imaging platform in a supine position, adjust the tubing for continuous inhalation of 1.5% isoflurane in oxygen (200 ml/min), and fix the inhalation tube with tape (**Figure 2A**). Sufficient anesthesia should be confirmed during the procedure by the relaxed posture of the mouse and the absence of any response to tail and toe pinches.
3. Place electrode gel only on the left top and right bottom electrode pads (right fore leg and left hind leg, also called lead II position) for electrocardiography. Lead II position provides better defined upright positive P-wave and QRS complex to determine heart rate. Secure the four paws to each pad using tape (**Figure 2A**). To prevent dryness of the eyes, apply 1 drop of ophthalmic balm into each eye.
4. Shave the abdomen from chest to bladder with a hair clipper. Then apply depilatory cream for 2 min and wipe it off with gauze and/or cotton swab to carefully remove any remaining hair. Be careful not to cut the nipples.
5. Insert the thermometer prelubricated with electrode gel into the female's rectum to monitor the body temperature (should be maintained at 37±0.5 °C by adjusting an infrared heat lamp placed above the platform). Heart rate should be 450±50 beats/min (bpm). Both temperature and heart rate are displayed on the physiology controller unit (**Figure 1B**).
   Note: Long anesthetization, hair loss and ultrasonic gel (although prewarmed) can lead to hypothermia, which can affect the female's heart rate. If the body temperature drops below 36.5 °C, stop the procedure and modify the position of the heating lamp to adjust the temperature and the heart rate. Wait a couple of minutes before proceeding again.

3. Embryo Identification

1. Gently press down on the naked abdomen to locate the embryos. Slowly and lightly spread them out to have most of embryos in a single layer under the abdominal surface. In each uterine horn, sacs are linearly connected to each other. Try to respect this order when spreading.
2. Mark each embryo on the naked abdomen of the female with a permanent marker with their anterior/posterior and dorsal/ventral directions. Knowing the orientation will facilitate locating the heart with the probe. Number the embryos in the right and left horns starting from the cervix (1, 2, 3… and 1', 2', 3'… respectively; **Figure 2B**).
   Note: (i) Avoid spreading the embryos with excessive force. Sketch their locations on a piece of paper to track them (**Figure 2C**). (ii) C57BL/6 females have ~8 fetuses per litter. However, 0-2 embryos in each litter are located beneath the others, making their imaging impossible. Exclude these embryos from the analysis and assess more litters if needed.

4. Heart Rate Measurement

1. Place a small amount of prewarmed ultrasound gel on the naked abdomen and spread it evenly. Avoid bubble formation. Add a larger amount of gel (~ 5 ml) on the specific area to image.
2. Hold the probe in contact with the thick gel layer (10 mm) and gradually move the probe toward the skin while looking for the beating heart. Once the beating heart is visualized on the screen, adjust the angle of the probe to have both ventricles in their largest size in the imaging plane (**Figure 2D**).
3. Begin acquiring image. With the forearm resting on the station, place the transducer on the ultrasound gel to obtain a live image on the viewing screen (**Figure 2D**). Maintaining the ridge of the transducer on the top and clicking the scanhead orientation marker (in the left top corner of the image) allow the coordination of the hand movement and area visualized on the screen.

4. Starting from the cervix, move to the closest embryo marked (1 or 1' in right or left uterine horn, respectively) to visualize the beating heart on the screen. Because focal depth is fixed, gently move the probe up/down and sideways without losing contact with gel to obtain the desired image plane.
5. Position the beating heart in the center of the screen within the region indicated by the yellow dashed line representing the focal zone for better data acquisition.
6. Acquire the live recording. Obtain the scout image and resume recording (**Figure 3A**). Once a minimum of 10 sec of stable recording is obtained, stop recording and save.
7. Proceed to the next embryo.
8. Once all the embryos are analyzed, press "Browse" on the keyboard to view the list of the recordings. Play each recorded M-mode tracing and perform multiple measurements (at least 5 per tracing) of the spacing between adjacent peaks (time/flow cycle) to obtain the average heart rate (**Figure 3B**).
   Note: Some protocols suggest using a stationary stand for the scanhead to avoid shaking. However, it restrains the angle of analysis, making the observation of lateral embryos difficult. It also slows down the analysis, as the stand has to be adjusted for each embryo. Holding the scanhead while settling the forearm on the station efficiently minimizes shaking. Training with 5-10 pregnant females is recommended to optimize the outcome.

5. Genotyping

1. Using clean surgical scissors and forceps, incise longitudinally the skin and muscle layer of the abdomen. Locate the sacs in each uterus horn and match the numbers. Cut open the yolk sac to expose the embryo in the order used above. If not in a linear arrangement, the sketch of the embryo positions on a piece of paper will be essential once the marks on the skin are no longer visible.
2. Cut only ~4 mm of tail for genotyping as genomic DNA is very efficiently extracted from embryonic tails.
   Note: (i) The surgical procedure may have to be performed in a separate location from the imaging room. However, it is important not to move the mouse and have the surgical procedure on the imaging platform in order to keep track of the numbered embryos. Consult the animal facility manager to optimize the surgical procedure. (ii) The pregnant female rapidly dies due to blood loss as the fetuses are removed for genotyping after imaging. After tail cut, the fetuses are placed 3 min on ice for anesthesia by hypothermia and then decapitated with scissors, as recommended by our Animal Care Committee.

Representative Results

The above method was used to assess the impact of the presence or absence of the serine protease furin in endothelial cells on heart rates of mouse embryos at E18.5 *in utero*. Furin belongs to the family of proprotein convertases (PCs) that cleave protein precursors after basic residues. Furin and its substrates are secretory proteins and cleavage can occur in the Golgi apparatus, endosomes or at the cell surface. Key substrates of furin include TGFb, and TGFb-like factors, such as the bone morphogenetic proteins (BMPs) that play an important role in heart development. Other members of the PC family can also activate typical substrates of furin by *in vitro* cleavage[6]. The key and unique role of furin during development is evidenced by the early death of furin-deficient embryos at embryonic day 11[7].

Because many of the defects exhibited by furin-deficient embryos suggested an important function of furin in endothelial cells, the role of the enzyme was examined in endothelial cell-specific knockout (ecKO) mice. *Furin*$^{flox/flox}$ mice that carry conditional *flox* alleles of the *Furin* gene[8] were crossed with *Furin*$^{flox/+}$mice carrying a transgene in which the expression of the Cre recombinase is driven by the endothelial cell-specific Tie2 promoter[9]. In endothelial cells, Cre recombines two *loxP* sites that flank exon 2 of *Furin* gene, which encodes the signal peptide and part of prosegment, and thereby generates inactivated *Furin* alleles. ecKO newborns die shortly after birth, confirming the essential role of furin in processing of endothelial cell precursors.

In utero analysis of heart rates in embryos at E18.5 showed that homozygous, but not heterozygous, ecKO embryos suffered from tachycardia (elevated heart rates; **Figure 4**). In agreement, subsequent magnetic resonance imaging revealed that ecKO embryos exhibited ventricular septal defects and/or valve malformations with some subcutaneous edema. Isolated septal and/or valve malformation cannot explain either heart failure or tachycardia. Although we cannot exclude other causes, the observed sudden death of homozygous ecKO newborns is likely due to cardiac failure. This finding was previously published[10].

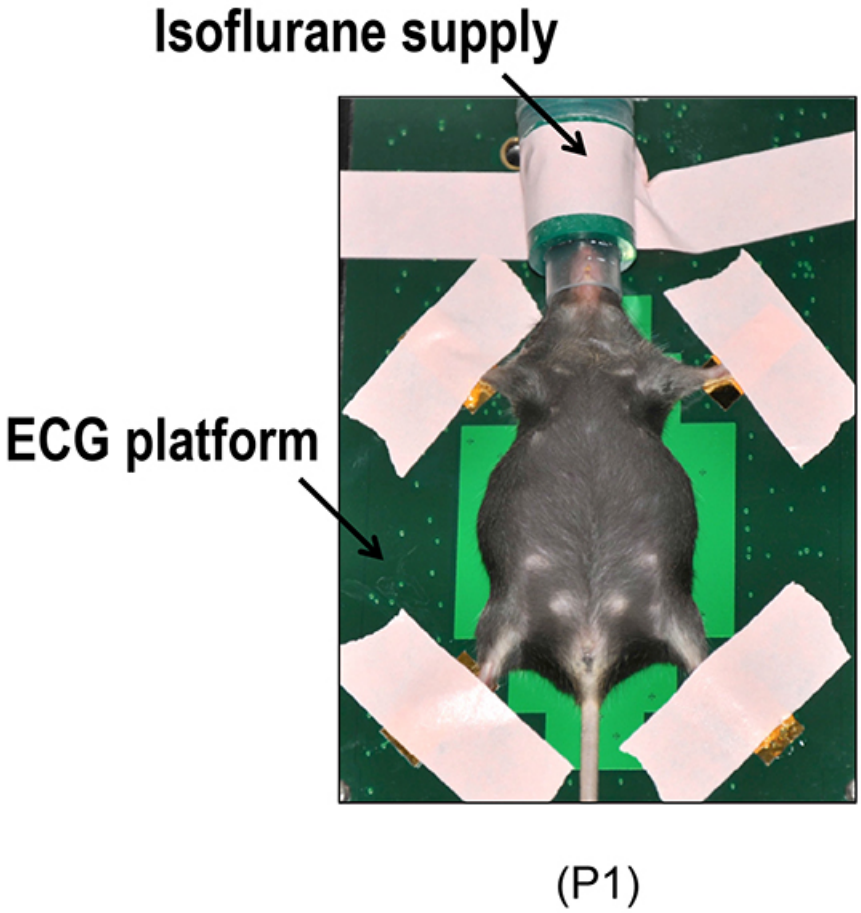

Figure 1. Overview of system set-up. (**A**) Ultra-high frequency ultrasound system with the scanhead in its holder is shown. (**B**) The station comprises an anesthetic system, the physiology controller unit and materials. Click here to view larger image.

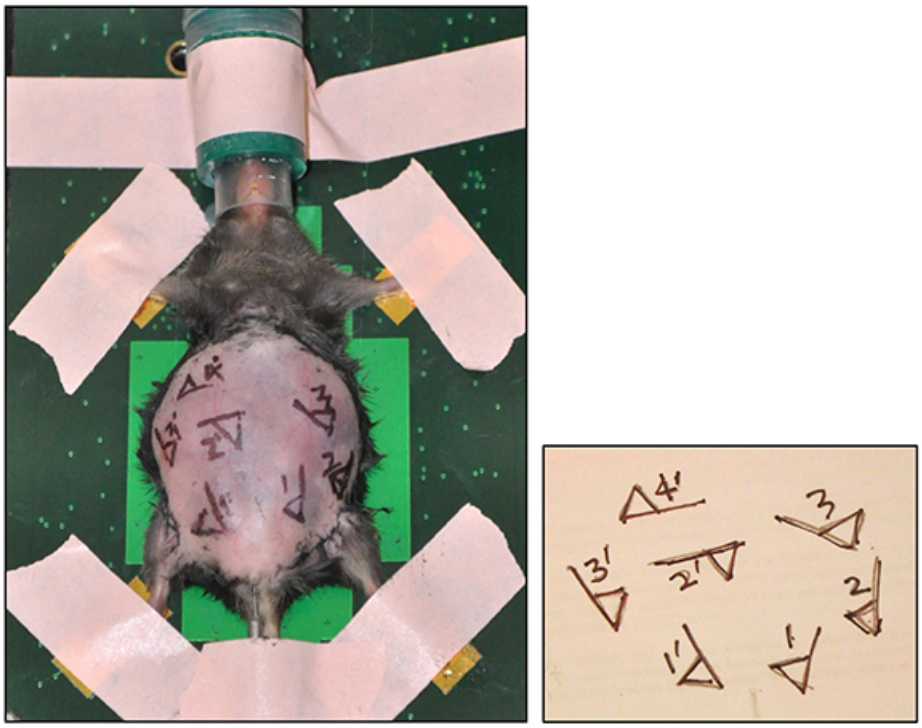

Figure 2. Mouse set-up. (**A**) The pregnant mouse is placed in supine position with isoflurane supply and restrained on the platform with tape. (**B**) After abdominal hair removal, the location of embryos is marked on the abdomen or (**C**) sketched on a paper. (**D**) The desired imaging plane is obtained by moving the scanhead that remains in contact with the ultrasound gel. The forearm is securely placed on the station to avoid shaking. Click here to view larger image.

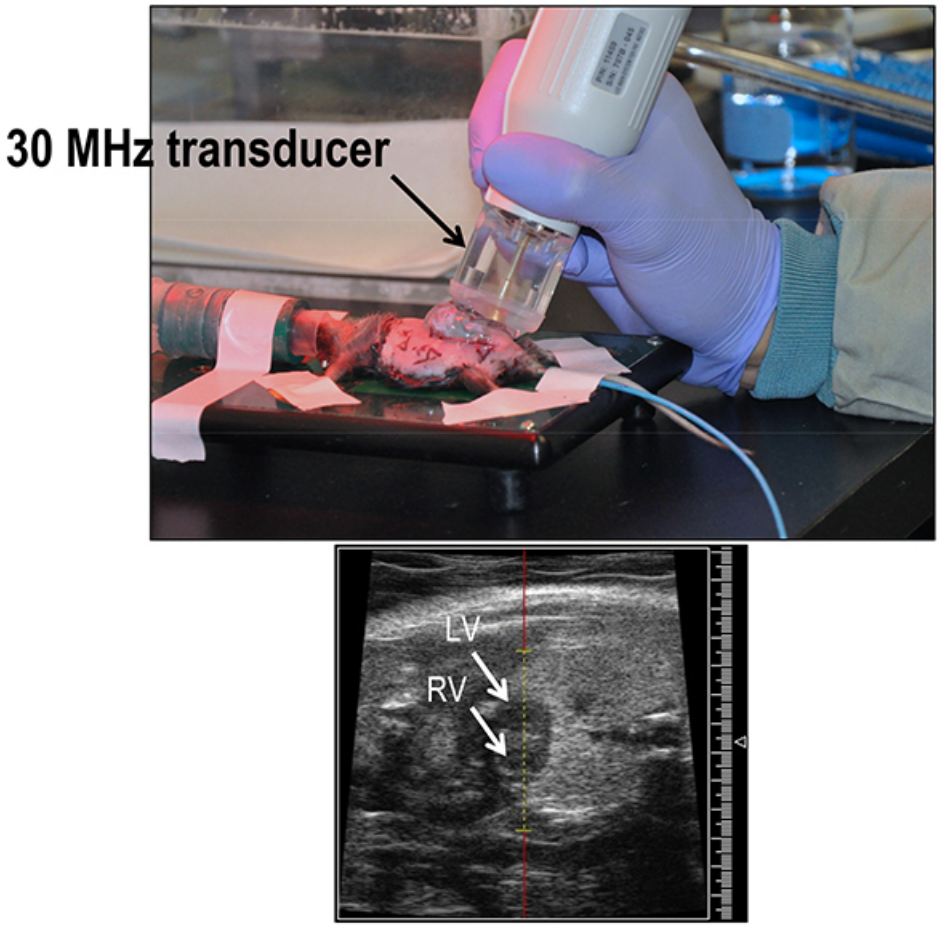

Figure 3. Representative assessment of heart rate. (**A**) A two-dimensional echocardiography image of an embryonic heart at E18.5 was obtained by positioning it within the focal zone centered on the yellow dashed line. The two ventricles are indicated by arrows. LV, left ventricle; RV, right ventricle. (**B**) Heart rate is calculated from repeated measurements between adjacent flow cycles in M-mode tracing. Click here to view larger image.

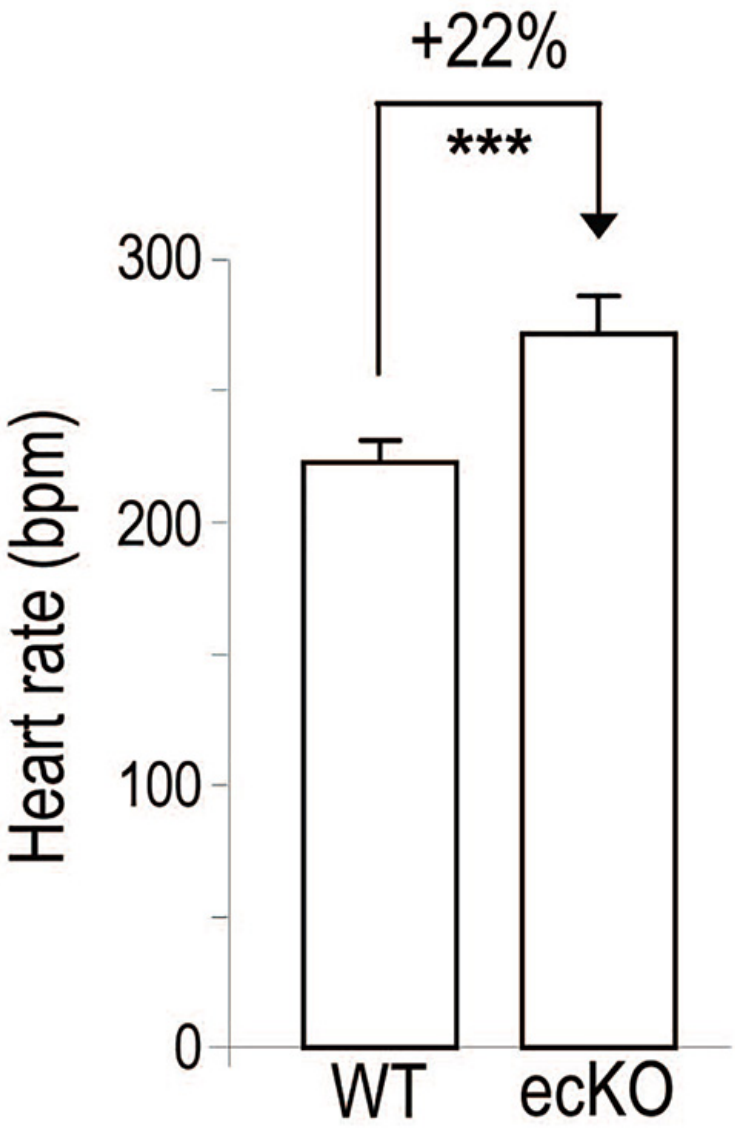

Figure 4. Representative data analysis of heart rate. *In utero* echocardiography (M-mode) of 9 WT and 7 ecKO embryo hearts at E18.5 was performed using a 30 MHz transducer. These embryos were obtained in 3 independent litters. P<0.0005 (***) was determined by a two-tailed Student's t-test and bars represent the mean + SEM. WT, transgene negative *Furin*$^{flox/+}$ or *Furin*$^{flox/flox}$ mice; ecKO, *Furin*$^{flox/flox}$ Tg(Tie2-cre)$^{+/0}$ mice. Click here to view larger image.

Discussion

M-mode echocardiography is an effective and simple method to measure *in utero* heart rates of mouse embryos. Commercially available transducers provide sufficient resolution to visualize small beating hearts. Thus, they allow a highly accurate heart rate measurement, as compared to other methods such as pulse measurement, and can substitute high resolution video microscopy. However, current tools do not allow the simultaneous analysis of all embryos, implying a tedious procedure to visualize each of the embryos. In addition, the relatively narrow field of view and the absence of depth of field (2D-image) require a manual adjustment only achieved by trained hands. Indeed, a proper training can greatly maximize the efficiency of this method.

In vivo measurements of embryonic heart rates (E18.5) offers a physiological evaluation of heart function by a noninvasive live imaging as, different from previous methods, it does not require laparotomy. Instead, marking the position of the embryos on the abdomen of the pregnant female ensures a proper tracking and preserves their physiological state. Thus, the procedure overcomes the major weakness of other imaging technologies, such as computed tomography and magnetic resonance imaging. Last but not least, this method is less costly.

A few critical steps in this method include keeping the body temperature and heart rate of the pregnant mouse stable by adjusting the position of the heating lamp and generating a proper rate of isoflurane to maintain the physiological state of embryos and acquire reliable data. Proceeding

in a consistent manner and in the shortest time possible is essential. Therefore, keeping the procedure simple and practicing prior to proceeding is highly recommended.

In conclusion, M-mode echocardiography is an effective method for assessing embryonic heart rates *in utero*. Abnormal heart rates are indicative of heart dysfunction and the method described will allow specialists, as well as nonspecialists, to screen for developmental defects leading to heart failure in mouse models.

Disclosures

No conflict of interest declared.

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