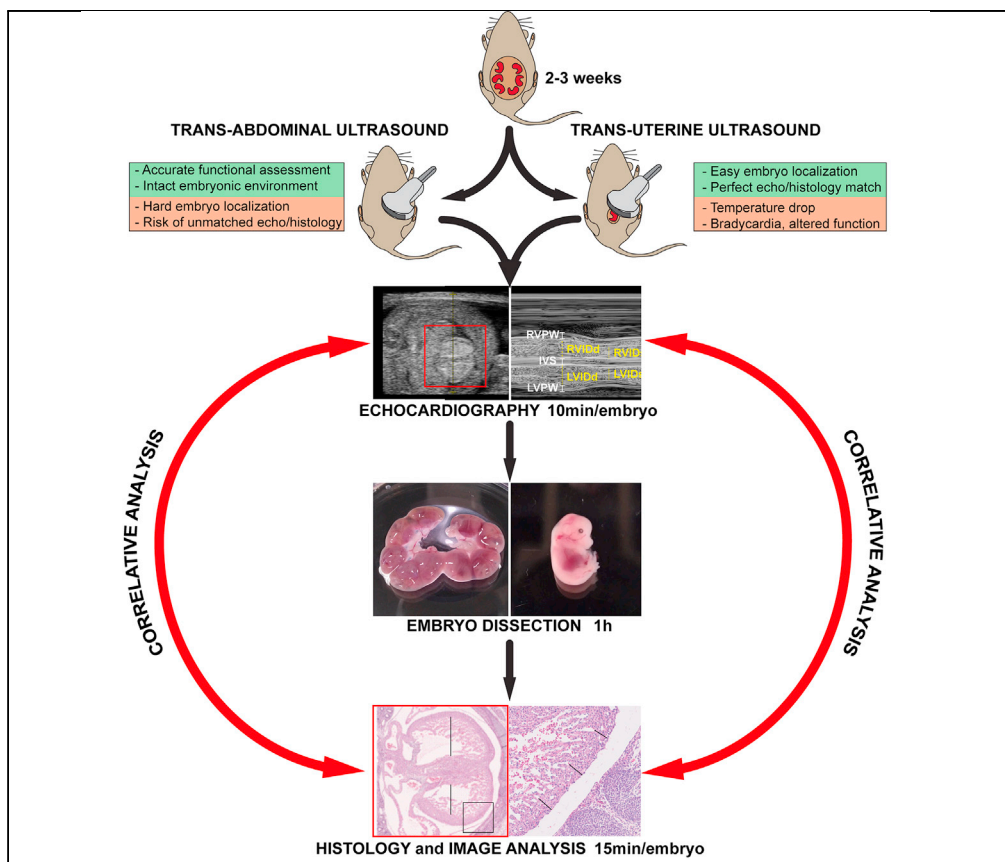


Protocol

Embryonic echocardiography for assessment of congenital and functional cardiac defects



Cardiac function and morphology by mouse fetal echocardiography can be assessed by scanning the uterus extracted from the abdominal cavity (trans-uterine ultrasound) or the womb (trans-abdominal ultrasound). Advantages of trans-abdominal ultrasound include (1) non-invasive longitudinal analysis at different stages, reducing animal use; and (2) maintenance of natural environment, diminishing perturbations on functional parameters, which are more frequent in trans-uterine conditions. Here we describe both approaches, explaining how to identify congenital cardiac defects and defining the correlation between echocardiography findings and histological analysis.

Ivan Menendez-Montes, Maria Villalba-Orero, Beatriz Escobar, Silvia Martin-Puig

ivan.menendez-montes@utsouthwestern.edu (I.M.-M.)
smpuig@iib.uam.es, silvia.martin@ufv.es (S.M.-P.)

Highlights

Detailed protocol of fetal trans-abdominal and trans-uterine ultrasound

Measurement of embryonic cardiac structural and functional parameters

Identification of congenital cardiac defects by echocardiography

Correlation of ultrasound findings with histological analysis

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Protocol

Embryonic echocardiography for assessment of congenital and functional cardiac defects

Ivan Menendez-Montes,^{1,2,7,8,*} Maria Villalba-Orero,^{1,3,7,8} Beatriz Escobar,¹ and Silvia Martin-Puig^{1,4,5,6,9,*}

¹Vascular Pathophysiology. National Center for Cardiovascular Research. Madrid, Spain

²Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, USA

³Centro de Investigación Biomédica en Red Cardiovascular (CIBERCV), Madrid, Spain

⁴Unidad Mixta de Investigación Cardiovascular, Universidad Francisco de Vitoria (UFV), Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain

⁵Instituto de Investigaciones Biomédicas Alberto Sols, (IIBM), Consejo Superior de Investigaciones Científicas & Universidad Autónoma de Madrid (CSIC-UAM), Madrid, Spain

⁶Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Madrid, Spain

⁷These authors contributed equally

⁸Technical contact

⁹Lead contact

*Correspondence: ivan.menendez-montes@utsouthwestern.edu (I.M.-M.), smpuig@iib.uam.es or silvia.martin@ufv.es (S.M.-P.)

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SUMMARY

Cardiac function and morphology by mouse fetal echocardiography can be assessed by scanning the uterus extracted from the abdominal cavity (trans-uterine ultrasound) or the womb (trans-abdominal ultrasound). Advantages of trans-abdominal ultrasound include (1) non-invasive longitudinal analysis at different stages, reducing animal use; and (2) maintenance of natural environment, diminishing perturbations on functional parameters, which are more frequent in trans-uterine conditions. Here we describe both approaches, explaining how to identify congenital cardiac defects and defining the correlation between echocardiography findings and histological analysis.

For complete details on the use and execution of this protocol, please refer to (Menendez-Montes et al., 2016) and (Menendez-Montes et al., 2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for the analysis of cardiac structure and function of embryonic (E14.5) mouse models by echocardiography. We have used this protocol specifically for cardiac characterization of *Vhl*-deficient embryos at E14.5 (Menendez-Montes et al., 2016) which presented dilated ventricular chambers, myocardial thinning and interventricular septal defects associated with cardiac dysfunction. In addition, we have also used this protocol to assess cardiac function in cardiac specific *Hif1a*-deficient mice (Menendez-Montes et al., 2021). This protocol can be applied to any mouse model at embryonic stages E14.5 and further.

Before starting, first you need to confirm the gestation of timed-pregnant females to be analyzed. In addition, you should have access to a high-resolution ultrasound machine for echocardiography (Vevo 2100, Visualsonics, Canada or similar device) with a 40–50 MHz transducer and an ultrasound probe manipulator, connected to a physiologic-controlled mouse heating platform and linked to an isoflurane-based anesthesia system (Liu et al., 2014; Golden et al., 2012; Moran et al., 2013). To maintain normal body temperature, apart from the warmed platform, it is useful to maintain the



warmed ultrasound gel and to use heat lamps. Additionally, an anesthetic gas scavenging system is also required to reduce the volatile gas from escaping into the room, which prevents operators from inhaling and reduces environmental contamination. Finally, for embryo processing at the end of the procedure, ice-chilled 4% paraformaldehyde, individual flat bottom tubes or plates and 1.5 mL small tubes for embryo genotyping should be prepared. For echocardiography analysis you will need to have access to the Vevo 2100 software from FUJIFILM VisualSonics Inc. Image J program from NIH (<https://imagej.nih.gov/ij/ge> J) will be required for histological image analysis of the embryo cardiac sections stained.

Experiments with mice and embryos followed the EU Directive 2010/63EU and Recommendation 2007/526/EC, enforced in Spanish law under Real Decreto 53/2013 and were approved by the authorized Environmental Department of Comunidad de Madrid, Spain, with reference numbers: PROEX 12/14 and PROEX 267/19.

Preparation of echocardiography station

⌚ Timing: 5 min

1. Turn on the physiologic-controlled animal heating platform and adjust the temperature to 38.6 Celsius degrees.
2. Turn on the heating lamp (120W infrared bulb).
3. Place the ultrasound gel tube on a thermostat device to keep it warmed before application.
4. Set up the Vevo ultrasound machine.
 - a. Turn on the system by pressing the switch located on the rear upper left corner.
 - b. Turn on the computer and log in with your credentials.
 - c. Connect the ultrasound probe and select the measurement package: cardiology
5. Set up the anesthesia system.
 - a. Refill the isoflurane reservoir up to the line indicated with MAX level. Make sure the cap is properly screwed back into the opening.
 - b. Check there is enough oxygen in the oxygen cylinder.
 - c. Open the oxygen cylinder manifold.

⚠ **CRITICAL:** Allow enough distance between the heat lamp and the animal to avoid overheating and risk of burns. A distance of 25–30 cm between the infrared bulb and the animal should be enough to provide heat without causing any burning damage.

Setting up dissection tools and embryo processing material

⌚ Timing: 15 min

6. Organize dissection material
 - a. Sterilize in advance surgical scissors, fine forceps and Vannas scissors with 70% ethanol for abdominal section procedure, uterus exposure and dissection of the embryos after echocardiography.
7. Prepare embryo collection tubes/plates and genotyping tubes.
 - a. Label 1.5 mL microcentrifuge tubes with the pregnant female code followed by E and numbers from 1 to 10 (later adjust to the total number of embryos according to each litter size). These tubes will be used for genotyping material collected from the dissected embryos.
 - b. Label flat-bottom tubes or a p12 well plate with the pregnant female code followed by E and numbers from 1 to 10 (later adjusted to the total number of embryos) in each well/flat-bottom tube. Add 2 mL of 4% paraformaldehyde (PFA) diluted in PBS in each well/tube and store at 4°C until embryo dissection. These wells/tubes will be used for embryo fixation prior to histological analysis.

Shaving of the abdominal area of pregnant female

⌚ Timing: 5 min

To perform trans-abdominal ultrasound in mice it is necessary to remove hair before the scanning because unshaved hair blunts the proper acquisition of echocardiographic images.

- Remove hair from the abdominal area of the pregnant female using depilatory cream and an ear swab right before performing the ultrasound or the day before.

Note: the use of an electric clipper before the depilatory cream facilitates an adequate depilation and improves echocardiography image acquisition.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|-------------------------------|---|
| Experimental models: Organisms/strains | | |
| Vhl ^{fl/fl} -Nkx2.5 ^{Cre} mouse line | Dr. Martin-Puig laboratory | https://doi.org/10.1016/j.devcel.2016.11.012 |
| Hif1a ^{fl/fl} -Nkx2.5 ^{Cre} mouse line | Dr. Martin-Puig laboratory | https://doi.org/10.1016/j.isci.2021.102124 |
| Chemicals, peptides, and recombinant proteins | | |
| Potassium chloride | Sigma-Aldrich | Ref. P9541-500G |
| Histosec pastilles | Sigma-Aldrich | Ref. 1116099025 |
| Paraformaldehyde 16% solution, EM grade | Electron Microscopy Sciences | Ref. 15710 |
| Hematoxylin Solution, Harris Modified | Sigma-Aldrich | Ref HHS3 |
| Eosin Y solution, alcoholic with Phloxine | Sigma-Aldrich | Ref. HT110316 |
| Ethanol, GPR RECTAPUR | VWR Chemicals | Ref. 20824,365 |
| Depilatory cream | Vichy/Others | N/A |
| Ultrasound transmission gel, Aquasonic 100 | Parker Laboratories | Ref. 01-08 |
| Isoflurane | IsoVet – B. Braun VetCare | IsoVet |
| Other | | |
| Microtube 1.5 mL | SARSTEDT/Other | Ref. 72.690.001 |
| Flat bottom 12 well plates | Falcon/Other | Ref. 353043 |
| Bright field microscope | N/A | N/A |
| Binocular scope with upper or bottom lamp | N/A | N/A |
| Disposable Histology Molds – Peel-A-Way, Truncated, 22mm square top, tapered to 8mm bottom | TED PELLA, INC. | Ref. 27116 |
| Embedding Cassettes – Macrosette with Covers | TED PELLA, INC. | Ref. 27158-1B |
| Vevo 2100 Imaging System (or similar device) | VisualSonics, Toronto, Canada | N/A |
| Physiologic-controlled mouse heating platform | VisualSonics, Toronto, Canada | N/A |
| Transducer with center frequency of 40–50 MHz | VisualSonics, Toronto, Canada | MS550S |
| Ultrasound probe manipulator | VisualSonics, Toronto, Canada | N/A |
| Heating lamp: Infrared bulb (120W) | Braintree Scientific, Inc. | HL-1 120V |
| Bottle warmer | Philips Avent/Other | N/A |
| Rodent anesthesia unit | JD Medical/Other | VT-RAU |
| Oxygen supply | Linde Healthcare España/Other | N/A |
| Anesthetic gas scavenging system | Barin/Other | N/A |
| Ear swab | N/A | N/A |
| Adhesive tape | 3M /Others | N/A |
| Bottle with water | N/A | N/A |
| Fine scissors, sharp, straight, 22mm | Fine Science Tools | Ref. 14060-09. |
| Extra fine Bonn scissors, straight, sharp | Fine Science Tools | Ref. 14084-08 |
| Vannas-Tübingen Spring scissors, 5mm, straight | Fine Science Tools | Ref. 15003-08. |

(Continued on next page)

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|--------------------|----------------|
| Dumont #5 fine forceps | Fine Science Tool | Ref. 11254-20. |
| Fine forceps, mirror finish SS, straight, 13cm | Fine Science Tools | Ref. 11923-13. |
| Moria perforated spoon, 15mm | Fine Science Tools | Ref. 10370-18. |

Note: $Vhl^{f/f}$ - $Nkx2.5^{Cre}$ and $HIF1^{f/f}$ - $Nkx2.5^{Cre}$ mice are in the C57/BL6 background. For these protocols we have analyzed embryos at embryonic day 14.5 (E14.5) and included males and females in the analysis.

△ **CRITICAL:** isoflurane vaporizer must be calibrated regularly (usually every 12 months) to maintain optimal flow according to each manufacturer's recommendations.

△ **CRITICAL:** PFA solutions and isoflurane are toxic by inhalation. Always handle PFA solutions inside a fume hood and use gloves and proper personal protective equipment. Isoflurane use during mouse anesthesia requires a filter and an exhaustion system attached to the echocardiography station to avoid inhalation from the operators and to reduce environmental contamination.

STEP-BY-STEP METHOD DETAILS

Mouse breeding and embryonic gestation

⌚ **Timing:** 2–3 weeks (for E14.5 pregnancies)

This part of the protocol includes all the necessary steps to breed the females, check the vaginal plug and establish timed pregnancies.

1. Start the breeding process by placing 1 or 2 fertile females in a cage with a single male of the appropriate genotype (Figure 1A). Consider that different mouse lines have different reproductive efficiencies. To maximize the breeding process outcome, we recommend adhering to the following guidelines, especially in difficult-to-breed strains:
 - a. Use females that have been pregnant before and efficiently delivered a litter.
 - b. Choose animals between 2 and 5 months old. Using too young (below 6 weeks old) or too old (above 30 weeks old) animals might reduce breeding efficiency.
 - c. Always introduce the females in the cage containing the male that should have been individualized at least 2 days in advance if coming from a stock cage.
 - d. Avoid adding more than 2 females per male at the same time.
2. Every day in the morning (the earlier the better) check the presence of a vaginal plug in the females.
 - a. Locate the female on a flat surface with the head pointing forward.
 - b. Lift the back part of the animal by the tail, exposing the vagina (Figure 1B).
 - c. Carefully, introduce a p200 sterile pipet tip 1–3 mm inside the vagina to check the presence of a vaginal plug (Figure 1C). The tip can be also used to gently expose slightly the vaginal opening to visualize the plug inside.

Note: Different mouse strains produce different types of plugs in terms of size, location and duration. The plug could fall down before the time it is checked. For that reason, we recommend exploring the females early in the morning.

- d. When the plug is observed, the female will be considered positive, the date recorded and established as embryonic day E0.5. It is recommended to separate the female into a new cage

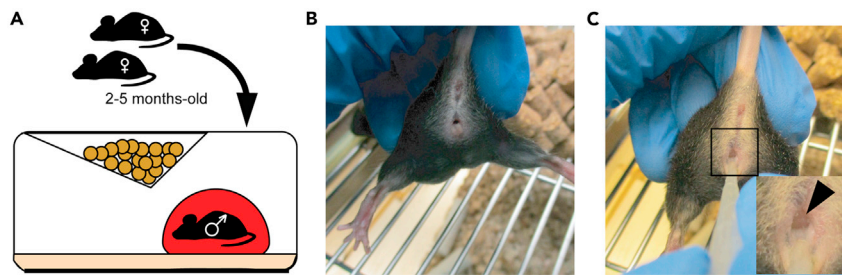


Figure 1. Mouse breeding and plug check

(A) Schematic of breeding strategy.

(B) Representative image showing normal aspect of C57B6/N female genitalia.

(C) View of C57B6/N female genitalia with a positive vaginal mating plug (black arrowhead on inset).

without the male. If no plug is observed, the female should remain in the male cage until a positive plug is observed.

△ CRITICAL: If a female is negative for more than 7–10 consecutive days, it is recommended to separate the animals and use a different female for the plug breeding. However, the original female should be monitored for pregnancy since there is a possibility of getting false negatives (i.e. in case the plug is lost before plug check). We do not recommend using false negative females for echocardiographic analysis as the exact day of pregnancy might not be accurate. Instead, these pregnant females could be used to refresh the mouse colony.

3. Since a positive plug does not mean pregnancy, the positive females should be monitored for effective pregnancy. Several methods can be used depending on the stage of pregnancy:
 - a. For early stage pregnancy, monitoring the weight of the female might be helpful. The values could vary depending on the genetic background, e.g., C57BL6/N vs CD1. Generally, for B6N backgrounds an increase in body weight of 1.5g from day E0.5 to E8.5 is usually indicative of pregnancy.
 - b. For later stage pregnancies, closer to the echocardiography day (E12.5, E14.5 and so on), observe enlargement and roundness of the female's lower belly, indicative of pregnancy. Alternatively, hold the female exposing the abdomen and feel the developing embryos. However, this last option should be performed gently and only by experienced personnel, since excessive pressure could damage the embryos and/or cause abortions.

Animal preparation and anesthesia

⌚ Timing: 5 min

This step describes the anesthesia set up for the pregnant female and spatial disposition at the echocardiography stage. It also covers how to position the female for trans-abdominal echocardiography (detailed in next section) and the necessary steps to expose the embryos for trans-uterine echocardiography.

4. Turn on the anesthetic gas scavenging system.
5. Turn on the isoflurane anesthesia system.
 - a. Make sure the anesthesia flow is directed to the induction chamber.
 - b. Turn on the oxygen flow meter and establish an oxygen flow of 0.4–0.6 mL (Figure 2A).
 - c. Set the isoflurane vaporizer to a 2.0%–2.5% for anesthesia induction (Figure 2B).
6. Place the female in the induction chamber and wait until movement stops. This step usually takes between 2 to 3 min.
7. Transfer the anesthetized female to the mice heating platform that has been prewarmed at 38.6°C and switch the anesthesia flow to the nose adapter located in the platform (Figure 2C).

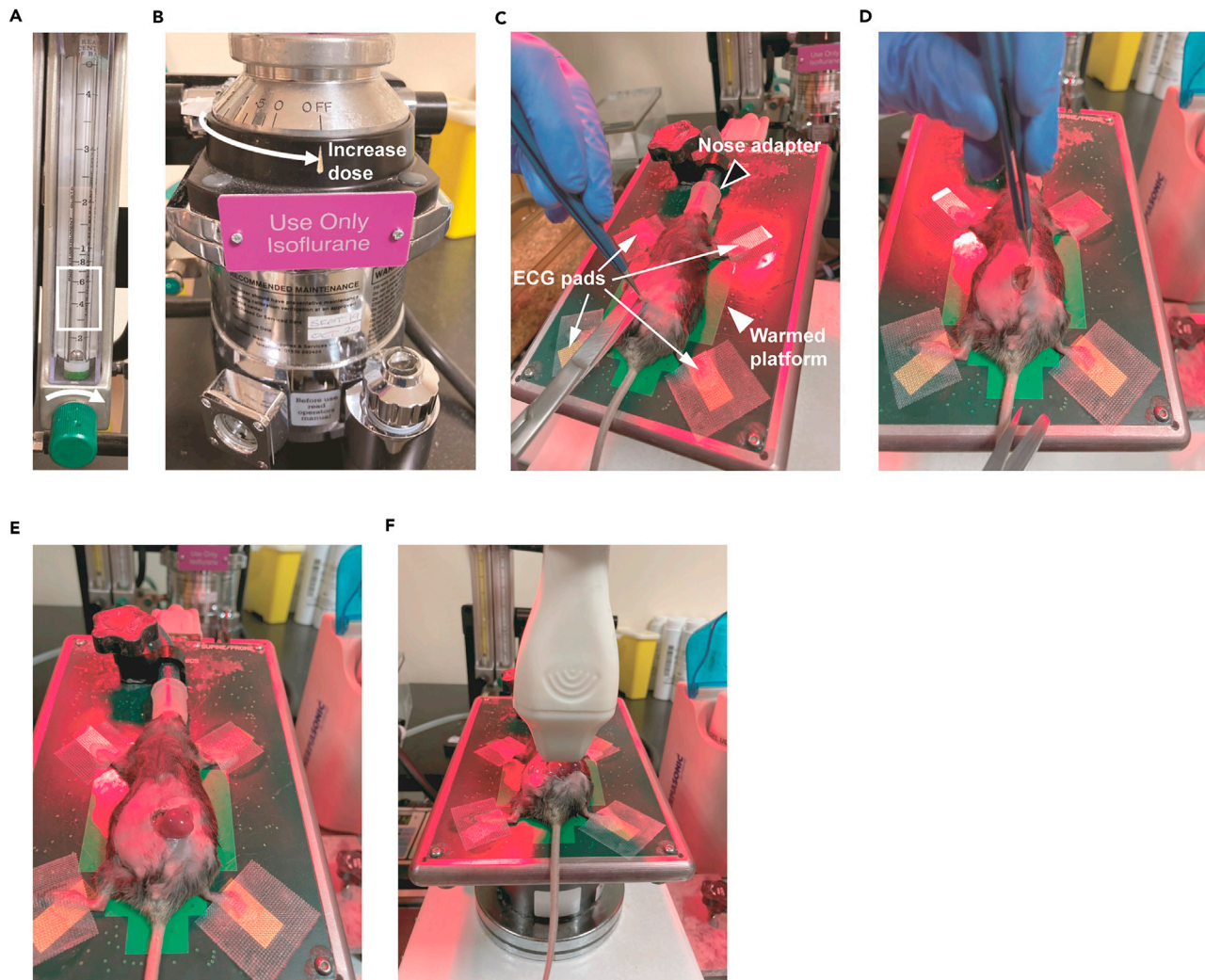


Figure 2. Fetal echocardiography by trans-uterine ultrasound

(A) Oxygen flow meter showing appropriate opening direction (white arrow) to administer oxygen to the anesthetic circuit up to around 0.4–0.6 mL (white square), the usual oxygen range used in mice.

(B) Isoflurane vaporizer in closed position (OFF) and white flow indicating head's vaporizer opening direction to dispense anesthesia.

(C) Pregnant female placed in the warmed platform (white arrowhead), showing mouse head inside the anesthetic nose adapter (black arrowhead) and the paws attached to the electrode pads (ECG pads, white lines).

(D) The skin of the caudal part of the abdomen is grasped and lifted to pull it upwards to open the abdominal cavity. A small incision is performed to localize the embryo closer to the ribs on the left lateral side (left uterine horn).

(E) Embryo outside the abdomen inside the uterus.

(F) Individual embryo inside the uterus but outside the abdomen covered with warmed gel and with the echography transducer on top ready to begin a direct trans-uterine ultrasound.

- a. Place the female in supine position and gently introduce the nose into the nose adapter.
 - b. Using tape, gently attach the rear and front paws of the animal to the electrodes pads and add a small volume of water underneath to improve the ECG signal.
 - c. Adjust the isoflurane dose (usually it varies between 1.5%–2.5%) to avoid spontaneous movements but maintaining pedal reflex (light anesthesia plane). This ensures to scan the hearts maintaining physiological conditions as much as possible.
 - d. Adjust the heating platform to maintain the mouse temperature in normothermia (36.8°C–38°C).
8. For trans-uterine echocardiography embryos should be exposed by opening the abdominal cavity of the pregnant female to expose the uterus.

9. For trans-abdominal echocardiography, which allows non-invasive analysis and prevents potential cardiac function alterations associated with temperature drop in ex vivo analysis, proceed from step 4 to next section (See [problem 2](#)).

Troubleshooting 1

Troubleshooting 2

△ CRITICAL: For this surgical approach anesthesia must be increased to avoid pedal reflex, which ensures a deep anesthesia plane. Always check full absence of the pedal reflex or the blinking reflex in the pregnant female to make sure anesthesia is complete and effective.

- Increase isoflurane dose to avoid pedal reflex (usually around 2.5%–3.0%)
- Gently sterilize the abdomen of the pregnant female with 70% ethanol.
- Using fine forceps, grasp the skin on the lower central part of the abdomen and lift it pulling upwards ([Figure 2C](#)).
- Using sterile surgical scissors positioned parallel to the plane of the animal, localize the embryo closer to the ribs on the left lateral side and make a small incision to create a hole in the skin ([Figure 2D](#)).
- Keep pulling slowly with the forceps, introduce the tip of the scissors and make a cut on the top left lateral side of the abdomen until the embryo pops up ([Figure 2E](#)).
- Immediately cover the exposed embryo with warmed gel to protect body temperature ([Figure 2F](#)) and proceed to echocardiography acquisition as detailed in next section.

Note: When using the scissors, always locate the tip facing upward to avoid damaging any abdominal structure or the embryos inside the uterus.

Note: The embryo should remain inside the uterus during the acquisition to minimize alterations on cardiac function and further drop of body temperature.

Echocardiography acquisition

⌚ Timing: 5 min per embryo

Here we describe how to perform a systematic embryo identification and imaging acquisition in order to minimize imprecisions and mistakes when associating trans-abdominal echocardiography analysis with the *postmortem* embryos collected for further histological characterization. In addition, we describe the direct ex vivo method of trans-uterine echocardiography of embryonic hearts.

10. For trans-abdominal echocardiography begin the ultrasound analyzing the embryos located at the left uterine horn scanning them from caudal to cranial. Afterwards, proceed with embryos of the right uterine horn ([Figure 3A](#)).

△ CRITICAL: Always begin the study from the embryo located closer to the female urinary bladder in each uterine horn to have a fixed reference that allows you to match a given set of acquired images with a certain embryo. The urinary bladder is easy to detect by ultrasound and is adjacent to the uterine bifurcation ([Figure 3A](#)).

- Cover the shaved abdominal region of the pregnant female with abundant warmed ultrasound gel.
- Approximate the probe to the gel and verify a clear echocardiographic view ([Figure 3B](#)).
- Search for the tail of the embryo of interest and slowly move towards the head until you find the beating heart ([Figure 3C](#)).

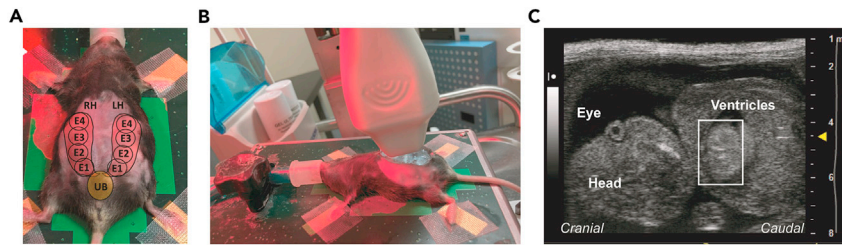


Figure 3. Fetal echocardiography by trans-abdominal ultrasound

(A) Pregnant female placed in the warmed platform with a scheme of the urinary bladder (UB) location at the point of uterine bifurcation, left and right uterine horns (LH, RH) and embryos from E1-E4 in each side.
 (B) Pregnant female abdomen covered with abundant warmed gel and the transducer positioned to visualize the embryos of the left uterine horn.
 (C) Representative echocardiography view of an embryo in cranio-caudal position, showing the head (recognized by visualization of the eye) and the heart with both ventricles (white square).

11. For trans-uterine echocardiography after performing an abdominal section, begin exposing the embryo located closer to the left ovary and more distal from the uterus bifurcation below the pregnant female left ribs. Next, proceed with the consecutive embryos until you reach the bifurcation and start over from the right ovary with embryos located in the right uterus horn.
 - a. Place the exposed embryo inside the uterus in supine position and cover the thorax with abundant warmed ultrasound gel.
 - b. Verify that no bubbles form in the gel as they might cause imaging artefacts.
 - c. Approximate the probe to the gel and verify a clear cardiac echocardiographic view.

Note: The strategy for embryo acquisition in trans-abdominal and trans-uterine analysis is different. Trans-uterine acquisition requires the exposure of the embryos and has a higher risk of body temperature drop after abdominal opening of the pregnant female. For that reason, when performing trans-uterine ultrasound, acquisition begins with the embryo located more distal of the uterine bifurcation, closer to the female ribs. In this way, the exposure of embryos out of the abdominal cavity is reduced, as the operator can gently place them back after each embryo acquisition and proceed from the ovary to the uterine bifurcation.

12. Once the heart of a given embryo is identified with the ultrasound probe in either trans-abdominal or trans-uterine procedures, continue with a common protocol for functional and structural data acquisition (Liu et al., 2017; Zhou et al., 2014).
 - a. Place the probe longitudinally from head to tail to identify the heart (Figure 4A). Angled to optimize a *long axis* view (equivalent to a longitudinal 4-chamber section of the heart). Once the heart is visualized and centered in the screen, both ventricles and the interventricular septum could be easily observed (Figure 4B).
 - b. In this view, use the bidimensional mode (2D mode) to subjectively assess cardiac function and morphology.
 - c. Apply color Doppler (CD) to identify the flow through the atrioventricular and semilunar valves in this plane (Figure 4C).
 - d. To evaluate heart rate, valvular function, and the presence of arrhythmias, utilize Pulsed Wave Doppler (PWD, Figure 4D).
 - e. In addition, CD mode is used to detect abnormal flows through wall septum defects. The Doppler window is placed in the area of interest to highlight normal (Figure 4E) or pathological (Figure 4F) flows, as in the case of ventricular septal defects (VSD).
 - f. Incline the probe to a frontal plane as needed (usually around 90 degrees) to obtain a *short axis* view (equivalent to a transversal 2-chamber section of the ventricles) for motion-mode acquisition (M-mode). Rotate the probe clockwise to improve the 2-chamber view if necessary. The line used to choose the ultrasound area for M-mode construction must divide both ventricles symmetrically (Figure 5A).

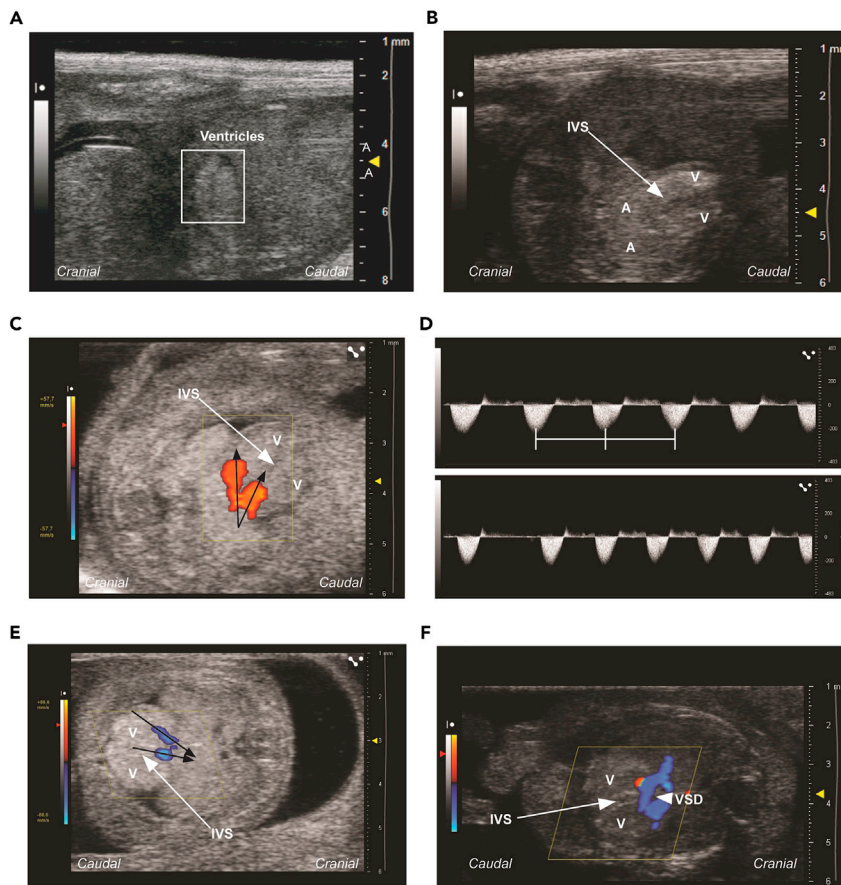


Figure 4. Basic echocardiographic analysis in a long axis view using the 2D mode

(A) Heart echocardiography view when the probe is positioned longitudinal to the embryo.

(B) Optimized long axis view of the heart visualizing the four chambers and the interventricular septum (IVS) by slight angulation of previous longitudinal position.

(C) Color Doppler echocardiography through the atrioventricular valves. Red color indicates flow towards the upper part of the screen and blue towards the lower part. Black arrows indicate the direction of the blood flow (from the atria into the ventricles).

(D) Pulse wave Doppler obtained positioning the sample in a semilunar valve and showing a regular rhythm (upper image) and an arrhythmia (lower image). In this image, the heart rate can be obtained positioning the heart rate ultrasound caliper in three consecutive waves (white lines).

(E) Color Doppler echocardiography view placing the area of interest in the IVS to confirm its integrity and lack of communication between ventricular chambers in a normal embryo. Black arrows indicate the direction of the blood flow (from the ventricles to the big vessels, (Aorta and Pulmonary artery)).

(F) Color Doppler echocardiography view placing the area of interest in the IVS to identify pathological septal defects in an abnormal embryo with ventricular communication (VSD, white arrowhead). A flow (blue area) crossing the IVS can be identified. Red color indicates flow towards the upper part of the screen and blue towards the lower part. A, atria; V, ventricle; IVS, interventricular septum; VSD, ventricular septal defect.

Echocardiography analysis

⌚ Timing: 5 min

In this step we describe how to perform a blind analysis off-line using the Vevo Workstation (Visualsonics, Toronto).

13. Perform structural analysis of both ventricles using de M-mode acquisition to calculate internal chamber dimensions at the end of the diastole (Figure 5B).

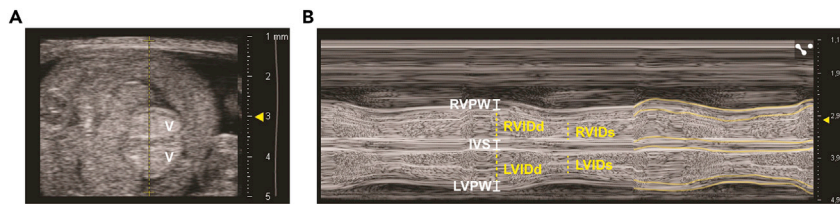


Figure 5. Basic echocardiographic parameters in a short axis view using the M-mode

(A) Short axis view of the heart visualized when the probe is positioned in a frontal plane to the embryo, and the M-mode line (yellow line) crossing the middle of the ventricles.

(B) Representative M-mode image of a short axis view showing the measures required to perform structural and functional assessment of the heart. V, ventricle; RVPW, right ventricular wall thickness; IVS, interventricular septum and LVPW, left ventricular wall thickness (in diastole); RVIDd, right ventricular internal diameter; LVIDd, left ventricular internal diameter (d, in diastole); RVIDs, right ventricular internal diameter; LVIDs, left ventricular internal diameter (s, in systole). The yellow lines shown in the right follow the endocardium and the epicardium's motion as a reference to trace the detailed measures.

- a. The internal diameter of left (LVID) and right (RVID) ventricles are measured from endocardium to endocardium, from the ventricular free wall to the septum at the end of the diastole (d) and systole (s)
- b. The left and right ventricular posterior wall thickness (LVPW, RVPW, respectively) and interventricular septum (IVS) can be measured at the end of the diastole from endocardium to epicardium.

Note: increased inner diameters could be associated with ventricular chamber dilation and generally correlates with poor cardiac contraction capacity. Increased thickness of LVPW, RVPW or IVS could be related with hypertrophic phenotypes, while thinning of these walls might reflect deficient proliferation or growth arrest and is also present when ventricles are dilated.

14. Determine cardiac function using M-mode images to obtain fractional shortening (FS), which represents the percentage of size reduction of the ventricle during each contraction or systole (s); and ejection fraction (EF), which represents the percentage of blood that is pumped in each systole in relation to the total volume of blood in the ventricle in diastole (d). The FS and EF are automatically calculated by the ultrasound software using the diastolic and systolic ventricular internal diameter measured (Wu et al., 2010).

- a. LVFS (%) is calculated as: $[(LVIDd - LVIDs) / LVIDd] \times 100$.
LVEF (%) is calculated as: $[(LV \text{ Vold} - LV \text{ Vols}) / LV \text{ Vold}] \times 100$.
Volume is calculated as: $[7 / (2.4 + LVIDd)] \times (LVIDd)^3$

Note: same calculations can be applied for the RV.

Note: contractility can be also assess subjectively identifying oscillatory peaks in the M-mode reflecting effective diastole (higher peaks) and systole (lower peaks) (Figure 5B)

Note: congenital cardiac defects associated with pathological chamber communication are subjectively assessed with the CD images in both atrial and ventricular septum as detailed in further sections (Figure 7).

Embryo dissection and histological validation

© Timing: 1 week

This part of the protocol describes how to dissect the embryos after echocardiography and its processing for histological analysis and morphological validation of echocardiographic assessment.

15. Embryo dissection:
 - a. In order to collect the embryos, the pregnant females should undergo euthanasia:
 - i. Once the last embryo has been acquired and before switching the anesthesia system off, sacrifice the female by bilateral pneumothorax.
 - ii. To this aim, perform an incision with the scissors into the second intercostal space, open the chest cavity and break the diaphragm. This procedure will make the lungs collapse.
 - iii. Wait 2–5 min before switching the anesthesia off and remove the female from the platform.
 - b. Excise the uterus in one piece. To keep the embryos matched with the echocardiography acquisition, it is recommended to keep the ovary in the right side of the uterus (last imaged embryo) and remove the ovary in the left end (first imaged embryo).
 - c. Place the uterus in a Petri dish filled with ice-cold PBS (Figure 6A).
 - d. Cut out the first decidua containing embryo #1 (Figure 6B) and transfer it to a clean Petri dish with ice-cold PBS (Figure 6C).
 - e. Using sterile fine forceps, under the binocular scope remove all the extraembryonic tissues and the yolk sac (Figure 6D).
 - f. Save the yolk sac in a 1.5 mL tube labeled with the embryo code for genotyping purposes.
 - g. For E14.5 or older embryos, it is recommended to only fix the thoracic region, to improve fixative penetration (Figure 6E).
 - i. Remove the head by cutting at the neck level.
 - ii. In the abdomen region locate a dark-brown area, the liver, and cut out the inferior part of the embryo at this level.
 - iii. In the remaining piece of the thorax, remove the abdominal organs and upper limbs and carefully open the diaphragm membrane with fine forceps without disturbing the heart or lungs.
 - h. Transfer the thorax of the embryo to a p12 well labeled with the embryo code and filled with 2 mL of 1M KCl solution. Incubate for 5 min. This treatment will relax the cardiac chambers to be in diastole (Figure 6F).
 - i. With a plastic pipette, remove the KCl solution and add 2 mL of 4% ice-cold PFA. Keep the plate on ice until all the embryos have been harvested.
 - j. Follow steps d. to i. with each of the remaining embryos matching the E number code between genotyping tubes and embryo collecting plates.
 - k. Fix the embryos for 12–16 h at 4°C with gentle shaking.
16. Genotyping:
 - a. Perform your preferred DNA extraction and genotyping protocol using the yolk sac saved in a 1.5 mL tube during each embryo dissection.
17. Histological processing:

Once fixed, cardiac morphology will be analyzed to correlate with echocardiography results.

 - a. After fixation, wash the embryos with PBS for 5 min 3 times.
 - b. After washes, place the embryos in 70% ethanol. Embryos can be stored in this solution at 4°C or immediately be processed for paraffin embedding.
 - c. Perform dehydration and paraffin embedding steps following general histological practices. In our experience, processing the embryos using an automatic processor gives better and more consistent results.
 - d. Once embryos are paraffin-embedded, orientate them in a histological mold. We recommend transversal sections at this gestational stage. To this aim, place the thorax of the embryo with the caudal part (closer to the liver) laying on the bottom of the mold filled with paraffin.
18. Sectioning and staining:
 - a. Obtain paraffin sections of 5 microns thickness using a microtome.
 - b. Store sections at 4°C to perform immunostaining or at 20°C–25°C for routine histological staining (H&E, Masson Trichrome, etc). For *in situ* hybridization, 10 microns sections should be stored at –20°C.
19. Morphological analysis by microscopy:

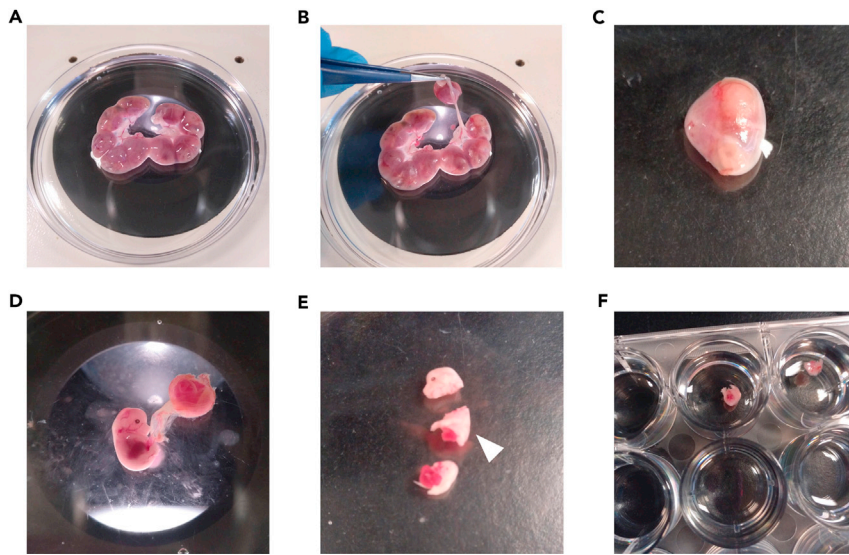


Figure 6. Midterm embryo collection, dissection and preparation of thoracic region for paraffin inclusion

(A) View of the whole uterus containing embryos at E14.5 in cold-PBS over a Petri dish.
 (B) Excision of the first decidua located in the more distal position relative to the uterus bifurcation on the left horn of the uterus.
 (C) Isolated decidua at E14.5, containing the embryo and the placenta.
 (D) View of an E14.5 embryo, still connected to the placenta through the umbilical cord.
 (E) Fragmented E14.5 embryo to isolate the trunk, allowing a better fixation of the thoracic region (white arrowhead).
 (F) Trunks containing the heart are incubated in P12 wells filled with ice-cold-4% PFA for fixation.

- a. Hematoxylin and eosin (H&E) stained sections can be analyzed using a bright field microscope. In our mouse models, we correlated the observations in the echocardiography acquisition with the H&E analysis, including the interventricular septum defects and the ventricular wall thickness reduction in VHL mutants compared to controls, as described in the next section.

Troubleshooting 3

Quantification of histological parameters

⌚ Timing: 1–2 h

This last part of the protocol describes how to quantify morphological parameters using ImageJ software and H&E stained embryo sections.

20. Acquire a full transversal section of the embryonic heart after H&E staining. Use an anatomical position where the ventricles are open and the atrioventricular valves are present (4-chamber view, [Figure 7A](#)).

Note: Tile scan or montage might be necessary to cover the full section at higher magnification (20× or 40×).

21. Load the image file into ImageJ.
22. Go to Analyze>Set scale and introduce the equivalence between pixels and microns. This will set up the scale of the image and will allow to obtain further measurements in microns.

Troubleshooting 4

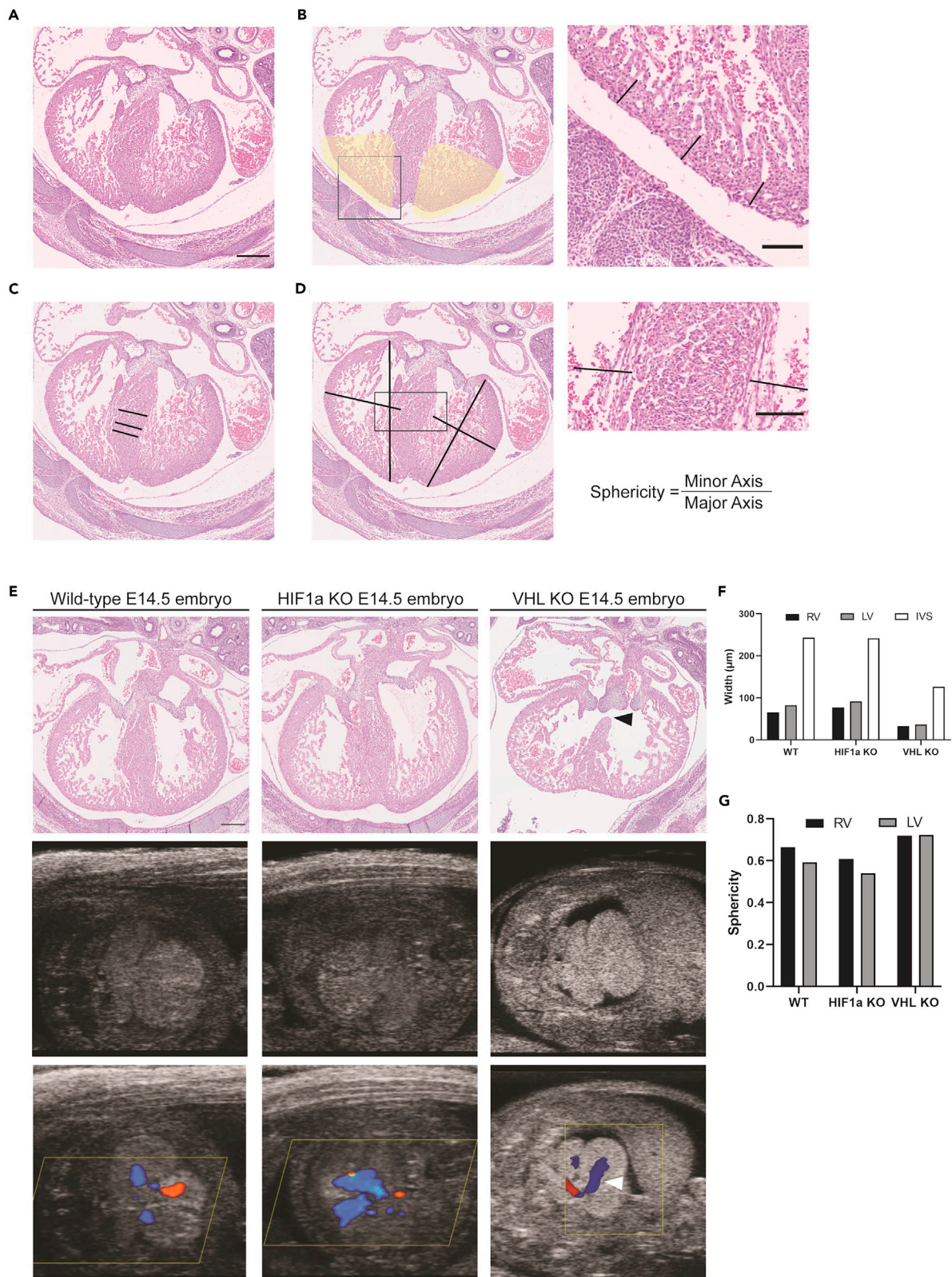


Figure 7. Histological analysis of midterm hearts: ventricular dimensions and correlation with echocardiography findings

(A) Representative H&E staining of a 4-chamber cardiac section of a E14.5 embryo to perform morphological measurements. Scale bar represents 300 μm (100 μm on insets).
 (B) Section shown in A with the area to determine compact myocardium thickness highlighted in yellow. Inset shows a detail of the three length measures.
 (C) Section shown in A with measure lines to calculate IVS thickness length at the middle point of the septum.
 (D) Section shown in A with measure lines of minor and major chamber axis, from the epicardium to the septum endocardium at the midplane of the chamber and from the valvular region to the apex on each ventricle, respectively. Inset indicates a detail on how measurements should be taken up to the septum and not to the trabeculations.
 (E) Representative H&E staining of control (left), HIF1 KO (middle) and VHL KO (right) E14.5 cardiac sections in a 4-chamber view. Scale bar represents 200 μm . Note that 2D echocardiography (middle) and Doppler-color images (bottom) are comparable with the phenotype observed by classical histology analysis (top panels), revealing a VSD in the VHL KO (arrowhead in right top and bottom panels).
 (F and G) Quantification of right and left ventricular compact myocardium and IVS thickness (F) and relative right and left ventricle sphericity (G) of a representative control (WT), HIF1a KO and VHL KO E14.5 embryos. RV: right ventricle, LV: left ventricle, IVS: interventricular septum.

23. Using the straight-line tool, draw a line from the epicardium to the base of the trabeculae, covering the whole thickness of the compact myocardium (Figure 7B).
 - a. Perform the measures in the bottom outer quarter of the ventricles within the highlighted area in the figure.
 - b. Make 3 measurements per ventricle on the lateral wall as indicated in the inset (Figure 7B). The average of these 3 length measures represents the relative compact wall thickness in microns.
24. Using the same tool, measure the relative width of the interventricular septum in its central area from one endocardial edge to the other side of the septum (Figure 7C).
25. Average the 3 length measures of each area. This is the mean value per animal. Perform biological replicates as needed.
26. For the quantification of chamber sphericity, indicative of dilatation (Figure 7D):
 - a. Measure the *major axis* of the chamber from the intersection of the valve with the ventricular wall on the basal region to the most apical region of each chamber, at the epicardial level.
 - b. Measure the *minor axis* of the chamber from the epicardial level to the septum endocardium at the midplane of the chamber.
27. The sphericity is calculated as: *minor axis length*/*major axis length*.

Note: Values closer to 1 indicate more spherical shape (chamber dilatation). This parameter should correlate with ventricular inner diameter and volume determined by echocardiography.

EXPECTED OUTCOMES

A complete fetal echocardiography analysis of heart structure and function in C57/BL6 E14.5 embryos may successfully provide the following parameters from the M-mode acquisition, with the indicated standard values (Yu et al., 2008).

RVPWd: 0.3mm

IVSd: 0.3mm

LVPWd: 0.3mm

RVIDd: 0.7mm and RVIDs: 0.4mm

LVIDd: 0.8mm and LVIDs: 0.4mm

FS: 44%

EF: 80%

However, cardiac dimensions and functional parameters are significantly variable between different mouse strains, sexes and the developmental stage of analysis. For that reason, it is essential to always acquire control individuals in parallel to the cases, ideally littermates for embryonic studies as described here, in order to normalize each echocardiographic parameter of cases relative to the control group.

In addition, a subjective assessment of the ventricular contractility is recommended to validate the FS (%) and EF (%) obtained in the M-mode analysis. Low ventricular contractile motion should correlate with reduced FS (%) and EF (%) and vice versa. Moreover, a complete evaluation of the heart with the CD to identify flow abnormalities through the cardiac valves and through pathological communications, as VSD, and at least one PWD acquisition, placed in a semilunar or atrioventricular valve to obtain the embryo heart rate and to evaluate the presence of cardiac arrhythmias should be recorded.

To identify embryo alterations, measurements obtained should be compared with the control littermates and other control embryos of similar gestational stage. Interpretation of the results obtained may require an expert opinion. The following list shows a general interpretation of common abnormal measurements.

Increased PWd indicates cardiac hypertrophy.

Decreased Wtd indicates a smaller cardiac size if it is accompanied by a decrease in IDd; and indicates cardiac dilatation if it is accompanied by an increase in IDd.

Increase in IDd indicates cardiac dilatation.

Increased FS or EF indicates higher contractility.

Decreased FS or EF indicates reduced contractility (systolic dysfunction).

Decreased heart rate indicates bradycardia.

Increased heart rate indicates tachycardia.

The presence of irregular waves indicates the presence of a cardiac arrhythmia (using PWD).

The presence of flow through the interventricular septum indicates VSD (using CD).

The described echocardiography protocol has been validated by morphological analysis using histological approaches (Figure 7). Our protocol recapitulates the phenotype observed in the histological analysis in the cardiac-specific VHL KO (myocardial thinning and IVS defect) (Figure 7E). The combined histological analysis also allows the quantification of compact myocardium thickness (Figure 7F) and ventricular sphericity (Figure 7G). Moreover, morphological parameters determined by echocardiography correlate with those measured on histological sections (Figure 7H), such as decreased compact myocardium thickness (associated with proliferative defects, among others) and increased sphericity (associated with dilatation of the ventricular chambers).

QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical analysis used for determining differences in the echocardiography assessment does not require any specific statistical approach. Statistical test choice (t-test, ANOVA, etc) will be

determined by the specific experimental design (usually we use t-test to compare 2 genotypes at a given developmental stage). Sample size must be determined according to the *confident level* (usually we applied a 95% which corresponds to an alpha risk of 0.05), the *statistical power* (usually we applied an 85% which corresponds to a beta risk of 0.15), the *standard deviation* of main parameter which is evaluated and the *minimum expected difference* between groups. Usually, to detect relevant differences between embryo conditions a sample size of $n = 8$ may be required as quantitative echocardiography data at this stage commonly shows low dispersion.

LIMITATIONS

Regarding trans-abdominal ultrasound, the main limitation of this protocol is the risk of unmatching the echocardiography data of a given embryo with the histological analysis if embryo dissection is not performed using reference tips as embryonic position relative to ovary, uterus bifurcation or bladder location.

As discussed in the text, the main limitation of trans-uterine ultrasound is associated with inaccurate cardiac function assessment due to temperature drop after sectioning the pregnant female abdomen that usually causes bradycardia and contractility abnormalities.

TROUBLESHOOTING

Problem 1

The progressive drop in temperature associated with abdominal cavity opening might result in bradycardia and the development of cardiac arrhythmias which may be more patent in the last embryos analyzed.

Potential solution

To minimize this problem, we recommend performing trans-abdominal echocardiography whenever possible.

Problem 2

When performing trans-abdominal echocardiography, the major challenge is to ensure all embryos has been scanned and the precise location (E1, E2, etc) to correlate the ultrasound phenotype with successive ultrasounds of the same embryos or with the histological assessment and genotype.

Potential solution

To ensure proper correlation between analyzed embryos by trans-abdominal ultrasound and histology/genotyping during the dissection of the embryos after echocardiography acquisition, we recommend to count the total number of embryos on each uterus horn from the uterus bifurcation to the ovary before taking it out of the female. In addition, we suggest to keep the ovary in the right side of the uterus and remove the ovary in the left end. In this way you will have a reference between echocardiography acquisition and anatomical location.

Problem 3

Sometimes, when dissecting out and saving the yolk sac, maternal contaminant tissue can be taken together, especially small fragments of uterine wall or placenta. This can lead to errors in genotyping.

Potential solution

To minimize this problem, we recommend using another part of the embryo for genotyping. For example, a piece of the embryo tail or limbs can be used once all the extra-embryonic and maternal structures have been removed.

Problem 4

Depending on how the image has been acquired, the information about the scale (microns/pixel) is not included in the metadata and does not show up when opening the image on ImageJ.

Potential solution

If this happens, the information can be found in the microscope software once the camera and the objective are selected. However, it is always recommended to save the images in the raw format from the microscope, that usually can be opened on ImageJ. A second option, when working with uncalibrated images, is to use scale bar. Since the real length of the scale bar is known, it can be measured with the “line” tool on ImageJ to obtain its length in pixels. Then, both values are entered in the “Set scale” window.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Silvia Martín-Puig (smpuig@iib.uam.es).

Materials availability

This protocol did not include any newly generated tool, software or material. Mouse strains analyzed and previously reported in ([Menendez-Montes et al., 2016, 2021](#)) might be available under special request to the Lead Contact, Silvia Martín-Puig (smpuig@iib.uam.es).

Data and code availability

This study did not generate novel unique data or code resources.

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AUTHOR CONTRIBUTIONS

I.M.-M., M.V.-O. and B.E. performed experiments and data analysis. I.M.-M. and S.M.-P. designed the protocol and I.M.-M. and M.V.-O. prepared the figures. I.M.-M., M.V.-O. and S.M.-P. wrote the manuscript. S.M.-P. designed and supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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