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Corresponding Author:	Alejandro Giraldo University of Reading Reading, Berkshire UNITED KINGDOM
Corresponding Author's Institution:	University of Reading
Corresponding Author E-Mail:	a.giraldoramirez@reading.ac.uk
Order of Authors:	Alejandro Giraldo
	Jesús Talavera López
	Gavin Brooks
	María Josefa Fernández-del-Palacio
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TITLE:

Transthoracic Echocardiographic Examination in the Rabbit Model

AUTHORS:

Alejandro Giraldo[1]*, Jesús Talavera López[2]*, Gavin Brooks[1], María Josefa Fernández-del-Palacio[2]

[1]Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, United Kingdom

[2]Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, Murcia, Spain

* These authors contributed equally

Corresponding Authors:

Alejandro Giraldo (a.giraldoramirez@reading.ac.uk)

Jesús Talavera López (talavera@um.es)

Email Addresses of Co-Authors:

Gavin Brooks (g.brooks@reading.ac.uk)

María Josefa Fernández-del-Palacio (mjfp@um.es)

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SHORT ABSTRACT:

Here we describe, step by step, a detailed protocol for performing echocardiography in the rabbit model. We show how to correctly obtain the different echocardiographic views and imaging planes, as well as the different imaging modes available in a clinical echocardiography system routinely used in human and veterinary patients.

LONG ABSTRACT:

Large animal models such as the rabbit are valuable for translational preclinical research. Rabbits have a similar cardiac electrophysiology compared to that of humans and that of other large animal models such as dogs and pigs. However, the rabbit model has the additional advantage of lower maintenance costs compared to other large animal models. The longitudinal evaluation of cardiac function using echocardiography, when appropriately implemented, is a useful methodology for preclinical assessment of novel therapies for heart failure with reduced ejection fraction (e.g. cardiac regeneration). The correct use of this non-invasive tool requires the implementation of a standardized examination protocol following international guidelines. Here we describe, step by step, a detailed protocol supervised by veterinary cardiologists for performing echocardiography in the rabbit model, and demonstrate how to correctly obtain the different echocardiographic views and imaging planes, as well as the different imaging modes available in a clinical echocardiography system routinely used in human and veterinary patients.

INTRODUCTION:

Longitudinal evaluation of cardiac function in large animal models is a robust research methodology commonly used for the assessment of the effects of novel therapies for treating ischemic and non-ischemic cardiomyopathy. Amongst the several cardiovascular imaging techniques available for preclinical research, echocardiography has been used extensively because of its non-invasive and portable characteristics. In experienced hands, echocardiography is also a very reproducible imaging technique to study cardiac anatomy as well as systolic and diastolic function of the heart.

Large preclinical animal models such as pigs, dogs and rabbits, are paramount for preclinical translational research[1-3]. Indeed, the potential benefit of novel therapies such as cardiac regenerative medicine in the setting of cardiomyopathy requires extensive hypothesis testing in large preclinical models before they can be considered for human use[2,4]. Compared to other large preclinical models, the rabbit model offers some advantages, including its low maintenance cost, which is comparable to that of mice and rats. However, in contrast to mice and rats, the Ca^{+2} transport system and cardiac electrophysiology are similar in rabbits as those of humans, and those of other large animal models such as dogs and pigs, thus increasing the translational potential of the rabbit model[1,5]. Therefore, the rabbit, as a large experimental preclinical model, has an exceptional balance of cost and reproducibility for preclinical translational research.

The rabbit has the additional benefit of its amenability for echocardiographic imaging using clinical ultrasound units routinely used in human and veterinary patients, thus taking advantage of the superiority of harmonic imaging and state-of-the-art technology. For this, sector transducers (also known as phase array) of relatively high frequency (up to 12 MHz), such as those used in neonatal/pediatric cardiology, are preferred. Echocardiographic examination in the rabbit preclinical model allows the complete evaluation of systolic and diastolic function using multiple views and different modes available in modern echocardiographic units (e.g. continuous wave Doppler (CWD), pulsed-wave Doppler (PWD), and Tissue Doppler imaging (TDI)).

Echocardiography is an operator-dependent technique and therefore requires extensive training and core knowledge of the technique in accord with international guidelines. Part of this training can be facilitated with the visualization of videos explaining in detail how different echocardiographic views can be obtained. The achievement of high competency in echocardiographic imaging, as well as development of a standardized protocol and correct technique, are essential to minimize the influence of the operator and to generate reliable quantitative data, as required in rigorous scientific research.

Some considerations are necessary regarding the system and laboratory setup used for echocardiography in rabbits and other large animal models. For a standard transthoracic echocardiographic evaluation of cardiac function, the ultrasound system must include the following modalities: bi-dimensional mode (B-mode or 2D), motion mode (M-mode), color Doppler, as well as CWD, PWD and TDI. Moreover, the machine should have full cardiac analysis and measurement software installed, as well as sufficient internal hard drive space to store

enough high quality digital still images and video loops for offline analysis. Some systems use linear array transducers; however, for the best imaging of the heart, phased array sector transducers with a small scan head diameter are preferred, because these allow an easier passage of the ultrasound waves through the narrow intercostal spaces. For rabbits, we use relatively high frequency transducers (up to 12 MHz). The position of the animal for imaging is of utmost importance to acquire good quality images. Thus, both right and left lateral recumbent positions are recommended to obtain all standard imaging planes during an echocardiographic examination. For this, a table with a notch that coincides with the cardiac area of the chest is advisable (**Figure 1A**). This notched table facilitates the access with the transducer to the area of the chest that will be scanned, and therefore allows free mobility of the hand of the operator whist maintaining the best scanning position of the animal. Positioning the animal in a lateral recumbent position results in a fall of the heart towards the transducer and elevation of the lungs, as well as widening the access window of the ultrasound beam through the intercostal spaces, thus improving overall imaging quality (**Figure 1A**). The echocardiographic examination should be performed in a blinded fashion and following the guidelines of the Echocardiography Committee of the American College of Veterinary Internal Medicine and the American Society of Echocardiography/European Association for Cardiovascular Imaging[6-8].

Part of our scientific team is associated with the Cardiology Service of a Veterinary Teaching Hospital that attends daily to veterinary patients (e.g. dogs and cats), for which it has the relevant training and accreditation in veterinary cardiology and echocardiography, and its different imaging modalities, as well as extensive experience in imaging different sizes of animal patients and thoracic conformations with this technique. In addition, we commonly use echocardiography for longitudinal evaluation of cardiac function in a rabbit model of cardiomyopathy induced by anthracyclines[9]. Here, we describe a step by step echocardiography protocol for evaluation of cardiac function using a clinical ultrasound unit in a large preclinical model such as the rabbit. This protocol is adapted for current international guidelines[8], and includes practical recommendations based on our own experiences in clinical and experimental settings.

PROTOCOL:

The experiments described herein were approved by the Ethical Research Committee of the University of Murcia, Spain, and were performed in accordance with Directive 2010/63/EU of the European Commission. The steps described were performed under standard operating protocols that were part of the plan of work and have not been performed solely for the purpose of filming the accompanying video to this paper.

1. Preparation of the rabbit

1.1. Before proceeding, start by injecting a combination of ketamine (10 mg/kg) homogenized in the same syringe with medetomidine (200 µg/kg) to anaesthetize the animal, which will reduce the stress of the procedure for the rabbit.

NOTE: The use of anesthesia also reduces the heart rate in a predictable manner, thus reducing inter-individual variability, and has the added benefit of improving overall imaging quality. As shown in the video, cover the head with a surgical blanket to help keep the animal calm during the injection of anesthesia.

1.1.1. Verify that the animal is completely anesthetized within 10–20 min, by confirming the presence of muscle flaccidity, absence of palpebral reflex, mandibular movements and sniffing. The presence of the latter two signs (mandibular movements and sniffing), are in turn the earliest signs of reduced anesthetic depth. Even though it is rarely needed, re-dosing should be considered (e.g. half the initial anesthetic dose combination), if a long delay is anticipated in order to complete the procedure.

NOTE: Whilst the animal will quickly fall asleep within the first ~5 minutes following the injection, it is recommended to allow a deeper plane of anesthesia before attempting to manipulate the animal. This delay will avoid distressing the rabbit, which will otherwise likely produce tachycardia and adversely affect the imaging accuracy and reproducibility of certain parameters during the echocardiographic examination (e.g. mitral valve inflow analyses).

1.1.2. Once the animal is anaesthetized, use a hair clipper to remove the hair from the skin of the thorax. Start below the neck line and continue to the level of both right and left hypochondriac regions, as well as the sub-xiphoid region in the middle line (**Figure 1B**).

1.1.3. Shave 1–3 cm^2 of the internal face of the right forelimb, as well as the mediotibial regions of both right and left hindlimbs (**Figure 1B**).

1.2. After placing the rabbit on a thermal blanket or heating pad to avoid hypothermia during the procedure, apply a suitable conducting gel to the electrodes and position these in the shaved regions of the limbs. Fix the electrodes with surgical tape.

1.3. Verify that a correct ECG signal is displayed on the screen of the system; usually a simultaneous 1-lead electrocardiographic tracing is enough to synchronously monitor the heart rhythm during the whole echocardiographic study (**Figure 1A** and **Figure 1C**).

NOTE: In addition to heart rate, monitor respiratory rate as well as temperature. Respiratory rate can be monitored visually or through the incidence of thoracic movements in the echocardiographic image, whilst temperature should be monitored via rectal probe. These parameters should be monitored at the beginning, then every 10 min and at the end of the procedure. Rabbits do not tend to vomit during anesthesia[10,11]; therefore, fasting of the rabbits is not routinely recommended before an echocardiographic examination.

[Place **Figure 1** here]

2. Parasternal long axis (sagittal) view of the heart

2.1. To obtain a parsternal long axis (PSLAX) view of the heart, place the rabbit in the right lateral recumbent position, with the forelimbs outstretched away from the thorax, with surgical tape (**Figure 1A** and **Figure 1C**).

2.1.1. To achieve the best imaging quality possible, it is important to keep the skin of the thoracic region as flat as possible to increase the penetration and improve overall imaging quality whilst imaging the animal. For this, hold the forelimbs away from the thorax with one hand, whilst using the free hand to identify any skin folds and pockets, flatten these from top to bottom, and move any skin folding away from the chest towards the lateral side and back of the rabbit. This is particularly important for older and larger rabbits whose excessive skin and subcutaneous fat tissue could reduce image quality.

NOTE: The cardiac area of the chest should be positioned over the cutout section in the table. However, keep in mind that, in this position, the abdomen has a natural tendency to move towards the notch, and creates a positive pressure that displaces the heart cranially, which then interferes with good echocardiographic imaging. To prevent this, it is important that the abdomen rests completely on the table and, to achieve this, it is useful to gently move the abdominal organs towards the caudal region of the animal through gentle massaging (**Figure 1A** and **Figure 1C**).

2.2. For echocardiographic imaging, hold the transducer with the right hand, whilst using the left hand to operate the controls of the echocardiography system as shown in **Figure 1D**.

2.2.1. To maintain good skin contact, apply undiluted ethanol to the skin and then enough ultrasound transmission gel to the head of the transducer.

2.3. Next, position the transducer closely to the skin of the right hemithorax, at the level of the second to third intercostal space and about 1–3 cm away from the right parasternal line, with the transducer orientation mark pointing to the right shoulder of the animal and at an angle of approximately 30° relative to the midline (**Figure 2A**). This should produce an image of the right PSLAX of the heart (see Representative Results).

2.4. Once the 2D cardiac images are displayed on the screen, the next step is to adjust the ultrasound unit controls to obtain optimal images. The main ones are:

2.4.1. Depth and zoom controls: Use these controls to optimize the area of interest. The depth of the image must be adequate so that the cardiac structures can be seen on each image. Use the zoom tool for better assessment of structures of interest, e.g., integrity of valves and leaflets.

2.4.2. Total gain and time-gain compensation (i.e., gain settings at different depths in real time): Control gray scales and gains manually to minimize background noise and to maximize the delineation of cardiac structures. These parameters are especially important in rabbits because of the poor echogenicity of the ventricular myocardium.

2.4.3. Dynamic range or compression: Use this control to adjust the number of shades of gray that are displayed by the image. Set the dynamic range so the blood pool is dark and the tissue is bright. This will result in better endocardial border definition, which is important to obtain left ventricular volumes.

2.4.4. Sector width: Begin the examination with a wide sector (90°) and after an overview of the heart, reduce the sector width if specific areas need to be better imaged. Decreasing the sector size improves the temporal resolution by increasing the frame rate. This is especially important when 2D echocardiography is used to guide Doppler examination.

2.5. To maintain the position of the transducer whilst imaging the rabbit, and to reduce the fatigue of the operator, use the index finger to anchor the hand to the table or the chest of the animal, whilst the other fingers hold the transducer (**Figure 2A**).

2.6. Obtain two main imaging planes of the heart in the right PSLAX view.

2.6.1. Find an imaging plane which sections the heart longitudinally and where all four chambers of the heart (two atria and two ventricles) can be identified; also, when a wide field of view is used, the apex of the heart should also come into view on the left side of the image (see Representative Results section).

2.6.2. Perform subtle movements of the transducer, such as sweeping, rocking and rotation, relative to the intercostal space as well as the craniocaudal and dorsoventral angle of the ultrasound beam to obtain the other imaging plane of the parasternal long axis view (**Figure 2A,B**). In the other imaging plane, the left ventricular outflow track (LVOT) and the aorta can be identified (see Representative Results).

2.7. Image orientation: Note that the base of the heart will be on the right side of the sector image.

2.8. After obtaining the appropriate imaging planes, use B-mode to evaluate overall function of the heart, and use color Doppler to assess blood flow across all valves as well as the integrity of the interventricular septum (IVS).

NOTE: Always save images of the different views and planes for offline analysis.

[Place **Figure 2** here]

3. Parasternal short axis view of the heart

3.1. With the transducer at the same location in the chest while displaying a well-aligned PSLAX, perform a counter clockwise rotation of the transducer of approximately 90° (**Figure 3A**) to obtain a right parasternal short axis (PSSAX) view. This time, the transducer orientation mark should be pointing towards the left shoulder of the rabbit.

NOTE: To help maintain the transducer in the same location of the chest whilst rotating the transducer, use the left hand to perform the rotation from the cord of the transducer as shown in **Figure 3B**.

3.2. In the parasternal short axis view, obtain three imaging planes by sweeping the transducer along the axis of the heart: the mid-ventricular, the mitral valve, and the high base with the pulmonary artery (PA) and the aortic valve (AoV) in view.

3.2.1. In the mid-ventricular imaging plane, which sections the heart at the papillary muscles and chordae tendineae level (Figures 3C), visualize the right ventricle (RV) at the top and the left ventricle (LV) at the bottom of the image (see Representative Results).

3.2.2. Use B-mode to evaluate radial and circumferential contraction and relaxation of the LV, and check for regional wall motion abnormalities.

3.2.3. Use M-mode and with the help of the track ball move the cursor in real time over the 2D image, and then place the cursor in the middle of the LV, between both papillary muscles, perpendicular to the IVS and left ventricular free wall (FW) (**Figure 3C**). Once the M-mode images are displayed on screen, store images for offline analysis. In rabbits with high heart rates, use higher sweep speeds to better separate cardiac events during the cardiac cycle (e.g., 150 mm/sec).

3.2.4. By sweeping the transducer towards the cephalic region (**Figure 3D**), obtain a mitral valve (MV) plane. Use B-mode and M-mode to evaluate the integrity and motility of the MV leaflets. Place the cursor along the middle of the LV, perpendicular to the IVS (**Figure 3E**), to obtain detailed information regarding excursion of the MV in relation to the IVS.

3.2.5. Sweep the transducer further cranially to result in an imaging plane at the level of the high base (also known as AoV plane; **Figure 3F–H**), where the AoV and its leaflets, the right ventricular outflow track (RVOT), the PA, and the right and left atria (LA) can be identified (see Representative Results).

3.2.6. Image orientation: Note that the PA will be on the right side of the sector image.

3.2.7. To completely visualize the PA and its bifurcation, use a greater angulation and, sometimes, a cranial displacement of the transducer (an intercostal space).

3.2.8. Use B-mode for evaluation of the size and shape of these structures (e.g., left atrial size is increased in congestive heart failure), and use color Doppler and PWD to record the velocity of blood flow (outflow) at the PV level, by placing the sample volume just below the opening of the PV leaflets (**Figure 3G**). Finally, use M-mode and place the cursor along the AoV and LA (**Figure 3H**).

3.3. Use the following main controls and adjustments to obtain adequate color flow Doppler images:

3.3.1. With the color sector positioned in the area of interest, reduce the angle between the sector and the blood flow direction as much as possible.

3.3.2. Color sector width: Adjust this to the valve area, in order to increase the frame rate and improve the color flow information.

3.3.3. Baseline and pulse repetition frequency (PRF): Adjust the baseline on the color bar and the PRF, to allow higher velocities to be displayed. A number at the top and bottom of the color bar represents the maximum detectable velocity before color aliasing occurs.

NOTE: Aliasing is more frequent in color flow processing than spectral pulsed Doppler, because a portion of the pulses is assigned to obtain cross sectional images in detriment to the color flow Doppler information.

3.3.4. Color gain: First, increase this to the point that it just begins to create background noise, and then decrease to a level that optimizes color flow imaging.

3.4. Use the following main controls to obtain adequate spectral Doppler images:

3.4.1. Cursor position: Make this parallel to blood flow direction; at least, maintain at an angle < 30°.

3.4.2. Gate position: It is a marker in the cursor line corresponding to the sampling site. Place it after the aortic and pulmonary valves and at the leaflet tips of the atrioventricular valves.

3.4.3. Gate size: Use the minimum setting except to obtain small regurgitant flows.

3.4.4. Baseline: Select the baseline depending on the direction of the blood flow. Place it at the top when blood flows against the transducer (e.g. pulmonary and aortic flows), or at the bottom when the blood flows toward the transducer (e.g. atrioventricular valves flows).

3.4.5. Scale: Select this according to the velocity of the blood flow, usually, 25% higher than the obtained velocity.

3.4.6. Doppler gain: Use this to intensify the Doppler signals. Increase gain until the color displays.

3.4.7. Colorization of the Doppler signal: Use magenta color when the Doppler spectrum is weak because it makes the velocity sharper.

3.4.8. Wall filter: Use this to decrease the amount of low-frequency noise that is produced by the cardiac walls.

3.4.9. Sweep speed: Use higher sweep speeds to facilitate time measurements.

[Place **Figure 3** here]

4. Apical 4 chambers view of the heart

4.1. To obtain an Apical 4 chambers (AP4C) view, place the rabbit in the left lateral recumbent position with the forelimbs outstretched away from the thoracic region by means of surgical tape (**Figure 4A**). Maintain the skin of the thorax flat in a similar way as described above (Step 2.1.1). The cardiac area of the chest should be positioned over the cutout section of the table. Similarly, the abdomen should be well-supported on the table after moving caudally the abdominal organs through gentle massaging.

4.2. Apply ultrasound gel to the transducer, and then access the heart through the notch in the table and position it closely to the skin of the left hemithorax, at the level of the 4^{th}-5^{th} intercostal space with the midclavicular line, with the transducer orientation mark pointing towards the back of the rabbit (in the direction of the left scapula) (**Figure 4B**). In this way, the transducer is orthogonal with the apex of the heart and the ultrasound beam is directed towards the base of the heart.

4.2.1. From this position, if necessary, move the transducer upward one intercostal space at a time until the ~4th intercostal space (a maneuver often called “window shopping”).

4.2.2. Upon reaching the appropriate intercostal space (which may vary according to size and/or age of the rabbit), observe an image of the heart from the apex to the base of the heart, the typical heart shape where all four chambers can be seen, with the left and right ventricles at the top and both atria at the bottom of the image (see **Figure 4C,D** and Representative Results).

4.2.3. Image orientation: Note that the LV will be on the right side of the sector image.

4.3. Avoid foreshortening the apex in this view, so that the typical AP4C view of the heart should give a bullet shape image of the LV with the IVS in the middle (**Figure 4C,D**). If the apex is rounded, the LV is likely foreshortened; therefore, move the transducer downwards one intercostal space and/or tilt of the transducer.

4.3.1. Use B-mode to check for regional wall motion abnormalities and have a global view of the LV function. Use color Doppler to evaluate flow across the atrioventricular valves, and use PWD and position the sample volume at the level of the MV leaflet tips to obtain images of the MV inflow spectrum (**Figure 4C**).

4.3.2. Use TDI mode and place the sample volume at the septal and lateral sides of the mitral valve annulus (**Figure 4D**).

4.3.3. Use M-mode and place the cursor aligned with the lateral MV annulus to obtain the mitral annular plane systolic excursion (MAPSE). Store images in each of these modes for offline analysis of cardiac function.

[Place **Figure 4** here]

5. Apical 5 chambers view of the heart

5.1. Starting with the transducer at the same location as in AP4C view, perform a gentle tilting caudally (**Figure 4E**) until the LVOT and AoV come into view, this is the apical 5 chambers view (AP5C) of the heart (see Representative Results).

5.2. Use B-mode to evaluate the LVOT, the movement of the AoV leaflets, as well as the LV cavity size and function.

5.3. Use color Doppler mode for evaluation of outflow across the AoV, and use PWD to assess flow velocity across this valve by positioning the sample volume just behind the AoV (**Figure 4F**).

REPRESENTATIVE RESULTS:

Parasternal long axis view of the heart

Figure 5A shows an imaging plane of the right PSLAX view where the 4 chambers of the heart are clearly distinguished. You can identify in this view the right ventricle (RV), tricuspid valve (TV), IVS, LV, FW, as well as the mitral valve (MV). When the apex is clearly visible on the left side of the image in this view and the LV is not foreshortened, it is possible to estimate accurately the LV volume using the biplane method of disks (modified Simpson's rule) as shown in **Figure 5B,C**[8], which for accuracy should be combined with a similar measurement of the LV volume in the AP4C view, especially if the rabbit model used presents with wall motion abnormalities. **Figure 5D** shows the other imaging plane of the right PSLAX where the LVOT and the Aorta (Ao) also come into view. The location for placement of the calipers for accurate measurement of the LVOT is also shown in **Figure 5D**.

[Place **Figure 5** here]

Parasternal short axis view of the heart

In **Figure 6A**, a right PSSAX view of the heart at the level of the papillary muscles and chordae tendineae plane is shown. It is possible identify in this view the RV, IVS, LV, and FW, as well as the anterolateral (AL) and posteromedial (PM) papillary muscles (**Figure 6A**). In this view, the area trace tool is used to measure the circumferential area in end-diastole (CAd) (**Figure 6B**), and in end-systole (CAs) (**Figure 6C**), which allows the calculation of the total circumferential shortening area (CSA) by using the formula:

$$CSA=CAd-CAs/CAd\times100.$$

An example of an M-mode trace in the PSSAX at the papillary muscles level is shown in **Figure 6D**, where the placement of calipers, leading edge to leading edge, for the different measurements of the structures of the LV is also demonstrated. These measurements provide useful information regarding size of the LV structures. Thus, measuring the LV end-diastolic diameter (LVDd) and LV end-systolic diameter (LVDs) from three consecutive heart beats allows the calculation of the LV shortening fraction (%SF), using the formula:

$$SF\% = LVDd-LVDs/LVDd$$

as well as the LV systolic and diastolic volumes (LVVd, LVVs), using the Teichholz formula:

$$(7\times(LVD)^3)/(2.4+LVD)$$

The LV ejection fraction (LVEF (%)) is subsequently calculated according the formula LVEF=(LVVd-LVVs)/(LVVd×100).

An M-mode trace at the level of the MV plane in PSSAX view is shown in **Figure 6E**, where the location of the calipers for measurement of the E-point to septal separation (EPSS) of the mitral valve is also shown. An example of a PSSAX view of the heart at the AoV plane level is shown in **Figure 6F**, where the location of the calipers for measurement of the Aortic root diameter (AoD), as well as the left atrial dimension (LAD) are demonstrated.

An example of the PV outflow analysis using both color Doppler and pulsed wave Doppler is shown in **Figure 6G**. Note the blue colored outflow through the PV with color Doppler, which indicates that the flow observed is moving away from the transducer. Examples of how to quantitate the pre-ejection period of the PV (PEP PV), as well as the PV outflow using the volume time integral (VTI), are shown in **Figure 6H**.

[Place **Figure 6** here]

Apical 4 chambers view

An example of MV inflow using color Doppler in an AP4C view is shown in **Figure 7A**. Note the predominant red color of the MV inflow indicating that the flow is moving towards the transducer. Thus, a useful mnemonic to describe and learn how blood flows across the structures of the heart is the acronym BART (Blue Away, Red Towards the transducer). Using PWD, the MV inflow spectrum can be assessed as shown in **Figure 7B**, where the early (E) and late (A) filling waves during diastole are easily differentiated. Examples of myocardial tissue velocities of the MV annulus as assessed by TDI at both the lateral and septal walls are shown in **Figure 7C** and **Figure 7D**, respectively. The systolic component is denoted by the S wave, whilst the E' and A' waves correspond with myocardial movement of the mitral valve annulus during early filling (E') and late filling (A') components of diastole.

Apical 5 chambers view

Figure 7E shows an example of color Doppler positioned at the LVOT in an apical 5 chambers view. Note that, in line with the BART mnemonic described above, the blue color observed indicates that blood flow is moving away from the transducer. **Figure 7F** shows an example of how to quantitate the AoV outflow using PWD signal to evaluate the VTI of the AoV, systolic ejection time (ET) and pre-ejection period of the AoV (PEP AoV).

[Place **Figure 7** here]

Figure Legends:

Figure 1. Preparation and positioning of the rabbit for echocardiography. (**A**) Table with notch that coincides with the cardiac area to be imaged. (**B**) Remove hair from the chest. (**C**) Attach ECG electrodes to monitor the heart. (**D**) Positioning of the operator whilst preforming echocardiographic examination.

Figure 2. How to obtain a PSLAX view of the heart. (**A**-**B**) Positioning of the transducer to obtain the two different planes of the PSLAX view of the heart (see description in the text).

Figure 3. How to obtain a PSSAX view and its different imaging planes. (**A**) Position of the transducer to obtain a PSSAX view at the level of the papillary muscles. (**B**) Demonstration of the role of the left hand to help in rotating the transducer when switching from a PSLAX to a PSSAX view. (**C**) Location of the cursor of M-mode in the papillary muscles plane of the PSSAX view. (**D**) Position of the transducer to obtain a PSSAX view of the heart at the mitral valve plane. (**E**) Location of the cursor of the M-mode in the MV plane of the PSSAX view. (**F**) Position of the transducer to obtain the AV plane in the PSSAX view. (**G**) Demonstration of color Doppler and positioning of the PWD sample volume to evaluate the outflow of the PV. (**H**) Location of the cursor of the M-Mode in the AoV plane of the PSSAX view. LV = Left ventricle; RV = right ventricle; FW = LV free wall; AoV = aortic valve; RVOT = right ventricular outflow track; PV = pulmonary valve; PA = pulmonary artery; LA = Left atrium; RA = right atrium.

Figure 4. How to obtain the AP4C and AP5C views of the heart. (**A**) Positioning of the rabbit in left lateral decubitus for an AP4C view of the heart. (**B**) Position of the transducer to obtain an AP4C view of the heart. (**C**) Location of the sample volume at the MV leaflet tips to evaluate MV inflow. (**D**) Location of the sample volume for TDI analysis of myocardial velocities at the lateral side of the MV annulus. (**E**) Position of the transducer to obtain an AP5C view of the heart. (**F**) Location of the sample volume for PWD analysis of the outflow across the AoV. LV = Left ventricle; RV = right ventricle; MV = mitral valve; LA = left atrium; RA = right atrium; AoV= Aortic valve.

Figure 5. Imaging planes obtained in a PSLAX view of the heart. (**A**) Imaging plane demonstrating the 4 chambers of the heart. (**B**) End diastolic and (**C**) end systolic images, demonstrating Simpson's method for analysis of the LV. (**D**) Imaging plane where the LVOT and aorta come into view in the PSLAX view of the heart. LV = Left ventricle; RV = right ventricle; IVS = interventricular septum; Ao = aorta; LVOT = left ventricular outflow track; LA = Left atrium; RA = right atrium; MV = mitral valve; TV = tricuspid valve; FW = free wall of the LV; PC = pericardium.

Figure 6. Imaging planes obtained in the PSSAX view. (**A**) Representative image of a PSSAX view at the papillary muscles plane. (**B**) End diastolic and (**C**) end systolic tracing of the endocardial border to measure the total CSA. (**D**) M-mode trace obtained in a PSSAX view at the level of the papillary muscles. (**E**) An example of M-mode trace obtained in a PSSAX view at the level of the MV. (**F**) Representative 2D image of a PSSAX vie in the plane of the AV. (**G**) Color Doppler-guided PWD tracing of the PV outflow. (**H**) Demonstration of a VTI tracing using the PWD signal obtained from the PV outflow. LV = Left ventricle; RV = right ventricle; IVS = interventricular septum; FW = free wall of the LV; AL = anterolateral papillary muscle; PM = posteromedial papillary muscle; LVDd = left ventricular diameter at end-diastole; LVDs = left ventricular diameter at end-systole; PC = pericardium; EPSS = E-point to septal separation; AoD = aortic root diameter; LAD = left atrial dimension; MV = mitral valve; TV = tricuspid valve; PEP PV = pre-ejection period of the pulmonary valve; ET PV = ejection time of the pulmonary valve; VTI PV = volume time integral of the pulmonary valve.

Figure 7. The AP4C and AP5C views. (**A**) An example of color Doppler in an AP4C view. (**B**) Representative image of the PWD signal of the MV inflow in an AP4C, where E wave corresponds with early diastolic filling and A corresponds with atrial contraction component during diastole. (**C**-**D**) Representative images of myocardial velocity signals obtained from the lateral (**C**) and septal (**D**) segments of the MV annulus using TDI in an AP4C view. S corresponds with systole, whilst E' corresponds with early filling phase and A' with late filling phase during diastole. (**E**) An example of color Doppler signal obtained from the AoV in an AP5C view. (**F**) Demonstration of a VTI tracing using the PWD signal obtained from the AoV outflow. AoV = Aortic valve; VTI = volume time integral; PEP = pre-ejection period; ET = ejection time.

DISCUSSION:

We have described a protocol for the echocardiographic examination of cardiac function parameters in the rabbit, representing a large preclinical model[1-3]. The step by step methodology described herein should be considered guidance, which with a complementary study of the basic principles of echocardiography, and a basic knowledge of ultrasound imaging, will help the researcher to obtain, through practice and complementary and expert guidance, good quality data in a relative short period of time.

There are several critical steps to increase the value and reproducibility of the results whilst using the echocardiography protocol described here. First, ensure the skin of the thorax is hair free and clean; for this we recommend cleaning the skin with ethanol to remove excess of natural skin grease before applying ultrasound gel. Next, whilst it is possible to image the chest in a supine position, the lungs tend to inflate and reduce an already difficult to image chest wall with poor echogenicity, thus, a left or right recumbent position of the rabbit and the application of the transducer to the chest through the cut-out notch of a purpose built imaging table is the best way to improve overall imaging quality. Then, the researcher operating the ultrasound system should spend some time creating cardiac imaging presets with optimized imaging settings, which are essential to improve overall imaging quality in all views and will also shorten your imaging time at future imaging sessions. Some of the most important control settings to master are total gain and time-gain compensation, given the poor imaging of the chest of the rabbit (see step

2.4.2). It is also important to be systematic and always perform the echocardiographic examination in an orderly fashion. For this, getting into the habit of acquiring all the imaging views and imaging planes in the same sequence will avoid missing important information whilst performing the study. Furthermore, during imaging analysis it is recommended to perform all measurements in at least three consecutive cardiac cycles in the acquired images for each modality. Finally, the blinding of the observer during imaging as well as during the offline analysis is important to avoid bias and increase the value of the results for translational medicine. Taking into account all of the above considerations, together with the application of the principles of imaging and analysis according to current guidelines[7,8], will ensure the reproducibility of the research using longitudinal evaluation of cardiac function via echocardiography in a large animal model such as the rabbit.

Given the variability in body size and fat composition at different ages of the rabbits and the particular experimental settings, some variations of the technique will be required, such as subtle movements of the transducer (e.g., sweeping, rotation) relative to the intercostal space, in order to achieve the desired imaging planes. Therefore, the protocol described here must be interpreted as a starting point that should be adapted to the particular objectives of the research program involving this technique.

Whilst clinical echocardiography systems are widely available in most research centers, there are some limitations to the technique described herein. Indeed, the quality of the images obtained from echocardiographic studies depends to a large extent on the sophistication and technology of the ultrasound machine, the skills and expertise of the operator, and the individual patient characteristics. The minimum technical characteristics that the ultrasound equipment must meet were described in the introduction. Thus, inadequate equipment (e.g., a linear array transducer) constitutes a fundamental limitation for the use of the echocardiographic technique in the rabbit model. In addition, the echocardiographic technique and its results are strongly influenced by the operator. Therefore, an operator without enough experience and practical training could dramatically limit the obtaining of standardized images of appropriate quality. Similarly, inexperienced operators could also make mistakes in obtaining measurements even if they are performed on echocardiographic images of excellent technical quality. Furthermore, as mentioned above, some of the limitations are inherent to the rabbit model, such as age and, more specifically, by the size and body fat composition of the rabbits studied via echocardiography. In our experience, young rabbits weighing up to 2.5 kg have low subcutaneous and intra-thoracic fatty deposits. This phenotypic stage provides the best acoustic windows and offers crisper and sharper echocardiographic images and very few artefacts. As the size and body fat composition increase, the quality and accuracy of the echocardiographic study becomes limited, and the skills of the operator will ultimately play a fundamental role in achieving the best possible imaging under these circumstances.

We currently use echocardiography for longitudinal evaluation of cardiac function in a rabbit model of cardiomyopathy induced by anthracyclines and to test stem cell therapies for this condition[9,12,13]. The technique described here could also be used in other preclinical studies involving ischemia or valvular heart disease.

Another cardiovascular imaging technique is cardiac magnetic resonance (CMR), whose main advantage is better endocardial-myocardial definition, which translates into a more accurate estimation of LV volumes and systolic function[14]. However, CMR is limited by its high cost and lack of portability and therefore its limited availability in most research centers. Similarly, CMR has relative poor performance for the analysis of diastolic function, thus making echocardiography a better overall choice for longitudinal evaluation of systolic and diastolic function of the heart[15].

In our experience, the anesthetic regime used in the protocol described herein is safe and achieves reproducible results without significant depression of myocardial function attributable to the anesthesia[9]. However, it is important to standardize the anesthetic regime in each laboratory to ensure reproducible results for your particular experimental settings. After inducing anesthesia, in experienced hands the echocardiographic examination can be completed within 15 min.

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DISCLOSURES:

The authors have nothing to disclose.

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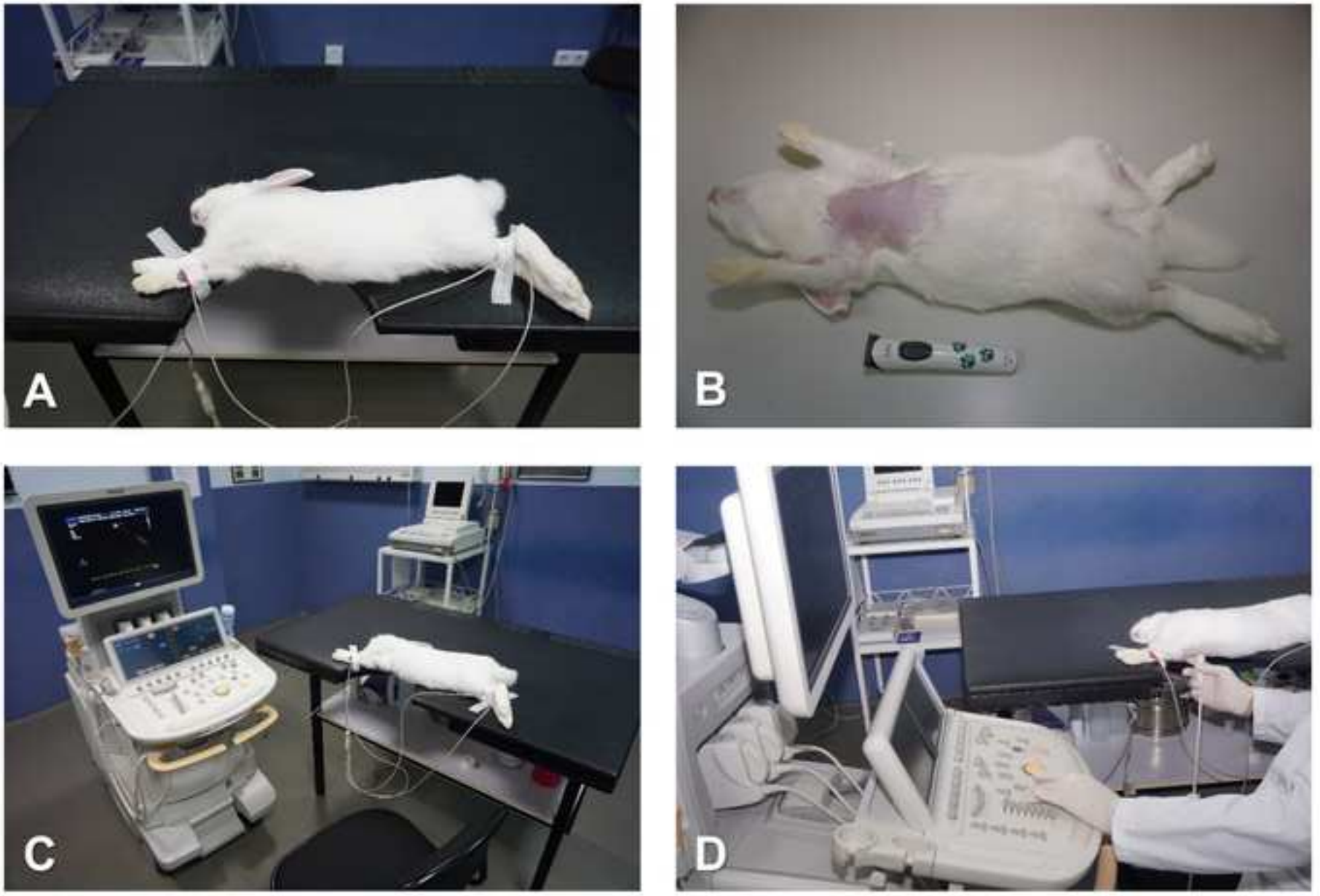

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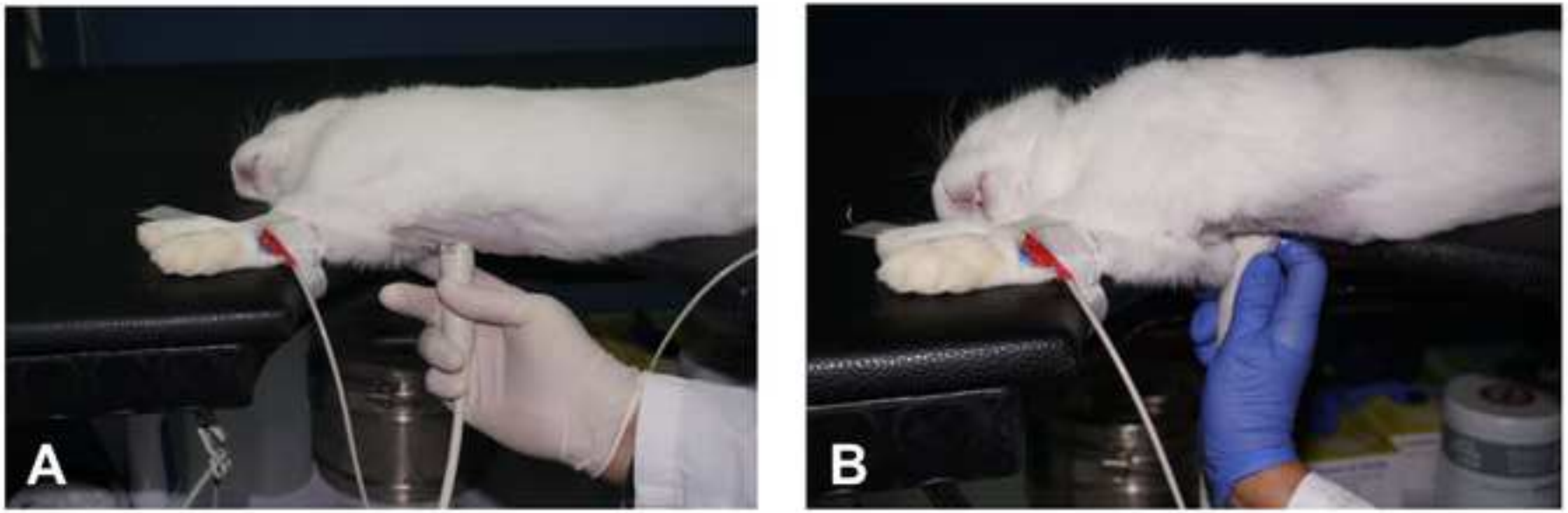

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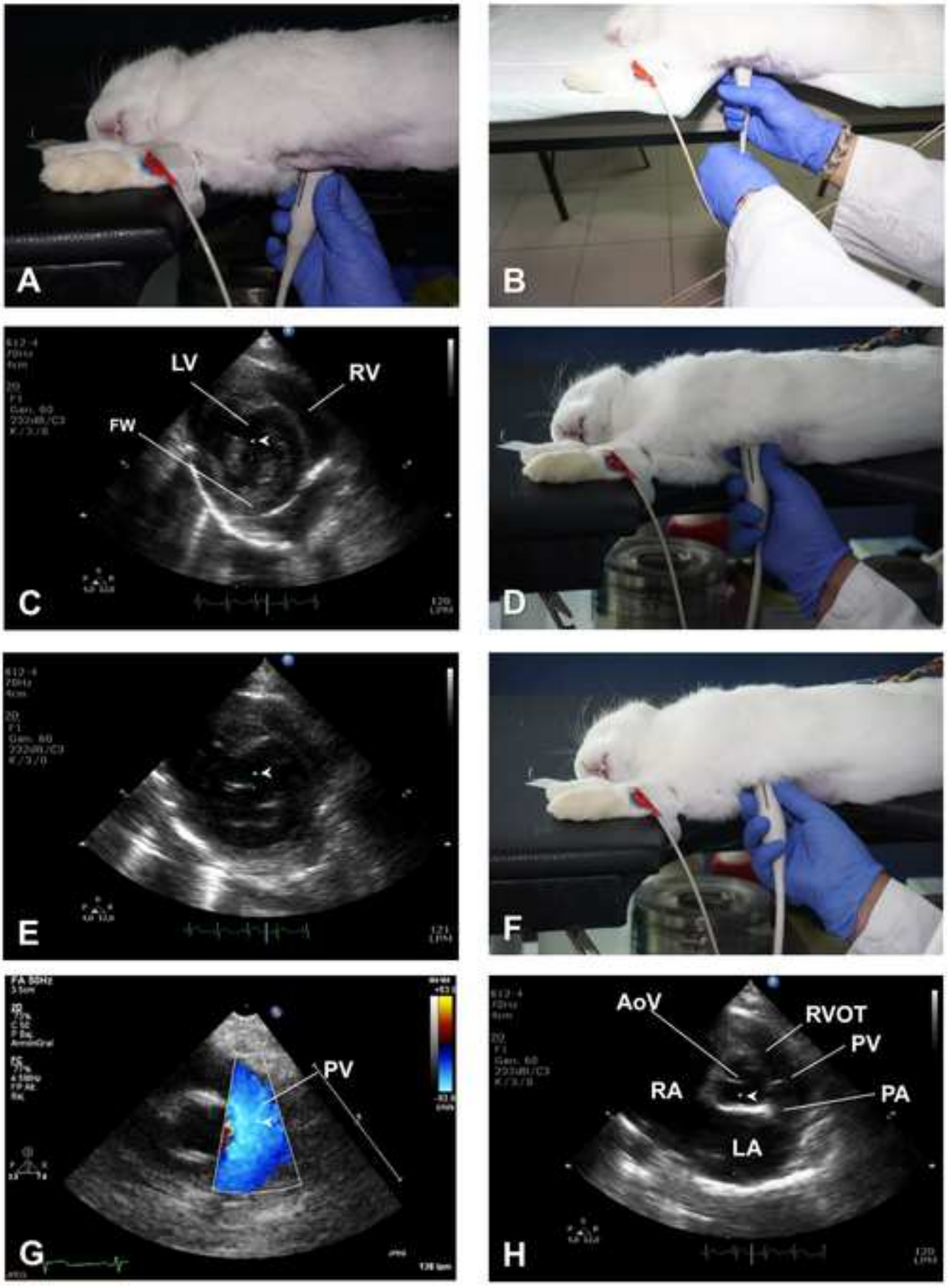

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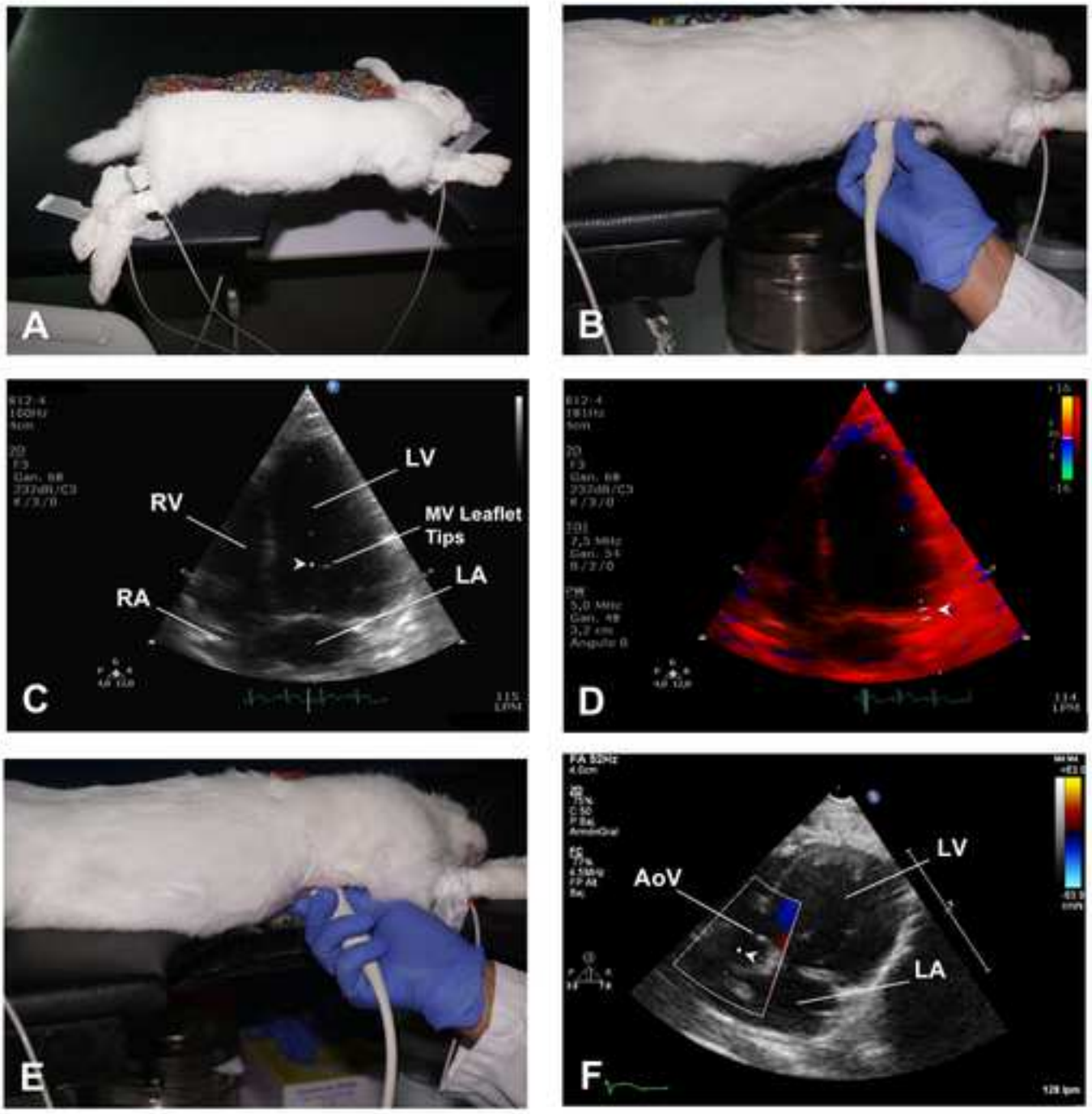

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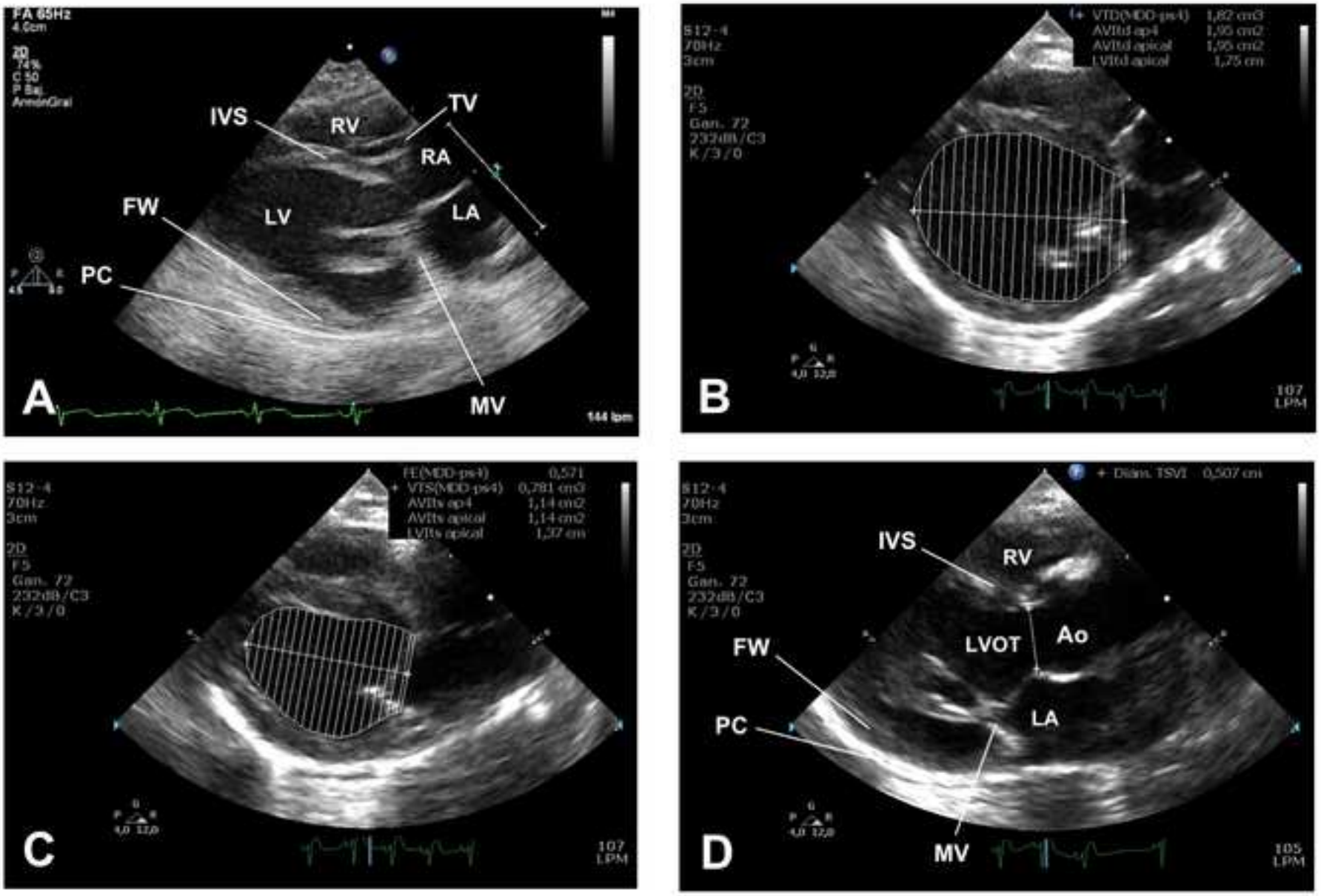

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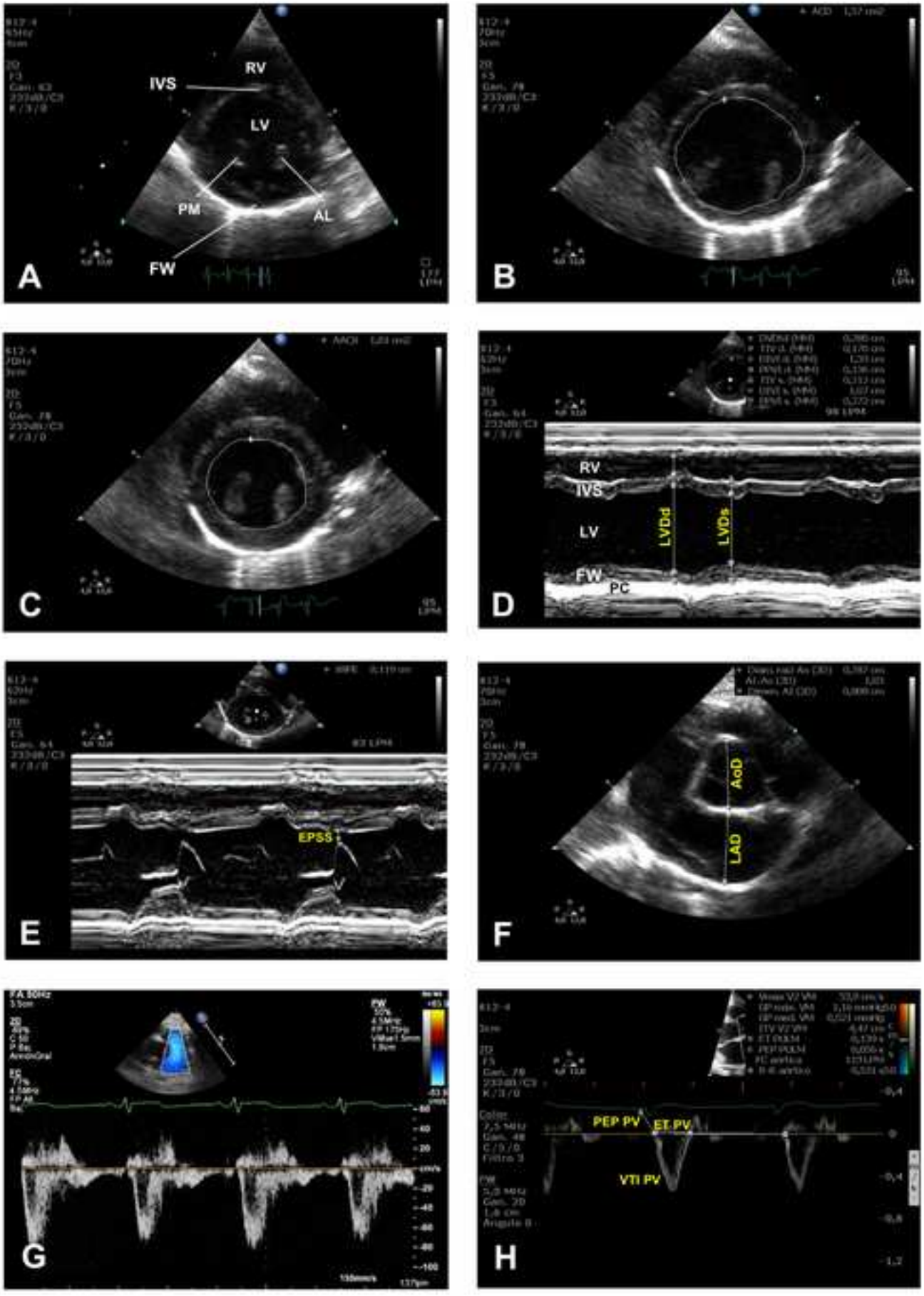

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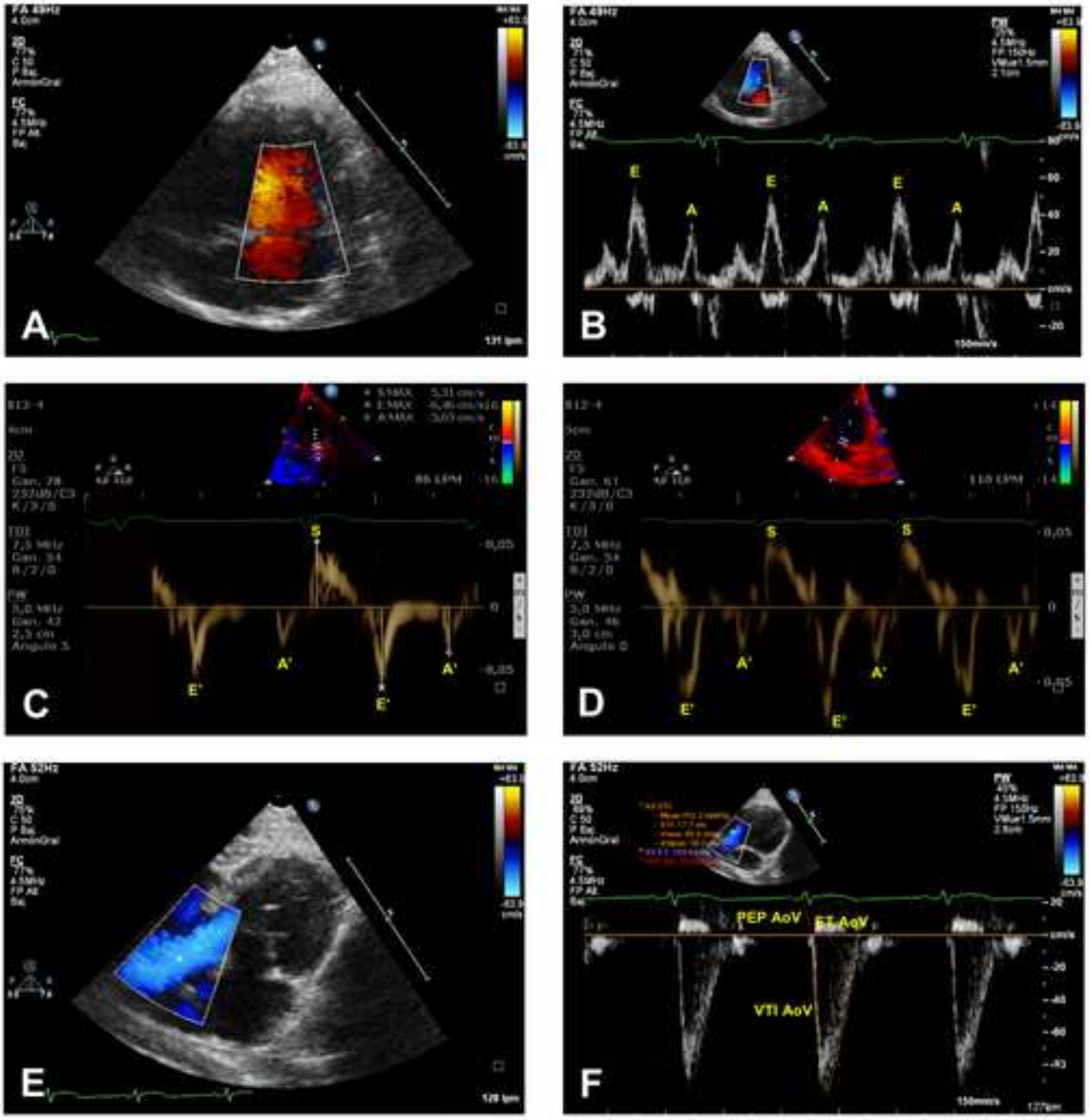

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Name:	Alejandro Giraldo
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Dear Dr. Steindel,

Thank you for your letter dated 13 March 2019 informing my co-authors and I that our manuscript requires minor revisions before formally being accepted for publication in *JoVE*.

In the enclosed revised version of our paper and video, we have addressed all of the Editorial and Reviewers' comments, and changes made in the manuscript are tracked to easily identify these throughout the text. I list below a detailed response to each of those comments. The response to the comments provided in the Veterinary Review Template are addressed directly in the corresponding template also uploaded through the submission system.

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Response: We value your suggestion, and have modified the title to “Transthoracic Echocardiographic Examination in the Rabbit Model”, this has been updated in the revised version of the manuscript as well as the Title cards located at time 0:00 and 14:42 of the video.

6. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Response: We value your suggestion; we have now rephrased the short abstract in the revised version of the manuscript (Lines: 42-45).

7. Please reference at least 10 previous publications.

Response: We appreciate your suggestion, during the review process we have now made changes to several sections of the manuscript which also required the addition of references, which now have a total of 15 references.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: We have made changes throughout the protocol to ensure that the actions are described in the imperative tense, and avoided as much as possible the use of phrases such as "could be", should be" and "would be". We have also made use of NOTES, where necessary, to increase the understanding of discrete steps (e.g. section 1.1 and NOTE: 1.1.1).

9. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Response: We have made changes throughout the whole protocol to ensure it contains mainly action items (Sections 1.1 to 5.3), and moved any discussion items about it to the Discussion section.

10. The long note at the beginning of the protocol should be moved to either the Introduction or the Discussion.

Response: We value your suggestion, we have moved the long note at the beginning of the Protocol section, to the Introduction section of the manuscript (Lines: 99-121).

11. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response: We have added more details to the protocol steps, making sure the step is clearly described and where necessary added details and clarification notes.

12. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have now made changes throughout the Protocol to ensure that, whenever possible, it does not contain personal pronouns such as "we", "you", "our".

13. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Response: We value your comment, and have therefore made changes throughout the Protocol, to ensure discrete steps of 2-3 actions per individual step are described.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

Response: The critical steps have now been added to the manuscript (Lines: 582-604).

b) Any modifications and troubleshooting of the technique

Response: Modifications of the technique in certain scenarios have been added to Discussion section (Lines: 605-610).

c) Any limitations of the technique

Response: The limitations of the technique are now described in the Discussion section (Lines: 611-630).

d) The significance with respect to existing methods

Response: The significance with respect to other cardiovascular imaging methods, namely cardiac magnetic resonance, was added to Discussion section (Lines: 635-640).

e) Any future applications of the technique

Response: The application of the technique has been added to Discussion section (Lines: 631-634).

15. Please do not abbreviate journal titles.

Response: We have now changed the references so that the journal tittles are not abbreviated.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the written protocol text and the audio narration. Ideally, the audio narration would be a word for word reading of the written protocol. Steps 2.1.1 and 2.3 are good examples of this.

Response: We have now increased the homogeneity between the written protocol and the corresponding narration throughout the whole protocol.

2. Please include some section title cards: Protocol, Representative Results, Conclusion, etc.

Response: We value your comment. We have changed the title cards to better match the sections of the paper and subsections within the protocol. For this the Section cards have been capitalized to match the style of the paper. In a similar way, subsections of the PROTOCOL section of the video have been numbered according to the different subsections of the protocol in the manuscript.

3. We recommend lowering the volume of the background music by 3 dB. It occasionally competes with the narrator and interview voices.

Response: We have adjusted the volume of background music throughout the video to ensure it doesn't compete with the narrator and interview voices.

4. 0:19, 0:24, 0:30 - These images should be scaled up to fill the frame.

Response: The images at 0:19, 0:24 and 0;30 have now been scaled up to fill the frame as suggested.

5. Many of the edits are page wipes, which can be a bit distracting. It is not required, but we recommend changing them to crossfades, so that the edits don't call as much attention to themselves and the viewer can focus more on the content.

Response: We have now changed the transitions to crossfades throughout the whole video as suggested.

6. 14:37 - This title card seems redundant with the following one. It should be removed.

Response: The tittle card located at 14:37 has now been removed as suggested.

Please upload a revise high-resolution video here:
https://www.dropbox.com/request/YTvdhxk8Ovu1ShYm9rWV?oref=e

Response: We have uploaded a revised version of the video in high-resolution, as indicated.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Accurate diagnosis of cardiac disease requires knowledge of normal heart parameters and useful standardized techniques. In the field of the cardiac translational medicine the knowledge of the most important imaging tool as echocardiography to analyze cardiac structures during systolic and diastolic function is a key and basic point to better apply in vivo study using large animal models. The paper entitled "Protocol for the Transthoracic

Echocardiographic Examination in the Rabbit Model" is a method article that explain a basic and standard ultrasounds views to obtain a complete echocardiographic examination. The choice of rabbit as animal model is an effective solution to compare in vivo scientific data for human purposes, indeed the rabbit is now considered a bridge between small animals such as mice and rats and real large models such as pig, sheep and goats. The paper is well presented, and all the materials and methods are given to the reader, clearly explained. Thanks to the video I think that the practical explanation of this procedure is effective and helpful to everyone who needs an echocardiographic guideline for rabbits. The anticipated results are reasonable and useful to readers and reference are appropriate. For my instance this article can be accepted for the publication.

Major Concerns:
None.

Minor Concerns:
I suggest revising minor error in the text to complete the publishing procedure:
Line 108: "This protocol is adapted for current international guidelines and includes practical recommendations based on our own experiences in clinical and experimental settings" (please insert a citation or explain which int. Guidelines as you later say in the line 139 to 142 (ACVIM, ASECHO and EACVI).

Response: We appreciate your suggestion, and therefore added the corresponding reference number to line 131 of the revised version of the manuscript.

Lines 123-124: "bi-dimensional mode (B-mode or 2D), motion mode (M-mode), color Doppler, spectral Doppler (CWD, PWD) and TDI". Change in bi-dimensional mode (B-mode or 2D), motion mode (M-mode), color Doppler (CWD), spectral Doppler (PWD) and Tissue doppler imaging (TDI).

Response: We value your suggestion, we have defined the acronyms for CWD, PWD and TDI in a previous paragraph (line 89 of the revised version of the manuscript), and therefore have now modified the text to reflect this change in line 103 of the revised version of the manuscript.

Reviewer #2:
Manuscript Summary:
This manuscript is a detailed protocol for performing transthoracic echocardiography in the rabbit as an animal model for medical and veterinary cardiovascular researches, the manuscript demonstrate how to obtain the different echocardiographic views and imaging planes, as well as the different imaging modes available in the clinical echocardiography systems.

Major Concerns:
The manuscript is well written and supported by a well overview video. but before accepting the manuscript for publication, some minor revision should be done on the manuscript.

Minor Concerns:
Line 247: It is recommended to use "craniocaudal" instead of "anteroposterior" in animal model.

Response: We value your comment, this is correct, we have now replaced "anteroposterior" with "craniocaudal" in line 268 of the revised version of the manuscript.

Line 416: talking about BIPLANE mode, it is necessary to estimate the left ventricular volume in left apical 4 chamber view, and either the right parasternal long axis image or the left apical two chamber view (average of volumes from two orthogonal planes). Here in current article you explained single plane but not biplane single plane method is no longer reliable if there are some wall motion abnormalities.

Response: We appreciate your suggestion; we have therefore added this clarification in lines 453-455 of the revised version of the manuscript.

Once again, on behalf of my coauthors, I would like to thank you for considering our article for publication in *JoVE*.

We look forward to hearing from you in due course.

Yours sincerely,

Alejandro Giraldo M.D., Ph.D.

Institute for Cardiovascular and Metabolic Research

School of Biological Sciences

University of Reading

Whiteknights

Reading

RG6 6UB

Email: a.giraldoramirez@reading.ac.uk

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center, Suite 200
Cambridge, MA 02140
www.JoVE.com

Title: Protocol for the Transthoracic Echocardiographic Examination in the Rabbit Model

URL: https://www.jove.com/video/59457/title?status=a61463k

Were animals used humanely and was the appropriate anesthesia or analgesia provided for potentially painful procedures?

Please provide additional comment, if necessary.

#	Time in the video	comment	Change in video required Yes/No	Change in text is sufficient Yes/No	Suggested Changes	Response
Example	2:20 – 2:34	Name of drug used for anesthesia is not mentioned	No	Yes		
1	n/a	What depth of anesthesia is sufficient for the procedure?	NO	YES	Describe how the depth of anesthesia is monitored, and what criteria would be used for additional doses of injectable anesthesia.	The appropriate depth of anesthesia was established by the presence of muscle flaccidity, absence of palpebral reflex, mandibular movements and sniffing. This clarification has been included in the text of the manuscript (see section 1.1.2.). Due to the short duration of the procedure (animal preparation +

						echocardiography = 20-25 minutes), a single dose was usually sufficient to complete it. If the procedure was delayed for some reason, the presence of mandibular movements and sniffing are usually the earliest signs of reduced depth of anesthesia state. Considering that it was a painless procedure, even in these cases re-dosing was rarely necessary.
2		Thermal blanket? The video shows only a table with an absorbent pad.	YES	YES	Procedure pads are not acceptable methods of providing external heat support.	Indeed, a thermal blanket/heating pad (green rectangle in the video) was always used during the echocardiographic procedure to avoid hypothermia. Sometimes it was placed under an absorbing cloth (time: 00:27) or directly under the animal (time 2:51-9:38). It is a blanket specially designed for use on animals that heats only the area of the blanket that comes into contact with the animal. Additionally, the animal was covered with a cloth to reduce the loss of temperature. This last procedure was not used during the recording of the video to facilitate the observation of the positioning of the animal. We have made the recommendation of the use of the

						thermal blanket more evident, by moving a comment from section 1.1.1. to a standalone section 1.2. in the protocol. The animal resting on a thermal blanket is already shown in the video (time: 0:17, 0:27, and 2:51-9:38).
3	n/a	Was the rabbit fasted before the procedure?	NO	YES	Fasting is not recommended for rabbits as they don't vomit, and they have prolonged gastric emptying times. If fasting was used, there needs to be a description on how intraoperative metabolic acidosis and hypoglycemia was prevented, and how postoperative gastrointestinal stasis was managed.	This is truly an important aspect that we forgot to mention in the text and since we do not routinely fast the rabbits for this procedure. Indeed, the animals were not subjected to any fasting before the procedures for the same reasons alluded to by the reviewer. We have added this to section 1.4 of the protocol as well as relevant references.
4	n/a	Procedure length	NO	YES	Describe the length (minutes) of the procedure.	As indicated above, the entire procedure (animal preparation and echocardiography) had an average duration of 20-25 minutes. As noted by the last conclusion of the video (time: 14:25), after inducing anesthesia, in experienced hands the echocardiographic examination can be completed within 15

						minutes. This has now been added to the discussion section of the manuscript (Lines 644-646).
5	00:14	Anesthesia is accomplished with one injection with Ketamine / Medetomidine mixed in the same syringe?	NO	YES	Describe how the K/M cocktail is prepared and administered in one syringe, or if it was administered with two injections.	Both drugs were loaded in the same syringe, homogenized and administered in the same injection shot to the patient. This is visible in the video at time 2:27. This has already been described in section 1.1 of the manuscript.
6	00:14	Anesthesia monitoring not described.	YES	YES	The patient must have at least temperature, respiration, SPO2 and heart rate monitored for any anesthetic events. It is OK if heart rate is monitored by ultrasound but that needs to be described.	Effectively, the heart rate was constantly monitored throughout the procedure by the ECG synchronous to the echocardiographic examination, as shown in the video (time: 3:06). The rabbits routinely received oxygen therapy through a mask (as shown in the video time: 3:11) and breathing was monitored visually, as well as through the incidence of thoracic movements in the echocardiographic image. The temperature was monitored via rectal probe. The monitoring of these variables was done, at the beginning of the procedure then every 10 minutes, as well as at the end of the

						procedure. This clarification was added to a note in section 1.3.1 of the protocol.

1. Please be specific in your comments. If possible, divide your comments into 2 categories:
   a) Absolutely not acceptable - for serious errors and deviations from the animal research standards.
   b) Improvement requires - for minor deviations, missing parts, etc....

   For each comment, please specify if the changes in video are required, or if only changes in the complementary text are necessary. **Obviously, changes in the video are more difficult so it is important to note if changes in the text are sufficient.** Please use the chart below to provide details on each issue (replace examples listed):