Journal of Visualized Experiments

Echocardiographic measurement of right ventricular diastolic parameters in mouse

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58021R2
Full Title:	Echocardiographic measurement of right ventricular diastolic parameters in mouse
Keywords:	Mouse, echocardiography, right ventricle, diastole, diastolic parameters, dysfunction, pulmonary artery banding
Corresponding Author:	Bakytbek Egemnazarov, M.D., Ph.D. Ludwig Boltzmann Institute for Lung Vascular Research Graz, AUSTRIA
Corresponding Author's Institution:	Ludwig Boltzmann Institute for Lung Vascular Research
Corresponding Author E-Mail:	Bakytbek.Egemnazarov@lvr.lbg.ac.at
Order of Authors:	Bakytbek Egemnazarov, M.D., Ph.D.
	Grazyna Kwapiszewska
	Leigh M. Marsh
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Stiftingtalstr. 24, c/o ZMF, Graz 8010, Austria.

TITLE:

Echocardiographic Measurement of Right Ventricular Diastolic Parameters in Mouse

AUTHORS & AFFILIATIONS:

B. Egemnazarov[1], G. Kwapiszewska[1,2], Leigh M. Marsh[1]

[1]Ludwig Boltzmann Institute for Lung Vascular Research, Graz, Austria

[2]Department of Physiology, Otto Loewi Research Center, Medical University of Graz, Graz, Austria

Corresponding author:

B. Egemnazarov (bakytbek.egemnazarov@lvr.lbg.ac.at)

Phone: +43-316-38572057

Email Addresses of the Co-authors:

G. Kwapiszewska (grazyna.kwapiszewska@lvr.lbg.ac.at)

Leigh M. Marsh (leigh.marsh@lvr.lbg.ac.at)

KEYWORDS:

Mouse, echocardiography, right ventricle, diastole, diastolic parameters, dysfunction, pulmonary artery banding

SUMMARY:

Here we describe and compare two positions for obtaining the apical four-chamber view in mice. These positions enable the quantification of the right ventricular function, provide comparable results, and can be used interchangeably.

ABSTRACT:

Diastolic dysfunction is a prominent feature of right ventricular (RV) remodeling associated with conditions of pressure overload. However, the RV diastolic function is rarely quantified in experimental studies. This might be due to technical difficulties in the visualization of the RV in the apical four-chamber view in rodents. Here we describe two positions facilitating the visualization of the apical four-chamber view in mice to assess the RV diastolic function.

The apical four-chamber view is enabled by tilting the mouse fixation platform to the left and caudally (LeCa) or to the right and cranially (RiCr). Both positions provide images of comparable quality. The results of the RV diastolic function obtained from two positions are not significantly different. Both positions are comparably easy to perform. This protocol can be incorporated into published protocols and enables detailed investigations of the RV function.

INTRODUCTION:

Diastolic dysfunction is a prominent feature of right ventricular (RV) remodeling[1] and is associated with pressure-overload conditions[2]. Echocardiography (EchoCG) can be used for the characterization of RV diastolic dysfunction[3,4]. Despite recent developments in small animal echocardiography, measurements of diastolic parameters are rarely reported. In contrast, measurements of the systolic function are widely used for the characterization of transgenic

mice[5], as well as for the evaluation of a treatment response[6].

This can be partly explained by the difficulties in the measurement of diastolic parameters from the apical four-chamber view. Visualization of the heart in this position can be facilitated by tilting the fixation platform LeCa or RiCr. Even if these manipulations are used, echocardiographers do not report them in their manuscripts[4,7]. Therefore, it remains unclear whether these manipulations provide comparable results. Moreover, this also precludes a development of standardized nomenclature of this position for mice.

The aim of this study was to describe two positions for apical four-chamber view visualization and compare their results. To determine the differences between the two positions, we have utilized the mouse pulmonary artery banding (PAB) model, in which a tantalum clip leads to a partial occlusion of the pulmonary artery. This occlusion results in right ventricle remodeling and dysfunction. Full details of the PAB operation can be found in previously published work[3]. Sham-operated mice, where the clip was placed next to the pulmonary artery, were used for comparison. EchoCG investigations were performed three weeks post-operation using the imaging system with a 30-MHz scan head (see **Table of Materials** for both). Nomenclature for the description of the positions and orientations between the mouse and the ultrasound beam is used as described by Zhou *et al.*[7].

PROTOCOL:

The study was performed according to national regulations for animal experimentation and EU Directive 2010/63. Prepare equipment as described previously by Brittain *et al.*[8].

1. Mouse Preparation

1.1. Obtain 12- to 13-week-old male C57Bl6/J mice and house them with a 12-h light/dark cycle, at a constant room temperature, and with *ad libitum* access to standard laboratory chow and water, until the start of the experiment.

1.2. Anesthetize the mouse using general anesthesia approved by the Institute and check for the lack of response to the toe pinch. Under mild anesthesia with isoflurane 0.8% - 1.2%, fix the mouse on a heated platform. Apply electrode gel to its extremities for the continuous monitoring of its heart rate and temperature.

1.3. Depilate the mouse's chest hair using depilation creme. To reduce pressure on its thorax, do not apply the ultrasound coupling gel directly on the thorax; rather, apply a layer of the gel to the tip of the transducer.

2. Image Acquisition

2.1. Apical four-chamber view with a left and caudal tilt of the platform

2.1.1. After the mouse preparation, angulate the platform to the left at 10° - 15° and then caudally at 10° - 15°.

2.1.2. Position the transducer above the apex with the imaging plane ~45° to the coronal plane and the central axis of the ultrasound beam directed cranially, posterior, and to the left to obtain the apical four-chamber view. Press the **B-Mode** button to activate the B-mode/2-D image.

Note: The transducer can be held manually or fixed by a stage. The term “B-mode” comes from the imaging system that was used instead of the more familiar term “two-dimensional” (2-D) and is used throughout the protocol.

2.1.3. Look for the appearance of the following structures in the acoustic window: the left ventricle (LV), the left atrium (LA), the RV, the right atrium (RA), the mitral valve (MV), and the tricuspid valve (TV).

2.1.4. Manipulate the imaging plane in the coronal plane and rotate clock- and counterclockwise around the central axis until both ventricles are visualized at their longest dimension and both atria are visible. This is the four-chamber view (**Figure 1**).

2.1.5. Press the **Cine store** button to save the recording.

2.1.6. Press the **Scan/Freeze** button to pause the system.

2.2. Measurement of transtricuspid blood flow velocities

2.2.1. Press the **Scan/Freeze** button to activate the system.

2.2.2. Press the **Overlay** button several times to activate the sample volume for PW (pulsed wave) mode.

2.2.3. While keeping the obtained four-chamber view, use the trackball to position the sample volume at the opening of the tricuspid valves for the measurement of inflow velocities (E and A peak velocities).

2.2.4. Press the **PW** mode button for the measurement of inflow velocities (E and A peak velocities).

Note: Because tricuspid valves are difficult to visualize in this position, performing several measurements helps to align correctly the sample volume with the blood flow. Perform the Doppler sampling with the smallest incidence angle between the Doppler beam and the blood flow direction. The obtained blood flow profile should correspond to the following criteria: 1) an inflow profile similar to an M-shape with the first peak lower than the second; 2) a respiratory modulation with an increased amplitude at inspiration; 3) a maximal amplitude of velocities in several measurements (**Figure 2**).

2.2.5. Press the **Cine store** button to save the optimized recording.

2.2.6. Press the **Scan/Freeze** button to pause the system.

2.3. Measurement of the tricuspid annular plane systolic excursion (TAPSE)

2.3.1. Press the **Scan/Freeze** button to activate the system.

2.3.2. Switch to B-mode by pressing the **B-Mode** button. Some manipulations on the image might be necessary to regain the correct four-chamber view.

2.3.3. Press the **Overlay** button several times to activate the sample volume of the M-mode. Using the trackball, align the sample volume with the lateral part of the tricuspid annulus. By pulling the edges of the sample volume using the trackball, align the length of the sample volume to cover the entire amplitude of the cardiac movement during the cardiac cycle.

2.3.4. Press the **M-Mode** button to activate M-mode. Tricuspid annulus' movements should appear as a wave (**Figure 2**).

2.3.5. Press the **Cine store** button to save the recording.

2.3.6. Press the **Scan/Freeze** button to pause the system.

2.4. Measurement of tissue Doppler parameters

2.4.1. Press the **Scan/Freeze** button to activate the system.

2.4.2. Press the **B-Mode** button to activate B-mode.

Note: Some manipulations by angulation in the coronal plane and rotation clock- and counterclockwise around the central axis of the image might be necessary to regain the correct four-chamber view.

2.4.3. Press the **Overlay** button several times to activate the sample volume for TDI (tissue Doppler imaging). Using the trackball, align the sample volume with the lateral part of the tricuspid annulus, where the RV free wall creates an angle with the tricuspid valve. By pulling the edges of the sample volume using the trackball, adjust the sample volume to include both the systolic and the diastolic extreme positions of the annulus.

2.4.4. Press the **Tissue** button to activate TDI mode.

Note: Yellow tracing of the TDI recording appears corresponding to the following criteria: 1) a recording similar to an inverted M-shape; 2) clearly distinguishable E' and A' peaks during diastole

and S' peak during systole; 3) a maximal amplitude of velocities in several measurements (**Figure 2**).

2.4.5. Press the **Cine store** button to record an optimized image.

2.4.6. Press the **Scan/Freeze** button to pause the system.

2.5. Apical four-chamber view with right and cranial tilt of the platform

2.5.1. Angulate the platform to the right at 10° - 15° and then cranial at 10° - 15°. Perform the measurements as described in the previous sections for the LeCa steps (steps 2.1, 2.2, 2.3, and 2.4).

Note: During the investigation, isoflurane should be titrated between 0.8 - 1.2 to keep the mouse's heart rate at 400 - 440 bpm. In this range, separate peaks of transtricuspid blood flow and tissue Doppler (DTI) velocities are measurable. To avoid the effects of the heat loss on hemodynamics, the data are recorded, and the analysis is performed off-line. Only signals obtained at the end-expiration are used for analysis. Measurements of 3 - 5 heartbeats are averaged.

REPRESENTATIVE RESULTS:

The apical four-chamber view is difficult to obtain in mice. Therefore, manipulations of the platform position can help to visualize the heart by changing its position in the thorax. The tilting of the platform to the left and to the right improved the acoustic window and provided images of comparable quality in B-mode (**Figure 1**). After obtaining the correct positions, measurements in PW-, M-, and TDI-modes provided images of comparable quality (**Figure 2**). The measurement of diastolic parameters was performed on sham- and PAB-operated mice (**Table 1**). Both positions (RiCr and LeCa) gave similar results in the diastolic parameters (**Table 2**). Furthermore, the EchoCG investigations in both positions revealed similar differences between the sham and PAB groups (**Table 2**, Dunnet's test). Correlation analysis revealed a good agreement between values obtained from these two facilitated positions (**Figure 3**). As small groups of animals were used for this study, non-parametric tests have been applied[9,10]. Intra-observer variability for some analyzed parameters has been published previously[3].

FIGURE AND TABLE LEGENDS:

Figure 1: Representative images of the apical four-chamber view. The apical four-chamber view is enabled by tilting the mouse fixation platform to the left and caudally (LeCa) or to the right and cranially (RiCr). LA = left atrium; LV = left ventricle; RA = right atrium; RV = right ventricle.

Figure 2: Representative images of the TAPSE, TDI, and transtricuspid flow measurements obtained from two facilitated apical four-chamber view positions. TAPSE = tricuspid annulus plane systolic excursion; E' = early peak of right ventricular relaxation velocity; A' = late peak of right ventricular relaxation velocity; S' = velocity of the right ventricular contraction; E = early

peak of diastolic tricuspid inflow; A = late peak of diastolic tricuspid inflow. Note the change in the transtricuspid blood flow profile at the inspiration (Insp).

Figure 3: Correlation analysis of data obtained from two facilitated apical positions. Correlation analysis was performed using non-parametric Spearman's test.

Table 1: Characterization of the operated groups three weeks after the operation. RVFW = right ventricular free wall thickness; VTI = velocity-time interval.

Table 2: Comparison of the results obtained from the apical four-chamber view facilitated by the left caudal or right cranial platform tilt. EchoCG-derived RV functional parameters are shown. As every mouse was investigated in both positions, the signed rank Wilcoxon test was used for intra-group comparisons. § $p > 0.05$ between RiCr and LeCa. The Kruskal–Wallis test, followed by Dunnet's *post hoc* test, was used for multiple group comparisons. The results of two selected intergroup comparisons are presented in the table. * $p < 0.05$, ** $p < 0.01$. PAB = pulmonary artery banding; LeCa = left caudal tilt; RiCR = right cranial tilt; E = early peak of diastolic tricuspid inflow; A = late peak of diastolic tricuspid inflow; TAPSE = tricuspid annulus plane systolic excursion; e' = early peak of right ventricular relaxation velocity; a' = late peak of right ventricular relaxation velocity; S' = velocity of the right ventricular contraction; HR = heart rate; bpm = beats per minute.

DISCUSSION:

The echocardiographic RV function and dimension assessment from parasternal positions have been well described. In contrast, the apical position in mouse echocardiography has been neglected partly due to technical difficulties. Using a horizontal platform position, it is difficult to obtain a sufficient acoustic window for four-chamber view imaging. To facilitate the imaging of this position, the platform can be tilted to the left, a manipulation similar to the left-sided positioning of patients. This should result in a leftward and more superior positioning of the heart, thereby improving the acoustic window. Therefore, LeCa is our standardized position for apical visualization. However, in approximately 30% - 35% of mice, the image quality in this position can be insufficient. Here, imaging in the RiCr position can be helpful.

From these positions, transtricuspid blood flow velocities (E and A) and tissue Doppler velocities (E' and A') can be measured, providing information about the RV diastolic function. We observed a good correlation between TDI parameters obtained from the two positions. Less satisfactory was the correlation of E. In general, the visualization of the transtricuspidal blood flow profile was the most challenging part of the protocol presented here and exhibited the highest variability. The measurement of TAPSE and S' by tissue Doppler provided an estimate of the RV systolic function. However, in the light of recent findings, the physiological meaning of TAPSE is not clear[11]. We do not routinely measure the RV fractional area of contraction from the apical position because, in the conditions of pressure overload, the lateral part of the enlarged RV is partly covered by the sternum and not completely visible from this position[3]. Thus, the visualization of the apical position in mice enables the measurement of the parameters routinely used in the clinic and, thereby, delivers more information, which allows a more complete

functional characterization.

Strain, strain rate analysis, and speckle tracking echocardiography are novel modalities of cardiac ultrasound[12]. Its high sensitivity can detect cardiac dysfunction at initial stages[13] and has the power to predict mortality[14]; therefore, its application is also warranted in experimental studies. Unfortunately, in mice, the RV free wall is partly hidden behind the sternum's shadow, which might hinder the analysis of strain. Furthermore, stain analysis requires good image quality and visualization of the entire free wall.

The cardiovascular system responds quickly to changes in the posture by activating baroreceptor mechanisms[15]. Therefore, it could be expected that the cranial tilt of the platform would cause reflectory changes in the measured cardiac parameters. Indeed, both the head-up and the head-down tilt position caused a transient change in the heart rate and cardiac electric axis in mice[16]. While a 90° head-up tilt causes an increased heart rate, a 90° head-down tilt caused transient and statistically insignificant bradycardia. In contrast, we recommend tilting the mouse only by 10° - 15° in either direction. These mild changes in posture did not cause any measurable hemodynamic perturbances.

LV diastolic function in mice is another understudied area. Although not tested in this study, the protocol presented here should be able to be used for the quantification of the LV diastolic function.

Theoretical and practical limitations of the small animal EchoCG have been described in detail elsewhere[8]. In this protocol, measurements are performed at heart rates of 400 - 440 bpm. At this range of heart rate, measurements of the E and A velocity peaks, as well as of TDI indexes, are feasible. At higher heart rates, peaks merge, making quantification impossible. Since the physiological heart rate for mice is 500 - 600 bpm, the heart rate used in this protocol is rather low. Nevertheless, the measurements at this heart rate range appear reliable and enable distinguishing between physiologic and dysfunctional phenotypes[3].

We described a protocol for two positions facilitating the assessment of RV functional parameters from four-chamber views in mice. The positions provide comparable results and can be used interchangeably.

ACKNOWLEDGMENTS:
The study was funded by the Ludwig Boltzmann Institute for Lung Vascular Research.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES
1. Egemnazarov, B., Crnkovic, S., Nagy, B. M., Olschewski, H., Kwapiszewska, G. Right ventricular fibrosis and dysfunction: Actual concepts and common misconceptions. *Matrix Biology: Journal of the International Society for Matrix Biology*. **68-69**, 507-521 (2018).

2. Rain, S. *et al.* Right ventricular diastolic impairment in patients with pulmonary arterial hypertension. *Circulation.* **128**, 2016-2025, 1-10 (2013).

3. Egemnazarov, B. *et al.* Pressure overload creates right ventricular diastolic dysfunction in a mouse model: assessment by echocardiography. *Journal of the American Society of Echocardiography.* **28**, 828-843 (2015).

4. Crnkovic, S. *et al.* Functional and molecular factors associated with TAPSE in hypoxic pulmonary hypertension. *American Journal of Physiology. Lung Cellular and Molecular Physiology.* **311**, L59-73 (2016).

5. Shi, L. *et al.* miR-223-IGF-IR signalling in hypoxia- and load-induced right-ventricular failure: a novel therapeutic approach. *Cardiovascular Research.* **111**, 184-193 (2016).

6. de Raaf, M. A. *et al.* Tyrosine kinase inhibitor BIBF1000 does not hamper right ventricular pressure adaptation in rats. *American Journal of Physiology - Heart and Circulatory Physiology.* **311**, H604-612 (2016).

7. Zhou, Y. Q. *et al.* Comprehensive transthoracic cardiac imaging in mice using ultrasound biomicroscopy with anatomical confirmation by magnetic resonance imaging. *Physiological Genomics.* **18**, 232-244 (2004).

8. Brittain, E., Penner, N. L., West, J., Hemnes, A. Echocardiographic assessment of the right heart in mice. *Journal of Visualized Experiments*. (81), e50912 (2013).

9. Kitchen, C. M. Nonparametric vs parametric tests of location in biomedical research. *American Journal of Ophthalmology.* **147**, 571-572 (2009).

10. Yan, F., Robert, M., Li, Y. Statistical methods and common problems in medical or biomedical science research. *International Journal of Physiology, Pathophysiology and Pharmacology.* **9**, 157-163 (2017).

11. Guihaire, J. *et al.* Non-invasive indices of right ventricular function are markers of ventricular-arterial coupling rather than ventricular contractility: insights from a porcine model of chronic pressure overload. *European Heart Journal Cardiovascular Imaging.* **14**, 1140-1149 (2013).

12. Sareen, N., Ananthasubramaniam, K. Strain Imaging: From Physiology to Practical Applications in Daily Practice. *Cardiology in Review.* **24**, 56-69 (2016).

13. Thavendiranathan, P. *et al.* Use of myocardial strain imaging by echocardiography for the early detection of cardiotoxicity in patients during and after cancer chemotherapy: a systematic review. *Journal of the American College of Cardiology.* **63**, 2751-2768 (2014).

14. Sengelov, M. *et al.* Global Longitudinal Strain Is a Superior Predictor of All-Cause Mortality in Heart Failure With Reduced Ejection Fraction. *JACC: Cardiovascular Imaging.* **8**, 1351-1359 (2015).

15. Silvani, A. *et al.* Physiological Mechanisms Mediating the Coupling between Heart Period and Arterial Pressure in Response to Postural Changes in Humans. *Frontiers in Physiology.* **8**, 163 (2017).

16. Mohan, M., Anandh, B., Thombre, D. P., Surange, S. G., Chakrabarty, A. S. Effect of posture on heart rate and cardiac axis of mice. *Indian Journal of Physiology and Pharmacology.* **31**, 211-217 (1987).

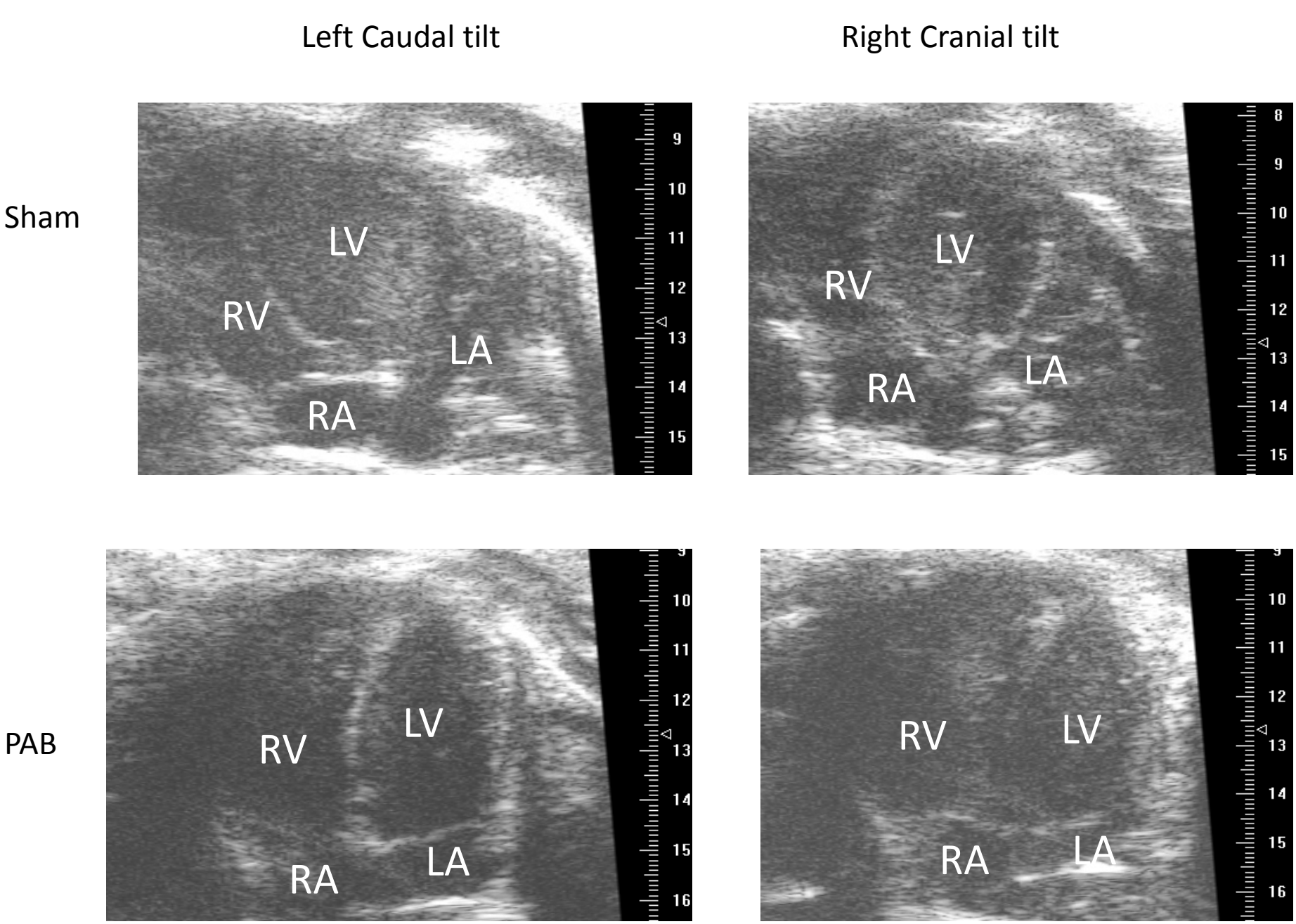


Fig. 1.

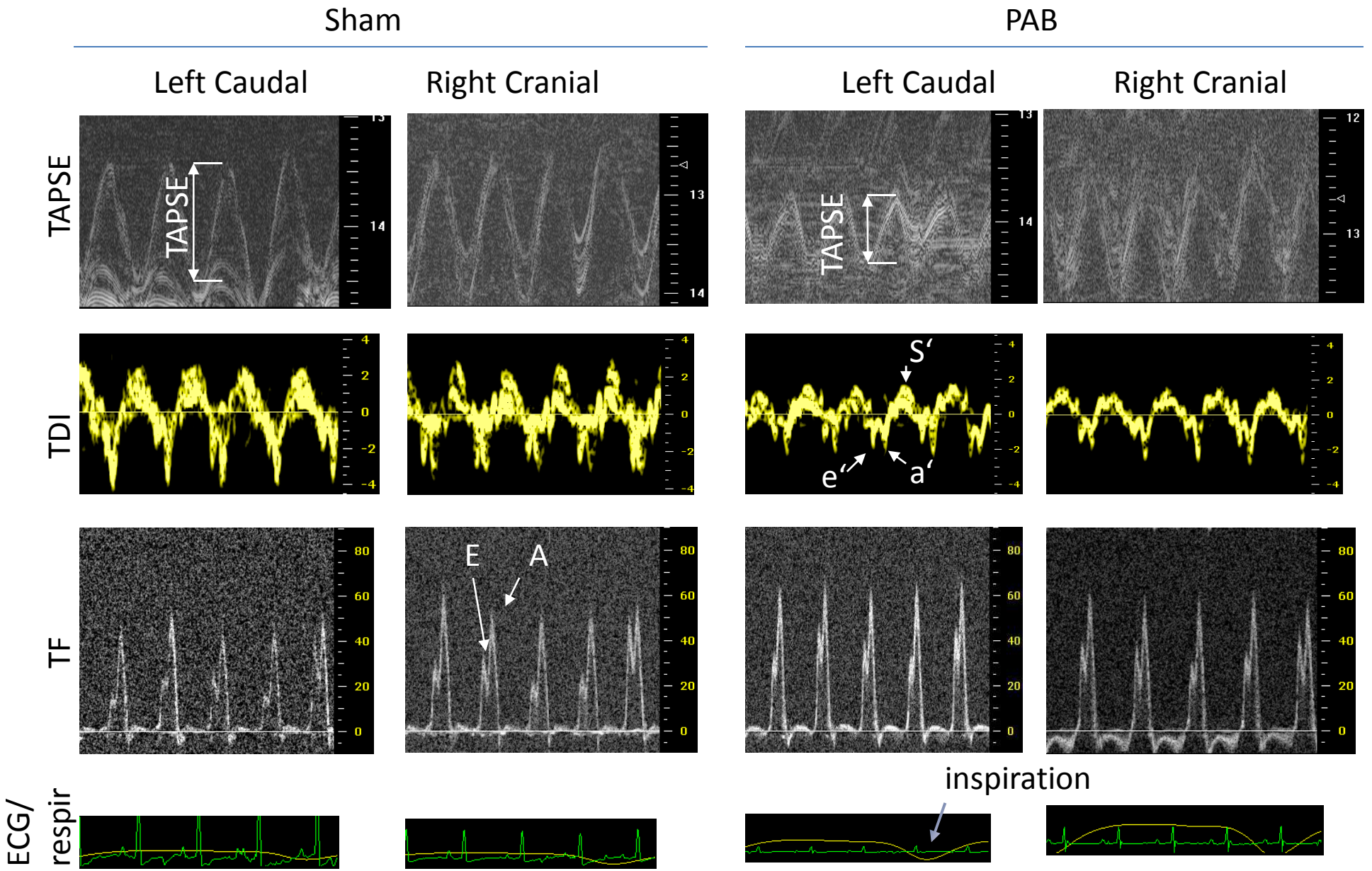
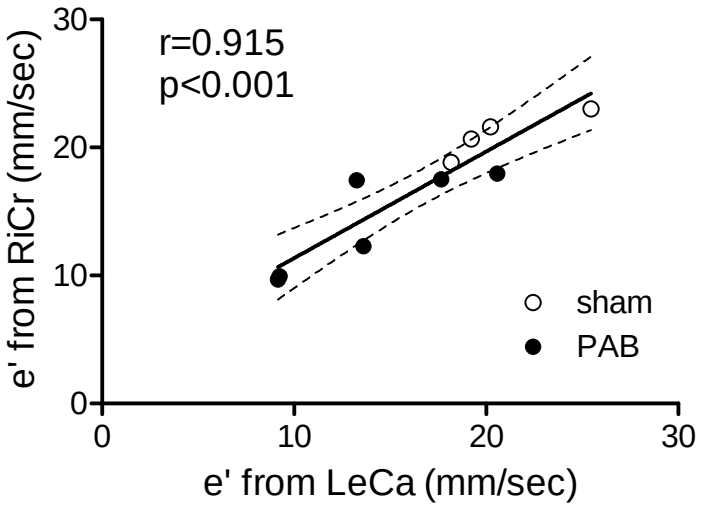
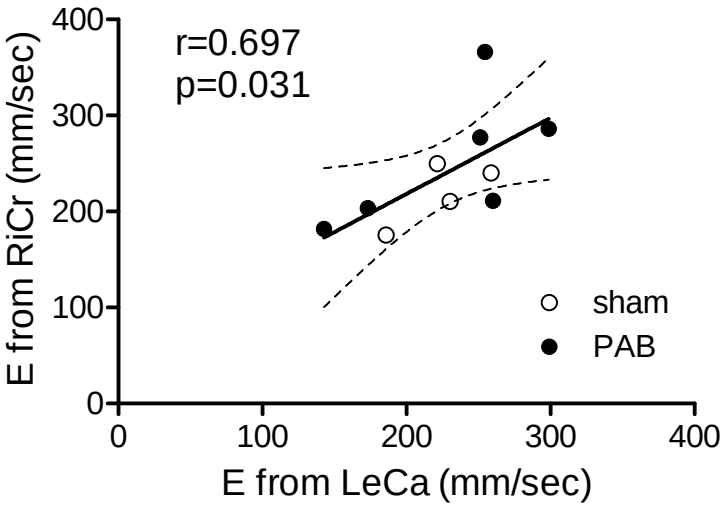
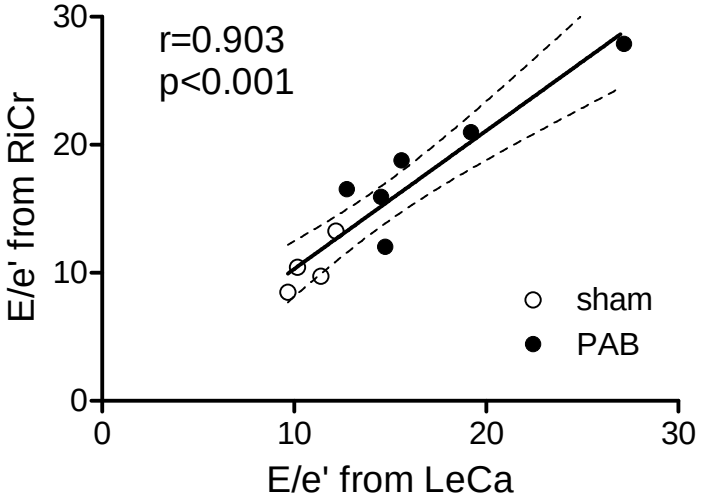


Fig. 2.







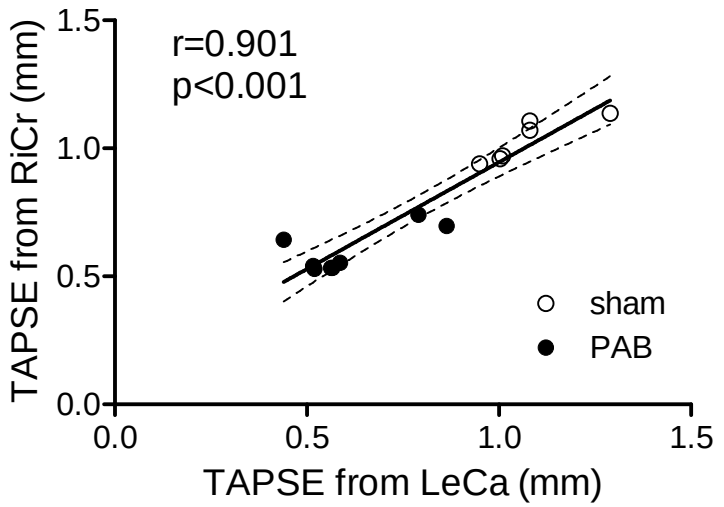


Fig. 3.

Table 1. Characterization of the operated groups 3 weeks after operation

Parameters	Sham	PAB	Mann Whitney test
	(mean ± SD)	(mean ± SD)	(p value)
Gradient velocity (mm/sec)		2.88 ± 0.15	
RVFW (mm)	0.19 ± 0.05	0.41 ± 0.1	0.006
Heart rate for VTI (beats per minute)	406 ± 39	413 ± 38	0.267
Stroke Volume (mcL)	38.9 ± 5.0	29.1 ± 8.6	0.016
Cardiac Output (ml/min)	15.7 ± 0.8	11.9 ± 3.4	0.043

Table 2. Comparison of the results obtained from apical four chamber view facilitated by the Right Cranial or Left Caudal platform tilt.

Parameter	Sham		PAB		Dunnet's post-hoc test	
	RiCr (mean ± SD)	LeCa (mean ± SD)	RiCr (mean ± SD)	LeCa (mean ± SD)	RiCr (Sham vs PAB)	LeCa (Sham vs PAB)
E (mm/sec)	222 ± 27	231 ± 30 §	251 ± 64	230 ± 59 §	n.s.	n.s.
A /mm/sec)	423 ± 50	404 ± 42 §	517 ± 74	491 ± 53 §	n.s.	n.s.
TAPSE (mm)	1.03 ± 0.08	1.08 ± 0.11 §	0.60 ± 0.08	0.61 ± 0.14 §	**	**
e' (mm/sec)	22.2 ± 2.4	20.1 ± 3.2 §	13.8 ± 3.7	13.9 ± 4.5 §	*	*
a' (mm/sec)	28.4 ± 4.4	26.9 ± 7.0 §	24.8 ± 4.9	26.2 ± 6.7 §	n.s.	n.s.
S (mm/sec)	23.8 ± 1.8	26.0 ± 3.8 §	18.4 ± 3.6	18.7 ± 6.3 §	n.s.	n.s.
E/e'	10.1 ± 1.7	11.7 ± 2.1 §	18.9 ± 5.0	17.3 ± 5.3 §	**	*
HR (bpm)	400 ± 33	401 ± 15 §	408 ± 41	409 ± 43 §	n.s.	n.s.

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
RMV-707B scan head 30 MHz	Visual Sonics	P/N 11459	mouse scan head
VisualSonics Vevo 770® High-Resolution Im	Visual Sonics	770-230	ultrasound machine
Veet depilation creme for sensitive skin	Veet	07768307	
Surgical tape Durapore 3M	3M Deutschland GmbH	1538-1	for fixation
Askina Brauncel cellulose swabs	B.Braun	9051015	
Aquasonic ultrasound gel	Parker Laboratories Inc.	BT025-0037L	
Electrode Gel	GE medical systems information t	2034731-002	apply to extremities for countinous ECG and heart rate monitoring
Thermasonic gel warmer	Parker Laboratories Inc.	82-04-20	to reduce heat loss warm up the ultrasound gel before use

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

Title of Article: ECHOCARDIOGRAPHIC MEASUREMENT OF RIGHT VENTRICULAR DIASTOLIC PARAMETERS IN MICE

Author(s): B. EGEMNAZAROV, G. KWAPISZEWSKA, LEIGH M. MARSH

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement (also known as CC-BY), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by/3.0/us/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and

(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

ARTICLE AND VIDEO LICENSE AGREEMENT – UK

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name: BAKYTBEK EGEMNAZAROV

Department:

Institution: LUDWIG BOLTZMANN INSTITUTE FOR LUNG VASCULAR RESEARCH

Article Title: Echocardiographic measurement of Right Ventricular diastolic parameters in mice

Signature: [signature] Date: 15.02.2018.

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy as a PDF to the *JoVE* submission site upon manuscript submission (preferred);
2) Fax the document to +1.866.381.2236; or
3) Mail the document to *JoVE* / Atn: *JoVE* Editorial / 1 Alewife Center Suite 200 / Cambridge, MA 02140

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use):

Dear Editor,

We thank for the support from the editorial team in the revision of our manuscript. In our revised manuscript, we have addressed all the critical points raised by reviewers. Please, find below our reply to reviewers' comments in the point-by-point fashion, a question *in italics* followed by our response.

Yours sincerely,

Bakytbek Egemnazarov on behalf of all co-authors.

Editorial comments:

1. The editor has formatted the manuscript as per the journal's style. Please retain the same.

We thank the editor for the work done.

2. Please address all the specific comments marked in the manuscript.

We introduced changes into manuscript according to comments.

3. For the protocol section, please specifically write how you perform your experiment with all the details in an imperative tense as if directing someone how to do the experiment.

In the revised version of the manuscript, we provide detailed description of every step during EchoCG paying attention to mentioning every button to press and expected action.

4. Please make the steps exactly how you perform the experiment with specific details.

We provide specific details to every step of the protocol.

5. The protocol should only be made up of action steps which should be numbered as 1. followed by 1.1 and 1.1.1 and so on. Please do not use paragraph style for this section. Also use complete sentences for the protocol section.

The protocol part is re-written in imperative tense using consecutive numbering.

Reviewer #1:

Manuscript Summary:

This revised manuscript by Egemnazarov B, et al. proposes an echocardiography-based assessment of right ventricle diastolic function in healthy and diseased mice. The authors addressed all the major concerns, as well as the majority of minor concerns raised by the previous review. The authors need careful and consistent editing throughout the manuscript, including the following minor concerns.

Major Concerns:
There is no major concern.

Minor Concerns:
Uncompleted work in response to the previous comment - References
References are not fully formatted for the journal style, e.g., lack of published year and capital letters in title.
We apologize for the missing the point. In the revised manuscript, we manually inspected every reference in our reference library and introduced necessary changes to match to the journal style.

New minor comment #1 - Text, Figure and Table editing
Comment #1-1: Already performed experiments/analysis should be described in the past tense, not in the present tense.

We thank the reviewer for drawing our attention to this point. In the revised manuscript, all sentences describing results are written in past tense.

Comment #1-2: Figure 3 "sham" → "Sham"

Changed accordingly.

Comment #1-3: Table 1 "parameters" → "Parameters"

Changed accordingly.

Comment #1-4: Table 2 "400±33" →"400 ± 33"

Changed accordingly

Comment #1-5: Table 2 "401±15" →"401 ± 15"

Changed accordingly

New minor comment #2 - Table 1
Comment #2-1: A unit of "heart rate for VTI" is missing.

Units (beats per minute) are added.

Comment #2-2: Is "mkl" correct?
Thank you for the remark. We corrected this mistake. In the revised manuscript, we use acronym “mcL” for microliters.

Reviewer #2:

Manuscript Summary:
The revised manuscript strengthened well, both the representative images and the data presentation of pathological conditions are valuable parts.
I have only one minor concern to add.

Minor Concerns:
-Table 2. and the statistical part is not clear. Are the data expressed as mean+SEM or SD?

We added a sentence explaining the reasons for selecting statistical tests with references to the part "Representative results". In the table 2, we added notifications that data are presented as *mean±SD.*

What does the p value of Krukal Wallis test tell us? Is it not more practical to show post hoc test p values to show differences between Sham and PAB groups?

We agree with the reviewer that the result Kruskal Wallis test alone is less informative, because it demonstrates only presence of significant differences between groups without specifying which ones. Therefore, we expanded the table and added results of Dunnet's post-hoc test between selected groups. In the revised version, the table 2 demonstrates that within groups every position delivers similar results (Wilcoxon test) and the results from both positions demonstrate differences between sham and PAB groups (Dunnet's test). This information is added to the "Representative results" part and expanded legend of the table 2.

The p value with 1.000 is not informative using rank tests.

We agree with the reviewer that this value delivers limited information; therefore, we have removed this information.

Was the data not following normal distribution?

With such small numbers of data points testing for distribution does not deliver reliable results. Therefore, we do not perform such test and routinely use non-parametric tests (Am J Ophthalmol. 2009 Apr;147(4):571-2; Int J Physiol Pathophysiol Pharmacol. 2017 Nov 1;9(5):157-163).

Both the statistics part and Table 2. legend might be more detailed and informative.

We expanded the legend of the table 2 by including more specific details about the tests used and results.