

APPENDIX 1. PROTOCOL FOR IMAGE ACQUISITION AND DATA ANALYSIS

Measurements: PAAT/ET, RVWTd and TAPSE

Image Acquisition

The echocardiography machine is Vevo 2100 and 3100 with color Doppler from Visualsonics (<http://www.visualsonics.com/>). The probe used is MS250 or MX250 respectively.

- Determine the body weight of the rat before initiating the echocardiography procedure
- Anesthetize the animal with isoflurane (1-3%) in a chamber and then place rat on the Vevo Imaging Station in the supine position and provide anesthesia via a nosecone. Titrate to maintain adequate sedation level (0.5-1.5 %).
- Secure the arms and legs of the rat with tape on the appropriate EKG lead positions on the stage and use electrode cream to ensure appropriate heart rate monitoring without artifact. Ensure hear rate is >300 beats per minute (bpm) during the echocardiography procedure.
- Place the temperature probe and ensure normothermia. Can use an extra heat lamp for additional heat source.
- Shave the chest of the rat using rodent clippers. Remove extra fine fur by applying Surgiprep depilatory crème for 1-2 minutes and then removing with a warm wet gauze.
- Apply pre-warmed echocardiography gel onto the shaved chest and the head of the transducer.
Tip: Can centrifuge in advance to remove air bubbles.
- Place the transducer vertically with the notch pointing towards the apex of the heart and obtain the two-dimensional (2D) parasternal long-axis view (LAX) in B-mode. Subsequently rotate the transducer 90° clockwise and obtain the short-axis view (SAX) at the level of the papillary muscle. Views:
 - Pulmonary Artery Acceleration Time (PAAT)¹. Pulsed-wave Doppler was used to measure PAAT. Locate an optimal location for PA flow measurement and place the

- pulsed-wave Doppler sample volume in the center of the color Doppler PA signal at the level where the medial aspect of the proximal aorta crosses the PA. PAAT (the time from the onset of systolic flow to peak pulmonary outflow velocity) and ET (the time from onset to completion of systolic pulmonary arterial flow) were measured.
- RVW Thickness (mm)¹. Modified right parasternal long axis imaging view. In M-mode place the ultrasonic beam positioned across the RV free wall perpendicular to the RV long axis at the level of the mitral valve annulus.
 - Tricuspid annular plane systolic excursion (TAPSE, mm)². In the subcostal 4- chamber view place the M-mode sample volume across the tricuspid valve near its attachment to the RV free wall.
- Obtain at least 3 consecutive cardiac cycles for each measurement with avoidance of beats that occur during breaths.
 - After successful recordings of desired measurements ensure removal of echocardiography gel, heating pad, and transducer. Release the rat by removing the tape and place back in the cage for monitoring until fully recovered.

Image Analysis

The echocardiography program used for analysis is Vevo LAB by Visualsonics (<http://www.visualsonics.com/>).

PAAT/ET:

- Measure the PAAT and PA ejection time (ET) from each Doppler spectral flow velocity envelope.

- PAAT is the time interval between onset of systolic ejection and the peak flow velocity (Suppl. Fig. 1 A, B).
- ET is the interval between the onset of PA ejection to the point of systolic pulmonary arterial flow cessation (Suppl. Fig. 1 A, B).
- Measure at least 3 consecutive cardiac cycles and calculate the average of three to account for respiratory variation in pulmonary flow.
- The ratio of the PAAT to ET is calculated by dividing PAAT by ET.
- Exclude measurement if doppler flow envelope of poor quality, including morphology, brightness and defined edges, also if heart rate of animal drops below 300 bpm.

RVWTd:

- Obtain the linear measurement of the RV free wall thickness at the end-diastole (Suppl. Fig. 1 C, D).
- Use the zoom mode provided by the software to improve border definition.
- Measure at least 3 consecutive cardiac cycles and calculate the average of three.
- Exclude measurement if RV wall borders are not clear or heart rate of animal drops below 300 bpm.

TAPSE

- Measure the linear base-to-apex shortening of the RV during systole (Suppl. Fig. 1 E, F).
- Use the zoom mode provided by the software to improve border definition.
- Measure at least 3 consecutive cardiac cycles and calculate the average of three.
- Exclude measurement if suboptimal 4 chamber view with unclear borders or if heart rate drops below 300 bpm.

References

1. Vitali SH, Hansmann G, Rose C, et al. The Sugen 5416/hypoxia mouse model of pulmonary hypertension revisited: long-term follow-up. *Pulm Circ.* 2014; 4: 619-29.
2. Hardziyenka M, Campian ME, de Bruin-Bon HA, et al. Sequence of echocardiographic changes during development of right ventricular failure in rat. *J Am Soc Echocardiogr.* 2006; 19: 1272-9.