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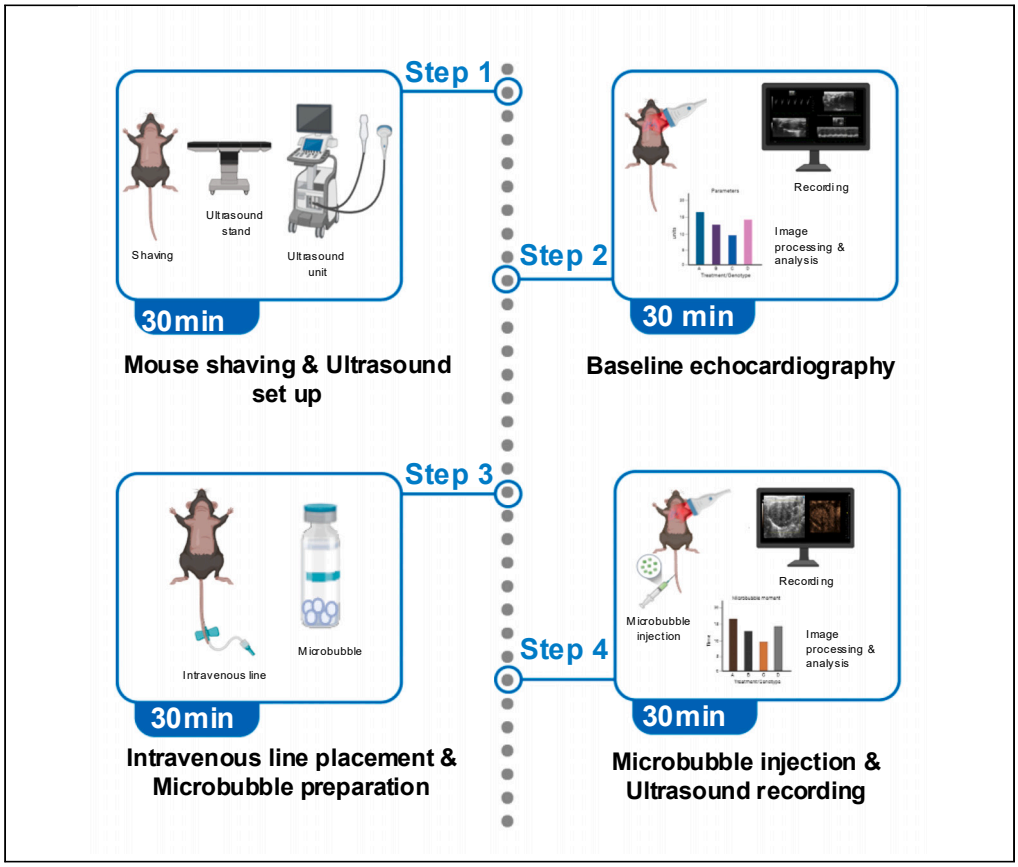
Protocol for monitoring intrapulmonary vasodilation in mice using contrast-enhanced echocardiography



Monitoring intrapulmonary vasodilation in animal models is vital for understanding pulmonary vascular physiology and disease mechanisms. Here, we present a protocol for the real-time visualization and quantification of pulmonary microvascular dynamics in living mice using contrast echocardiography alongside microbubble injection. We describe steps for mouse preparation, baseline echocardiography, microbubble administration, and imaging to capture dynamic changes in pulmonary vasculature. Closely resembling clinical technique used in humans, this methodology provides an effective tool for investigating the pathophysiology of diverse cardio-pulmonary vascular diseases.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for monitoring intrapulmonary vasodilation using contrast echocardiography

Instructions for monitoring cardiac functions by baseline echocardiography

Guidance on characterizing intrapulmonary vasodilation in hepatopulmonary syndrome

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Protocol for monitoring intrapulmonary vasodilation in mice using contrast-enhanced echocardiography

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SUMMARY

Monitoring intrapulmonary vasodilation in animal models is vital for understanding pulmonary vascular physiology and disease mechanisms. Here, we present a protocol for the real-time visualization and quantification of pulmonary microvascular dynamics in living mice using contrast echocardiography alongside microbubble injection. We describe steps for mouse preparation, baseline echocardiography, microbubble administration, and imaging to capture dynamic changes in pulmonary vasculature. Closely resembling clinical technique used in humans, this methodology provides an effective tool for investigating the pathophysiology of diverse cardio-pulmonary vascular diseases.

For complete details on the use and execution of this protocol, please refer to Dandavate et al.[1]

BEFORE YOU BEGIN

Importance of echocardiography

The gold standard method for detecting pulmonary vasodilation in humans is echocardiography combined with the injection of microbubbles (less than 10 μM diameter) into a peripheral vein.[2–4] In healthy patients, these microbubbles are trapped within the pulmonary circulation and absorbed by the alveoli[5] and do not appear in the left part of the heart.[2]

However, in the presence of pulmonary vasodilation, microbubbles bypass the pulmonary capture and reach the left part of the heart, a phenomenon detectable by echocardiography.[2] Currently, no established method exists for detecting pulmonary vasodilation *in vivo* in rodents aside from traditional histology and pathological examination. Here, we detail a method for monitoring *in vivo* intrapulmonary vasodilation in living animals, allowing real-time visualization and quantification of pulmonary microvascular dynamics. Our rodent protocol closely aligns with the clinical methodology used in humans, relying on contrast echocardiography alongside microbubble injection.

Recently, we applied this method to detect intrapulmonary vasodilation and diagnose hepatopulmonary syndrome in hepatic BMAL1 and HIF1α mutant mice.[1] This protocol enables the study of pulmonary vascular dilation in controlled and genetically modified animal models and can be expanded to test compounds that modulate pulmonary vascular physiology. Understanding the pathophysiology of intrapulmonary vasodilation and its progression in murine models of various cardiopulmonary pathologies is of clinical relevance and expected to guide the development of therapeutic interventions.

Ultrasound imaging

Ultrasound imaging is a powerful, non-invasive tool used in both humans and animals to observe the structure and function of internal organs.[6–9] This technique is painless and widely accessible, though it requires proper training to obtain accurate images and avoid biases in interpretation. The process involves emitting ultrasounds (i.e., sound at a frequency higher than 20 kHz) of a specific frequency from a transducer; these echoes are sent out in trains, reflect off organs, tissues and fluids and are analyzed to generate an 2D image in real-time, displayed using a gray scale to code the intensity of the returning echoes[7] Those images are called B-mode, for "brightness modulated".[7] Each body part has its own echogenicity, enabling their identification and detailed observation. Different transducers, varying in frequency, are available depending on the required depth and resolution.[10] Higher frequencies provide images that are more detailed, but the penetration becomes more limited.[7,11]

For small rodents like mice, ultra-high frequency transducers (between 30 and 40 MHz) are typically used, offering precision up to 30 μm.[10] The procedure is safe and painless, though various artifacts can occur, especially in the presence of air or gas.

Contrast-enhanced ultrasound (CEUS) has been developed as a technique that takes advantage of the specific properties of microbubbles, composed of gas encapsulated within a lipid shell.[11,12] As such, they provide a strongly reflective blood/gas interface, and they resonate in the ultrasound beam by contracting and expanding in response to the pressure changes of the sound wave.[7] They can be used to observe, monitor and even quantify specific parameters related to the vascularization and perfusion of organs in real time.[13] These microbubbles are injected intravenously, and their movement is tracked using a specific ultrasound-imaging program, enabling the detailed visualization of blood vessels and neovascularization. Their structure and composition can be modified according to the need of the experiment (here we used non-targeted microbubbles filled with neutral gas). They typically remain in the animal for up to 30 min, are safe, and provide a valuable tool for vascular ultrasound imaging.

Institutional permission

All animal experiments and procedures outlined in this protocol have been approved by the Weizmann Institute's Institutional Animal Care and Use Committee (IACUC). Prior to conducting this protocol, it is critical to obtain the necessary approvals from the relevant institutional authorities.

Preparation of ultrasound stand

Timing: ~5–10 min

Since the procedure is relatively brief and prolonged anesthesia with isoflurane can affect mouse hemodynamics, it is important to have the entire setup ready before placing the animal on the handling table.

1. Secure the anesthesia mask to the mouse handling table, ensuring it is ready to deliver 1–2% isoflurane.
2. Apply a drop of electrode gel to each of the four electrodes on the mouse handling plate to monitor electrocardiogram (ECG) and respiratory rate during the ultrasound measurement.
3. Prepare four pieces of surgical tape in advance to secure the mouse's paws to the electrodes on the handling plate.
4. Begin monitoring the ECG on the ultrasound screen immediately after the mouse is taped to the electrodes.

Microbubble preparation

Timing: ~5–10 min

The commercially available Lyophilized Vevo Micromarker non-targeted contrast agent is used for this protocol. Preparation of microbubble should be performed as specified below:

5. Resuspend a vial of Lyophilized Vevo Micromarker non-targeted contrast agent with 0.7 mL sterile saline using 1 mL syringe with 21G needle.

   Note: Resuspension of Lyophilized Vevo Micromarker should be performed just before the experiment. In addition, here, we used microbubbles from Vevo Micromarker, you can use them from another supplier as well (troubleshooting problem 1).

6. Gently agitate the vial in a top-to-bottom manner by hand for 1 min and let it wait for 10 min at room temperature.
7. The resulting final concentration of microbubbles in the vial is 2×10^9 microbubbles/mL.
8. Further dilute the stock solution 1:10 (15 μL from microbubble stock to 135 μL sterile saline) to have final concentration of 1×10^7 microbubbles/50 μL.
9. Use 50 μL (contains 1×10^7 microbubbles) of above microbubble suspension for tail vein administration in mice.

   Note: Dilutions with adequate and gentle mixing must be performed into an Eppendorf tube immediately prior to an injection to prevent microbubble destabilization. Any additional contrast agent removed from the vial should be discarded.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Vevo MicroMarker non-targeted contrast agent kit	ElsMed/FUJIFILM VisualSonics	#1193
Phosphate-buffered saline (pH 7.4)	Sartorius	02-020-1A
Heparin sodium	Panpharma	10542
Isoflurane	Novolog	NA
Depilatory cream	Nair	NA
Experimental models: Organisms/strains		
AlbCre^{+} mice (B6.Cg-Speer6-ps1Tg(Alb-cre)21Mgn/J) male, 3 weeks old	Jackson Laboratory	Strain #:003574
Bmal1$^{fl/fl}$ mice (B6.129S4(Cg)-Bmal1tm1Weit/J) male, 3 weeks old	Jackson Laboratory	Strain #:007668
Hif1α$^{fl/fl}$ mice (B6.129-Hif1atm3Rsjo/J) male, 3 weeks old	Jackson Laboratory	Strain #:007561
AlbCre^{+} *Bmal1*$^{fl/fl}$ *Hif1α*$^{fl/fl}$ mice, Male, 3 weeks old	Dandavate et al.[1]	NA
Software and algorithms		
NCL program	ElsMed/FUJIFILM VisualSonics	11953
Vevo LAB software (version 5.8.1)	ElsMed/FUJIFILM VisualSonics	VS-20034, https://www.visualsonics.com/product/software/vevo-lab
Other		
27G needle	Medharmony	150410
Syringe	Medharmony	160715
IV catheter	BD	NA
Sterile saline	Enzo	ALX-505-009-LD15
Ultrasound gel	Vetmarket	148140
Surgical tape	Superfarm	NA
Tube 0.025″ OD × 0.12″ ID	Instech	VAHBPU-T25

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sterile gauze pad 10 × 10	Sion Medical	030592Y
Vevo 3100	ElsMed/FUJIFILM VisualSonics	010017370
Transducer, 15–30 MHz, 30 mm, linear	ElsMed/FUJIFILM VisualSonics	MX250
Transducer, 25–55 MHz, 15 mm, linear	ElsMed/FUJIFILM VisualSonics	MX550D

MATERIALS AND EQUIPMENT

Mice

This method can be applied to any mouse strain. Here we used three to four months-old male AlbCRE and BHLKO mice were used. AlbCre+ Bmal1fl/fl Hif1αfl/fl (BHLKO) were generated by crossing AlbCre+ mice with Bmal1fl/fl, Hif1αfl/fl (Jackson Laboratories). Animals were housed in an SPF animal facility, at room temperature of ≈ 22°C, under a 12 h light-dark regimen (LD) and fed ad libitum.

Ultrasound requirement

In this protocol, we have used a Vevo 3100 ultrasound machine from Fujifilm VisualSonics. To perform the baseline echocardiography, we used a MX550D transducer, with a frequency of 40 MHz, a frame rate of 200-230 fps and gain of 30 dB. For the contrast enhanced ultrasound (CEUS), we worked with an MX250 transducer, with a frequency of 18 Mz, frame rate of 25 fps and contrast gain of 30 dB. The visualization of microbubbles requires the use of a specific recording program (NLC-nonlinear contrast, from Fujifilm Visual Sonic) Also, it needs to be visualized under relatively low frequency because the microbubbles resonate more effectively with those frequencies, generating strong echoes and enhancing the imaging of the blood flow. With higher frequencies, it may compromise the integrity of the microbubbles.

STEP-BY-STEP METHOD DETAILS

Here we provide detailed steps involved in mouse echocardiography with microbubble injection (Figure 1). Due to the IACUC requirements and the sensitivity of mice to isoflurane exposure (drop in blood pressure, bradycardia), each procedure requiring anesthesia should be performed on a different day, with sufficient time to allow the mice to recover from the procedure (Figure 1 and troubleshooting problem 2).

> ***Note:*** It is recommended to shave the mouse, the day before the ultrasound.

A day before ultrasound

> **Timing: 5–10 min per mouse**

This step describes how to remove fur from the mouse chest (Figure 1).

> ***Note:*** The first stage involves preparing the mouse for ultrasound (Figure 1). The mouse chest needs to shave from the neck to abdomen and on the sides to maximize the resolution and avoid any artifact caused by remnants of fur since we will be working with high frequency ultrasound. To shave the mice, depilatory cream and gauze pad (dry and wet) are recommended. Hair clipper can also be used, but it is less recommended, since the result is usually less thorough.

1. Anesthetize the mouse with 2% isoflurane in the induction box.
2. Once it is asleep, take mouse out from an induction box and apply enough amount of depilatory cream from mouse neck to the pelvis and on the side of the body.

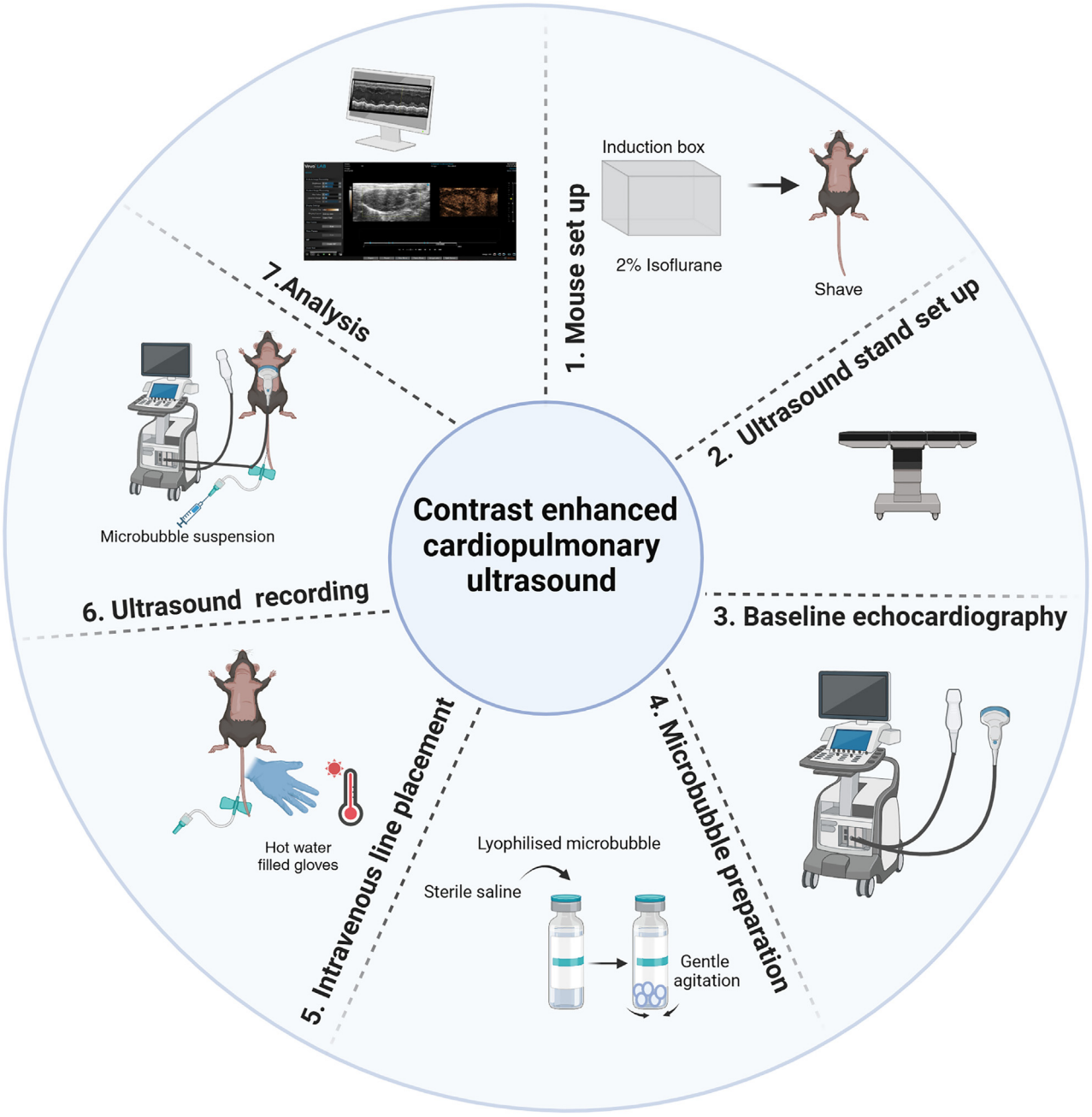

Figure 1. A schematic illustration of the major steps for cardiopulmonary contrast-enhanced echocardiography

Note: The sensitivity and compatibility of depilatory cream should be checked before use.

3. Return mouse in the induction box with the isoflurane delivery to keep it anesthetized, preventing it waking up and licking the cream.
4. Wait for 2 min.
5. Take out the mouse from the induction box and gently wipe out cream, first with a dry gauze pad and then with a wet pad.
6. Confirm the shaved status of the mouse.

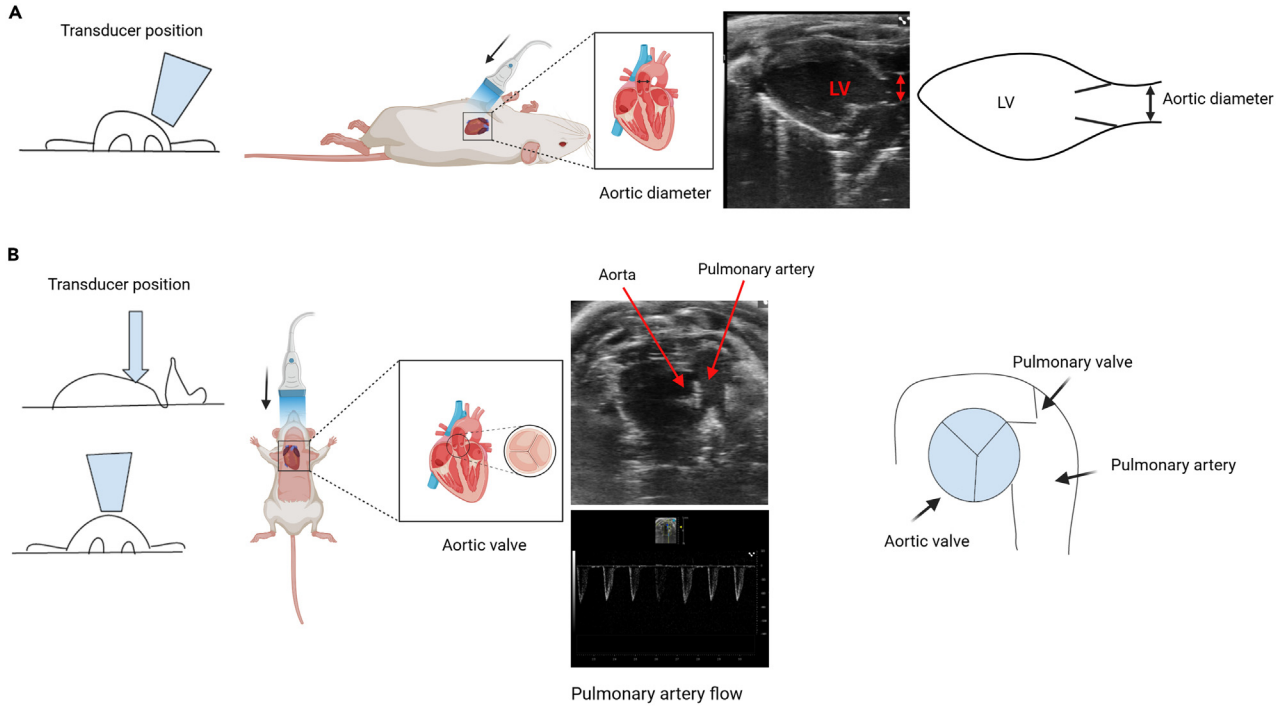

Figure 2. Transducer positioning

(A and B) A schematic representation depicting transducer positioning for the assessment of the Left Ventricle (LV) outflow tract diameter via the parasternal long axis view (A), and pulmonary flow via the parasternal modified short axis transaortic view (B) in albCRE mice with echocardiography.

7. Allow it to recover in a dry and warm cage placed either under a lamp or on a warming pad (troubleshooting problem 2).
8. Once the mouse is fully awake and moving, return to its cage.

⚠ CRITICAL: The presence of shreds of hair during ultrasound can lead to significant artifacts or distortions in the resulting images or recordings.

Baseline (echocardiography and abdominal ultrasound)

Timing: 15–30 min

This step describes how to perform baseline echocardiogram to validate health status of a mouse.

Before the experiment with CEUS, it is recommended to perform a complete echocardiogram to exclude any dysfunction or congenital defect in the mouse. An abdominal ultrasound can also be performed to validate the health status or any other anatomical issue of the mouse. In this part, we will focus on the heart, since it is the aim for the CEUS experiment (Figures 2 and 3). The echocardiogram shall be performed using the conventional views described.[9,14,15] Heart and respiratory rates must be monitored throughout the exploration, since isoflurane can cause a severe depression of the cardiovascular system and influence the heart function (troubleshooting problems 3 and 4).

9. Anesthesia and position of the mouse.
   a. Gently place the mouse in the induction cage and turn on the isoflurane flow to 2%.
   b. Once the mouse is anesthetized, quickly remove it from the induction cage.
   c. Position it on the warmed mouse handling table in a supine position and switch the anesthesia flow toward the mask.

A

	Mice (n=5)	SEM
Systolic parameter		
Ejection Fraction (%)	54.32	4.168
Fractional Shortening (%)	9.733	1.812
Stroke Volume (uL)	30.57	3.294
Cardiac Output (ml/min)	16.55	1.802
Heart rate (bpm)	541.4	17.26

Dimensions	**Mice (n=5)**	**SEM**
Volume (d, uL)	56.45	5.384
Volume (s, uL)	25.88	3.447
LVIDd (mm)	3.724	0.2347
LVIDs (mm)	2.527	0.2173
LA (mm2)	3.948	0.5425

Diastolic parameters	**Mice (n=5)**	**SEM**
E/E'	-30.9	3.308
IVRT (ms)	20.87	0.587
IVCT (ms)	14.95	1.281
AET (ms)	46.03	2.625
MVDecel (ms)	19.19	2.283

Global Parameter	**Mice (n=5)**	**SEM**
MPI	0.7499	0.05152

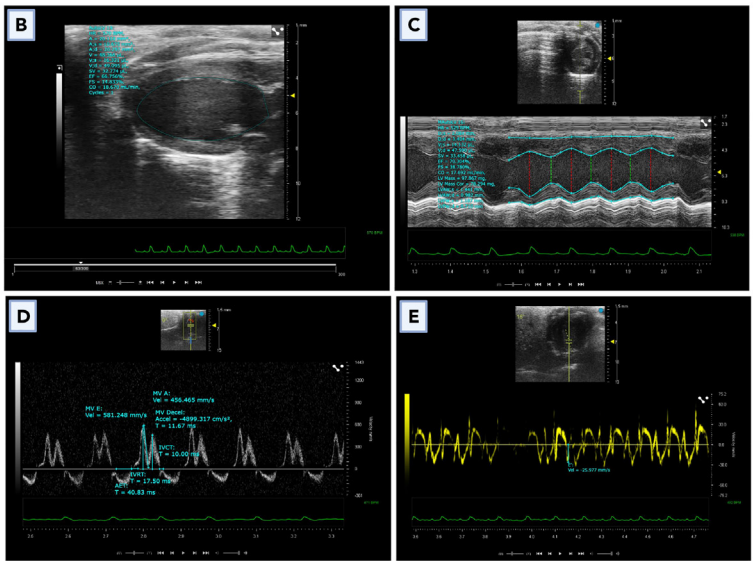

Figure 3. Baseline echocardiography

(A) List of baseline echocardiographic measurements for four-month-old male albCRE mice. Data represented as mean, *n* = 5 mice LVID = left ventricle internal diameter (d = in diastole and s in systole); LA = left atrium; IVRT = isovolumetric relaxation time; IVCT = isovolumetric contraction time; AET = aortic ejection time; MPI = myocardial Performance Index; Mvdecel = mitral valve flow deceleration (B-E) Representative baseline echocardiographic images showing LV systolic function measurement (B), M-Mode dimension measurement (C), diastolic parameters measurements (D), tissue Doppler imaging measurements (E) from albCRE mice.

d. Gently tape four paws on the electrodes in order to obtain the heart and respiratory rate monitoring.
e. Spread warmed ultrasound gel onto the mouse chest.

Note: It is recommended to also follow the temperature of the mouse in order to avoid hypothermia.

10. Left Ventricular (LV) parasternal long axis view in B Mode.

Note: To evaluate the systolic function, via the calculation of Ejection Fraction (EF), Fractional Shortening (FS), Cardiac Output (CO) and Stroke Volume (SV). LV volume (in diastole and systole), LV outflow tract diameter (LVOT) can also be measured this way (Figures 2 and 3A).

a. Position the transducer centrally on the mouse's chest, with the focal point directed toward the left shoulder (Figure 2A).
b. Adjust the transducer until the left ventricle, mitral valve, left atrium, and aorta are clearly visible (Figure 3B).
c. Save at least 3 cineloops.

11. Parasternal short axis LV view with M Mode.

Note: Measure anterior and posterior LV wall thickness (in systole and diastole), LV internal diameter (in diastole and systole), FS (M-Mode), heart rate (HR) (Figure 3C).

a. Staying in B-mode, rotate the transducer to 90 degrees on the right from the precedent position.
b. Visualize the left ventricle and the two papillary muscles supporting chordae tendinae of the mitral valve.

c. Place the M-Mode curser at the widest part of the left ventricle cavity and press play.
d. Save at least 3 cineloops.

12. Parasternal short axis transaortic view in B Mode.

Note: Measure the aortic diameter, pulmonary artery diameter, pulmonary artery flow to eliminate any sign of pulmonary stenosis (Figure 2B).

a. Move the transducer cranially until the aortic valve is visible.
b. Identify the aortic valve, left atrium.
c. Save at least 3 cineloops.
d. Move the transducer slightly further up cranially until the pulmonary artery is visible (Figure 2B).
e. Use Color Doppler to visualize the pulmonary flow and pulmonary valve.
f. Place the Pulsed-Wave Doppler sample after the pulmonary valve and press play.
g. Save at least 3 cineloops.

13. Aortic arch view.

Note: To access aortic flow to eliminate any signs of aortic stenosis.

a. Tilt mouse-handling table fully clockwise.
b. Position the transducer on the right side of the upper chest parallel to the body.
c. In B-mode, visualize the aortic arch and its branching arteries.
d. Use Color Doppler to visualize the blood flow in the aorta.
e. Place the Pulsed-Wave Doppler sample in the descending aorta and press play.
f. Save at least 3 cineloops.

14. Four chamber view with PW, color Doppler and tissue Doppler imaging.

Note: To record E and A wave speed, E′ wave speed, MV deceleration time, isovolumetric relaxation time (IVRT), Isovolumetric contraction time (IVCT) and aortic ejection time (AET) (Figures 3D and 3E).

Those parameters are critical to diagnose any diastolic dysfunction and provide information for the calculation of the Myocardial Performance Index, a parameter summarizing both systolic and diastolic function of the heart.

a. Place the transducer transversally to the mouse and below the ribcage.
b. Adjust the transducer position until the identification of four chambers of the heart (2 atria and 2 ventricles).
c. Visualize the left side of the heart.
d. Use the color Doppler to visualize the transmitral blood flow.
e. Place the sample of the pulsed wave Doppler at the tip of the mitral valve and press play.
f. Record 3 cineloops at least.
g. Move to tissue Doppler imaging function.
h. Place the sample Doppler on the base of the medial valve and press play.
i. Save at least 3 cineloops.

15. Calculations:
Calculations are performed either automatically with the Vevo Lab software (E/E′, MPI) or after measurements.
a. E/E′ to characterize the diastolic function.
b. MPI (myocardial performance index): $MPI = (IVRT + IVCT)/AET$

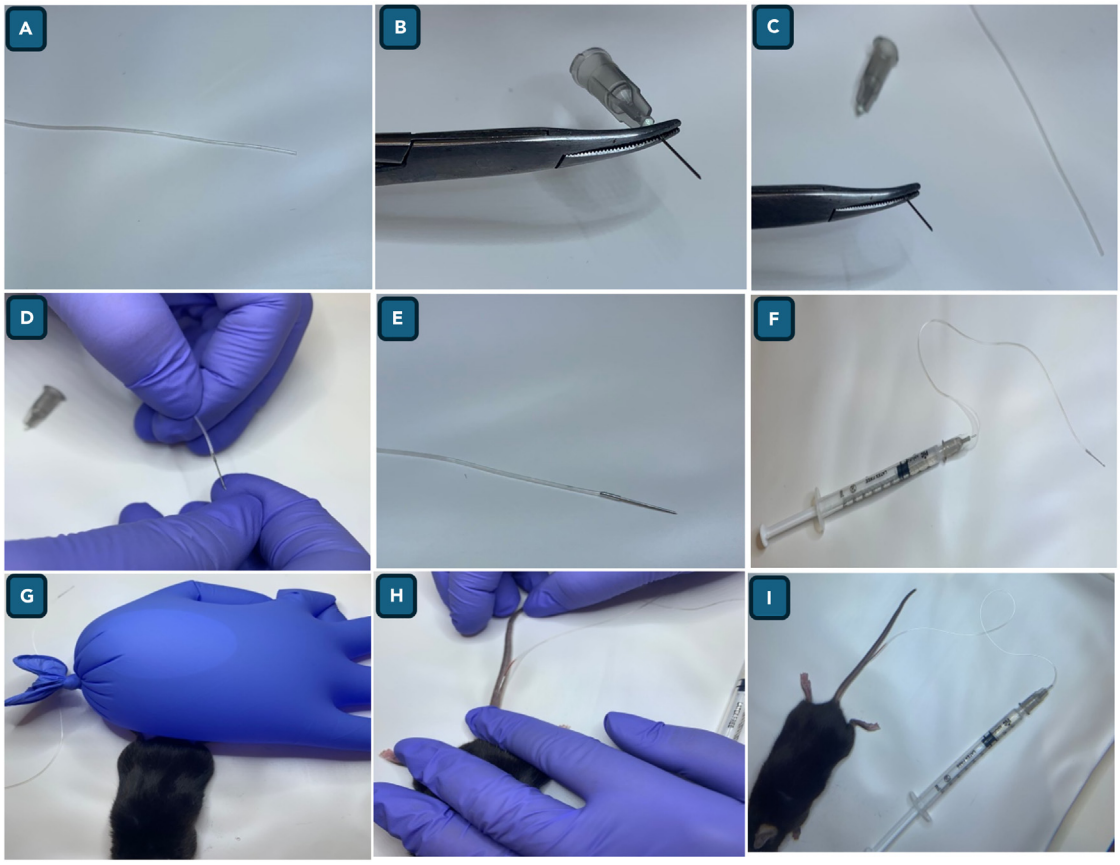

Figure 4. Images depicting the steps involved in mouse tail vein intravenous line placement

Needle preparation (A–C), handling and setup (D and E), PBS loading (F), needle insertion (G and H), and final placement confirmation (I).

c. Qp/Qs[16,17]: Qp and Qs are the pulmonary and systemic blood flow measurement respectively. To perform this calculation, you need to measure the velocity time integral from the pulmonary and aortic flow recordings (PAVTI and AoVTI, respectively), as well as the diameters of the left ventricular outflow tract (dLVOT) and the right ventricular outflow tract (dRVOT). Finally, the ration of Qp/Qs is calculated as:

$$Qp / Qs = \left(\pi * (dRVOT/2)^2 * PAVTI\right) / \left(\pi * (dLVOT/2)^2 * AoVTI\right)$$

For instance, basal echocardiographic measurements were performed in control albCRE mice for assessment of different parameters as in Figures 3A and 3B, troubleshooting problems 3 and 4).

Intravenous line placement

Timing: variable, 5–20 min

This step provides systematic explanation for how to place intravenous line in mouse tail for microbubble injection (Figure 4).

Intravenous-line placement is an essential procedure that must be completed just before the ultrasound recording. Follow the steps below for intravenous -line placement (Figures 4A–4I):

16. Prepare the Needle: Take a 27G needle and use forceps to break the needle off from the plastic part (Figures 4A–4C).

17. Assemble the Tubing: Insert the needle into one end of the tube, ensuring the pointed end is facing outward (Figures 4D and 4E).
18. Attach Syringe: Connect the other end of the tube to a needle attached to a syringe containing PBS (Figure 4F).
19. Anesthesia: Place the mouse in an induction chamber with 2% isoflurane to anesthetize it.
20. Prepare Mouse Tail: Warm the tail by placing it between two hot water-filled gloves to promote vasodilation, making the tail vein more visible (Figure 4G).
21. Insert the Needle: Gently insert the needle connected to the PBS-filled tube into the mouse's tail vein (Figure 4H).
22. Verify Placement: When blood is visible in the proximal part of the tube, gently inject PBS into the tail to confirm correct needle placement (Figure 4I, troubleshooting problem 5).
23. Mouse Transfer: Quickly transfer the mouse to the handling plate for further procedures.

Note: The use of a magnifying glass or binoculars can be very helpful to visualize the vein and facilitate the intra venous line insertion. Care must be taken when moving the mouse to the handling plate to avoid shifting the position of the intravenous line, as this could prevent proper injections from being administered.

Recording

Timing: variable, 5–7 min

This step describes ultrasound recording with tail vein injection of microbubble.

24. Place and install anesthetized mouse with the intra venous line onto the warmed mouse handling plate in a supine position.
25. Gently tape the paws to the electrodes to monitor the heart rate, ECG and respiratory rate.
26. Spread warm ultrasound gel on the mouse chest.
27. Using the handle, lower the transducer onto the center of the mouse chest and adjust it to obtain a left parasternal long-axis view with two heart cavities. Identify the left atrium (LA), left ventricle (LV), and left ventricular outflow tract (LVOT).
28. Turn on the Non-Linear Contrast (NLC) mode on the Vevo 3100 and move the rectangle of the measurement on the LV.
29. Check intra venous line patency by flushing 100 μL of saline and mark the time of injection, beginning and end using the option "add Frame Marker" in the NLC program.
30. Inject 50 μL of diluted microbubbles solution and mark the time of injection, beginning and end (Video S1, troubleshooting problem 1).
31. Once the microbubbles have appeared in the LV, save the cineloop (Videos S1 and S2).

Analysis

This stage provides stepwise explanation of image acquisition and data processing.

32. Open the Vevo Lab software (version 5.8.1 was used in this instance).

Note: During the recording of the microbubbles, the heart rate (HR) should be visible since the mouse's paws are taped on the mouse handling plate's electrodes.

33. Record the frame number at the time of injection and the first frame number where microbubbles are visible in the LV (Videos S1 and S2).

⚠ CRITICAL: To accurately document the timing of microbubble injections during your experiment, it's essential to insert a frame marker, a notation on the cineloop's timeline indicating when a critical event occurs. In the Vevo 3100 system, you can add a frame

marker by navigating to "General Control," selecting "More Control," and then choosing the "Add Frame Marker" option.

34. Open Vevo analysis software.
35. After launching the Vevo analysis software, document the animal's heart rate (HR).
36. Utilize the time cursor to locate and note the frame where the microbubbles are injected (Fi).
37. Similarly, identify and record the frame where microbubbles first appear in the left side of the heart (Fv).
38. Calculate the time difference (T) between Fi and Fv using the formula:

$$T = (Fi - Fv)X25$$

The system operates at a frame rate of 25 frames per second.

39. Then normalize the above time difference (Tn) with animal heart rate (HR) during this part of the experiment: $Tn = T/HR$.

For Instance:

Fv = 899. Fi = 888. HR = 624 bpm.

T =(899-888)x 25 = 275.

Tn = 275/624 = 0.441

Note: The time from injection until the appearance of microbubbles in the LV was normalized to the animal's heart rate during this time window to reduce the impact of heart rate variability between mice.

(troubleshooting: problems 1, 2, 3, 4, and 5).

EXPECTED OUTCOMES

Microbubbles are trapped in the pulmonary circulation and absorbed by the alveoli. However, in the presence of pulmonary vasodilation, microbubbles evade pulmonary capture and reach the heart faster, and can be detected by echocardiography (Figure 5). We previously reported that the microbubble reaches the left ventricle of the heart faster in three-month-old male BHLKO mice compared with same aged control AlbCRE mice supporting the presence of pulmonary vasodilation in these mice (Figure 6, Video S2).[1]

LIMITATIONS

The MX250 ultrasound probe, intended primarily for use in rats due to its lower frequency, provides a lower resolution compared to the MX550D, which is specifically designed for mice. The selection of a lower-frequency transducer is crucial for preserving microbubble integrity during imaging. This reduced resolution impacts image quality, making it more difficult to obtain highly detailed visuals.[7,11] While the use of this transducer still provides a correct image of key structures such as the left ventricle and left ventricular outflow tract , the smaller left atrium may be more challenging to visualize clearly, owing to both its size and the limitations in resolution.

Moreover, the effectiveness of echocardiography using this equipment is highly dependent on the operator's skill level.[18] Obtaining accurate and interpretable images requires extensive training, and the quality of the analysis can vary significantly based on the operator's experience and technique.

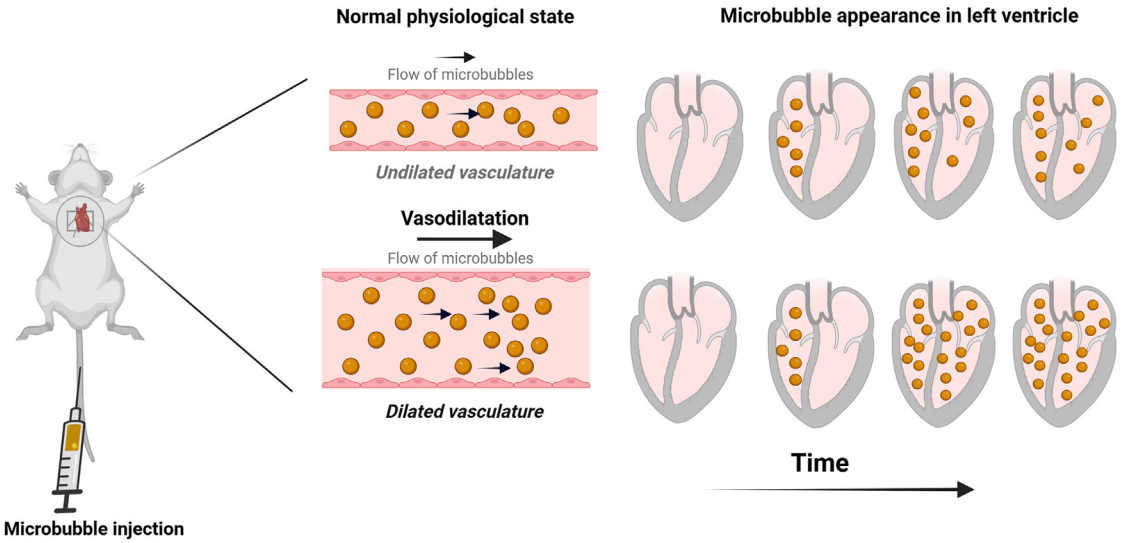

Figure 5. Principle of contrast echocardiography for monitoring pulmonary vasodilation

Microbubbles that are injected to the tail vein travel from the right part of heart through pulmonary vasculature and reach left part of heart. In the case of pulmonary vasodilation microbubbles appear earlier in the heart compared to normal physiological state.

This operator dependency underscores the need for proficiency in using the equipment to ensure reliable and consistent results.

TROUBLESHOOTING

Problem 1

Dilution reduces the stability of the contrast agent.

Potential solution

Prepare the contrast agent just before the beginning of the experiment. Any diluted contrast should be discarded if not used within the few hours from the time of the dilution, as should any unused stock solution.

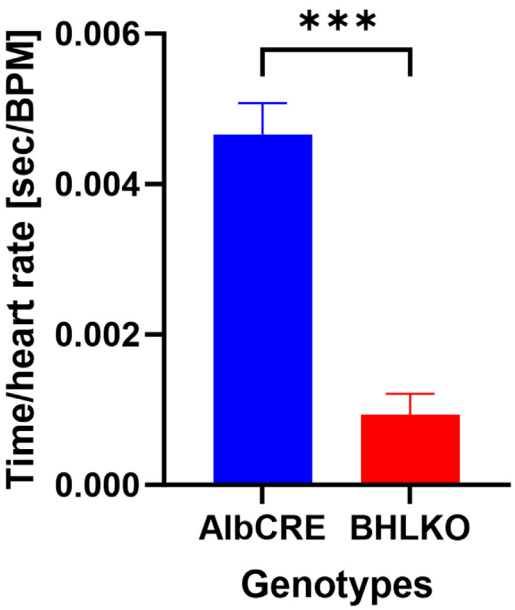

Figure 6. Echocardiography measurements in AlbCRE vs. BHLKO mice

Echocardiography measurements of the time taken for the microbubbles to reach the heart's left ventricle following tail vein injection, normalized by heart rate in AlbCRE vs. BHLKO mice. Data are presented as the mean ± SEM, $n = 4$ mice per genotype); ***$p < 0.001$ Student's t test. The data in the graph was adapted from Dandavate et al., 2024.[1]

Problem 2
Inadequate warming leads to bradycardia.

Potential solution
Ensure the mouse is properly warmed and monitored to prevent bradycardia and maintain normal physiological conditions. Use a warming plate during the experiment and continuously monitor vital parameters (heart rate, respiratory rate, temperature). If there is a significant drop in these parameters, exclude the results from the analysis.

Problem 3
Variations in the measurements.

Potential solution
Mice can exhibit significant biological variations in respiration rate and heart rate due to genetics, physiology, or sensitivity to anesthesia. To minimize the impact of these variations, it is recommended to use a larger sample size in the protocol.

Problem 4
The echocardiogram part of the baseline measurement requires training and experience, and the results can be user dependent.

Potential solution
It is recommended to train before the experiment to obtain correct views of the heart and thus trust the measurement performed. Also, using the same person to perform the imaging and analysis is advised, to avoid any inter-user bias.

Problem 5
The intravenous line must be properly positioned within the tail vein for the injection to be successful. The line's patency should be verified before administering the microbubble solution.

Potential solution
Use warm water-filled gloves or a warming lamp to promote vasodilation of the tail and improve vein visibility. If more than three attempts are needed to insert the line, it's recommended to stop the experiment for that mouse. Either exclude the mouse from the study or postpone the experiment by a week to allow the vein to recover and prevent peri-venous injection.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gad Asher (gad.asher@weizmann.ac.il).

Technical contact
Further information and technical questions on the protocol should be directed to and will be answered by the technical contact, Hanna Bueno-Levy (hanna.bueno-levy@weizmann.ac.il).

Materials availability
This protocol did not generate any unique reagents.

Data and code availability
This protocol did not generate any unique codes and datasets.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Conceptualizing and optimizing the protocol, N.B., H.B.-L., and G.A.; writing, N.B., H.B.-L., and G.A.; graphics, N.B.; pictures and movies, H.B.-L.; supervision and funding acquisition, G.A.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.103647.

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