Video Article

Contrast Imaging in Mouse Embryos Using High-frequency Ultrasound

Janet M. Denbeigh[1,2], Brian A. Nixon[1,2], Mira C. Puri[1,2,3], F. Stuart Foster[1,2]

[1]Department of Medical Biophysics, University of Toronto

[2]Sunnybrook Research Institute

[3]Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto

Correspondence to: Janet M. Denbeigh at Janet.denbeigh@utoronto.ca

URL: https://www.jove.com/video/52520
DOI: doi:10.3791/52520

Keywords: Developmental Biology, Issue 97, Micro-ultrasound, Molecular imaging, Mouse embryo, Microbubble, Ultrasound contrast agent, Perfusion

Date Published: 3/4/2015

Citation: Denbeigh, J.M., Nixon, B.A., Puri, M.C., Foster, F.S. Contrast Imaging in Mouse Embryos Using High-frequency Ultrasound. *J. Vis. Exp.* (97), e52520, doi:10.3791/52520 (2015).

Abstract

Ultrasound contrast-enhanced imaging can convey essential quantitative information regarding tissue vascularity and perfusion and, in targeted applications, facilitate the detection and measure of vascular biomarkers at the molecular level. Within the mouse embryo, this noninvasive technique may be used to uncover basic mechanisms underlying vascular development in the early mouse circulatory system and in genetic models of cardiovascular disease. The mouse embryo also presents as an excellent model for studying the adhesion of microbubbles to angiogenic targets (including vascular endothelial growth factor receptor 2 (VEGFR2) or $\alpha_v\beta_3$) and for assessing the quantitative nature of molecular ultrasound. We therefore developed a method to introduce ultrasound contrast agents into the vasculature of living, isolated embryos. This allows freedom in terms of injection control and positioning, reproducibility of the imaging plane without obstruction and motion, and simplified image analysis and quantification. Late gestational stage (embryonic day (E)16.6 and E17.5) murine embryos were isolated from the uterus, gently exteriorized from the yolk sac and microbubble contrast agents were injected into veins accessible on the chorionic surface of the placental disc. Nonlinear contrast ultrasound imaging was then employed to collect a number of basic perfusion parameters (peak enhancement, wash-in rate and time to peak) and quantify targeted microbubble binding in an endoglin mouse model. We show the successful circulation of microbubbles within living embryos and the utility of this approach in characterizing embryonic vasculature and microbubble behavior.

Video Link

The video component of this article can be found at https://www.jove.com/video/52520/

Introduction

Contrast-enhanced ultrasound imaging makes use of microbubble contrast agents to visualize and characterize the vascular environment. These agents enable noninvasive assessment of the microcirculation, vascularity and cardiovascular function. In addition, modification of the bubble surface can result in targeted microbubble binding to endothelial biomarkers, as demonstrated in preclinical applications of angiogenesis, atherosclerosis and inflammation [1,2] making molecular ultrasound imaging of vascular events possible. Contrast enhanced ultrasound can therefore be used to identify the complex and diverse environments that influence healthy and diseased vascular states[3-5].

In the past number of years, interest in the utility of microbubble imaging has extended to the versatile mouse embryo model. As a model of mammalian development, introduction of microbubbles into the embryonic vasculature enhances physiological study of the developing circulatory system (*e.g.*, blood flow, cardiac output) and in cases of transgenic and targeted mutant mouse models of cardiac disease [6,7], may yield insights into how genetic factors alter cardiovascular function. In fact, quantitative and qualitative 2D analyses of embryonic brain vasculature have already been achieved[8]. Furthermore, the mouse embryo presents as an excellent model for examining the binding of targeted microbubbles to vascular markers *in vivo*. Bartelle *et al.*[9], for instance, have introduced avidin microbubbles into embryo cardiac ventricles to assess targeted binding in Biotag-BirA transgenic embryos and examine vascular anatomy. The generation of heterozygous and homozygous mouse models can be also be used as a surrogate for tumor model studies aiming to define the quantitative nature of molecular ultrasound - an important benchmark in translating this technique to the clinic.

Microbubbles are most frequently introduced to the embryonic circulation via intra-cardiac injections into single embryos exposed through a laparotomy[8-10]. *In utero* injections, however, face a number of challenges. These include injection guidance, the need to counter motion in the mother and exteriorized embryo, maintenance of hemodynamic viability in the mother and exteriorized embryos, addressing long-term effects of anesthesia and complications due to bleeding[11]. Therefore, the goal of the investigation was to develop a technique for injecting microbubbles into isolated living late-stage embryos[12]. This option offers more freedom in terms of injection control and positioning, reproducibility of the imaging plane without obstruction, and simplified image analysis and quantification.

In the present study, we outline a novel procedure for the injection of microbubbles into living murine embryos for the purposes of studying microbubble kinetic behavior and of studying targeted microbubble binding to endogenous endothelial surface markers. Non-linear contrast specific ultrasound imaging is used to measure of a number of basic perfusion parameters including peak enhancement (PE), wash-in rate and

time to peak (TTP) in isolated E17.5 embryos. We also demonstrate the validity of the embryo model for assessing the quantitative nature of molecular ultrasound in an embryonic endoglin loss of function transgenic mouse model, where endoglin is a clinically relevant target due to its high expression in vascular endothelial cells at sites of active angiogenesis[13]. The adhesion of endoglin-targeted (MB_E), rat isotype IgG_2 control (MB_C) and untargeted (MB_U) microbubbles is evaluated in heterozygous endoglin (*Eng*$^{+/-}$) and homozygous endoglin (*Eng*$^{+/+}$) expressing embryos. Analysis of the targeted binding reveals that molecular ultrasound is capable of differentiating between endoglin genotypes and relating receptor densities to quantifiable molecular ultrasound levels.

Protocol

NOTE: The experimental procedures performed in this study were approved by the Animal Care Committee at Sunnybrook Research Institute (Toronto, Ontario, Canada). Procedures for the humane treatment of animals must be observed at all times. It is assumed that the investigator is trained in the basic operation of an ultrasound imaging system. This protocol works best with two people.

1. Animal Models

1. Mate CD-1 male and female *Mus musculus* to obtain wild type embryos for perfusion studies.
2. For molecular imaging studies, generate Eng+/- mice by homologous recombination using embryonic stem cells of 129/Ola origin as described by Bourdeau *et al.*[14].
    1. Backcross B6- *Eng*$^{+/-}$ mice, kindly acquired from Dr. Michelle Letarte, to the CD-1 background. Use *Eng*$^{+/-}$ CD-1 backcrossed embryos, as well as their *Eng*$^{+/+}$ littermate controls. Mate the mice to produce staged embryos.

2. Experimental Preparation

1. Initiate mating of mice and check plugs daily. Embryonic day 0.5 is defined as noon of the day a vaginal plug is observed.
2. Prepare embryo media by adding 50 ml of fetal bovine serum, 5 ml of 1 M HEPES and 5 ml of Penicillin-Streptomycin (10,000 units penicillin, 10,000 µg streptomycin) to 500 ml Dulbecco's modified Eagle's medium (DMEM) with high glucose (4,500 mg/L). Wrap the bottle in foil and keep chilled at 4 °C.
3. Centrifuge clear ultrasound gel in 50 ml conical tubes at 140 x g for 20 min. Draw gel up into 30 ml syringes. Fill additional (marked) 30 ml syringes with phosphate buffered saline (PBS) and set aside. One litter of embryos will generally require between 2-3 syringes of ultrasound gel and 4 syringes of PBS.
4. Pull approximately 30 glass needles and gently fasten to a strip of plasticine in a 100 x 20 mm cell culture dish. This ensures that the needles are separated and secure during handling. Using a glass puller, use the following settings on 1 x 90 mm glass capillaries with filament: Heater 77, Sub magnet 55, Main magnet 70.
5. Prepare ten to twelve dissection plates (enough to ensure each embryo within a litter will receive its own dish), using a 1:8 volume ratio of curing agent to base. Pour 40 ml of base into a 50 ml conical tube. Add 5-6 ml curing agent and stir with wooden stick.
    1. Pour into 60 x 15 mm culture dishes, filling each to approximately half. Let stand O/N to set.
6. Attach female luers to 400 mm long pieces of polyvinyl chloride (PVC) tubing (inner diameter: 0.79 mm). The tubing must reach from the syringe pump to the ultrasound stage comfortably.

3. Experimental Set-up

1. Arrange the imaging platform under the surgical microscope with a 3D motor and linear array 21 MHz transducer.
    1. Orient the platform so that the rail mount is on the left, with the stage positioned directly underneath the microscope.
    2. Attach the 3D motor to the ball joint of the extending arm of the platform. Screw the quick release post of the transducer clamp into the quick release mount on the motor, facing forward, and position the transducer head in the clamp, fastening the latch.
    3. Secure the transducer connector in the active port on the front panel of the cart. Turn the machine on.
2. Initiate the 21 MHz ultrasound transducer, selecting the 'cardiology' setting for molecular imaging studies, and 'general' for perfusion studies. Shift all time-gain-compensation sliders to the exact middle position. For 'Contrast Mode', set the following parameters: Frequency: 18 MHz, transmit power: 4%, (0.39 MPa), Contrast Gain: 30 dB, Foci: 6 & 10 mm, Contrast Mode: Nonlinear, Burst time: 100% at 0.1 sec, Beam width: Wide.
3. Aliquot 40 ml embryo media each into four 50 ml conical tubes. Place on ice.
4. Heat 1 L of water in a 2 L beaker on a hot plate. Maintain at 45 °C. Add one syringe of ultrasound gel and PBS to the beaker to preheat. Maintain this temperature by monitoring with a thermometer at all times.
5. Set aside ten to twelve 1 ml syringes and ten to twelve 21 G needles for the injection of microbubbles.

4. Surgical Procedure

NOTE: Have an assistant prepare the bubbles (stage 5) while the surgeon commences the surgical procedure. The protocol described here has been adapted from Whiteley *et al.*[15]

1. Collect E16.5 or E17.5 embryos from the pregnant female mouse following cervical dislocation.
NOTE: The effects of anesthesia on embryos have not yet been studied in detail[16]. Additional methods, including decapitation, may be appropriate options for the sacrifice of the pregnant mice.

1. Cut open the abdomen to reveal the uterus & embryos. Carefully and quickly remove the uterus by cutting at the tips of the uterine horns (severing the ovarian and uterine vessels) and severing at the vagina and bladder.
2. Using splinter forceps, transfer the uterus to a tube of 40 ml of ice-cold embryo media as quickly as possible without damaging the embryos. Discard the mouse carcass.

2. Relocate to microscope and stage. Deposit the uterus into a 100 x 20 mm cell culture dish. Transfer the uterus into a new dish filled with 50 ml fresh embryo media.
3. Using sterilized (70% ethanol spray) fine forceps, gently tear and pull away the outer membranes of the uterus starting at one end. Expose the placental decidua, parietal yolk sac and Reichert's membrane to separate and remove from the underlying visceral yolk sac.
4. Dissect out the embryos one by one, keeping the visceral yolk sacs intact and handling the placenta as gently as possible[17]. Take care to avoid rupture of the yolk sac as the embryos pop out immediately.
    1. Use the perforated spoon to move the embryos to another dish kept on ice with fresh embryo media. Keep the embryos chilled until needed. Change the media in the embryo dish every 1-1.5 hr. With these conditions embryos are viable for up to 4 hr after dissection.

5. Microbubble Preparation

NOTE: Perform molecular imaging with ultrasound using both targeted and untargeted microbubbles. A single experiment can require as many as 3 separate vials of microbubbles: i) antibody targeted microbubbles, ii) control isotype antibody targeted microbubbles and iii) untargeted microbubbles. The contrast agent comes as a dry-freeze powder and must be reconstituted with saline before injection. There are ~2 x 10^9 microbubbles in each untargeted microbubble vial and ~8.8 x 10^8 microbubbles in the target ready vials. The microbubbles are stable for up to 3 hr after reconstitution.

1. Reconstitution of Target Ready Vials
   NOTE: Examples of target ready preparations: endoglin targeted microbubbles (MB_E, with biotinylated rat MJ 7/18 antibody to mouse endoglin; in house hybridoma facility); vascular endothelial growth factor receptor 2 (VEGFR2) targeted microbubbles (MB_V, with biotin anti-mouse CD309); rat isotype IgG_2 control antibody targeted microbubbles (MB_C, biotin rat isotype IgG_2 control targeting mouse IgG2a).
    1. Dilute the antibody solution (20 µg) into 1 ml (final volume) of saline. Keep on ice.
    2. Fill syringe with 1 ml saline, removing all air bubbles. Attaching a 21 G needle, inject the solution slowly into the microbubble vial.
    3. Slowly withdraw the plunger, removing 1 ml of air, and withdraw the needle. Gently agitate. Let stand 5 min on ice.
       NOTE: Ultrasound microbubbles can be destroyed under high-pressure environments.
    4. After the elapsed time, fill a new syringe with the antibody dilution and add to the appropriate vial. Gently agitate. Let the mixture (now 2 ml) stand for 10 min. Keep on ice. Assuming complete surface conjugation, the average number of bound ligand for the microbubbles is approximately[18] 7,600 ligands/μm^2.

2. Reconstitution of Untargeted Vials
    1. Fill syringe with 1 ml saline, removing all air bubbles. Attaching a 21 G needle, inject the solution slowly into the microbubble vial.
    2. Slowly withdraw the plunger, removing 1 ml of air, and withdraw the needle. Gently agitate. Let stand 5 min. Add an additional 1 ml saline, as described above. Gently agitate and let stand 10 min on ice.

3. Coulter Counting
    1. Gently agitate the desired microbubble vial and draw ~50 µl of the solution into a 1 ml syringe using the 21 G needle. Inject the microbubbles into an empty 2 ml microcentrifuge tube.
    2. Pipette a 10 µl sample of microbubble stock solution into 10 ml of diluent.
    3. After gentle mixing, assess the concentration and size distributions of the microbubble population by using coulter counting (see List of Materials). Count a minimum of three 50 µl measurements (per vial sample) using a 30 µm aperture.

4. Preparing Microbubbles for Injection
   NOTE: The assistant performs this step when 'Injection of Microbubbles into Embryos' (step 6) is commenced. Each embryo is injected once, with the type of microbubble chosen for each injection to be randomly selected by the assistant such that all types of microbubble are evenly distributed throughout the procedure but given in a random order. Ensure the surgeon is blind to the type of bubble being injected.
    1. Determine the volume of stock bubble solution required to produce a final concentration of 1 x 10^8 MB/ml in a volume of 400 µl (calculate the volume using the stock concentrations measured in 5.3.3). Aliquot the corresponding volume of saline into an empty microcentrifuge tube.
    2. Gently agitate the selected microbubble vial and draw an excess volume (~50 µl greater than that calculated above) of the solution into a 1 ml syringe using the 21 G needle. Inject the microbubbles into an empty microcentrifuge tube.
    3. Pipette the necessary volume of microbubble stock solution and add to the aliquot of saline. Mix by stirring gently with the tip of the pipette.
    4. Draw the diluted microbubble solution into a clean syringe using the 21 G needle. Removing the needle, eliminate any air bubbles from the syringe and attach the luer and tubing. Slowly push the solution to the end of the tubing making sure not to generate any air bubbles.
    5. Insert the syringe into a syringe infusion pump set to dispense the microbubbles at a rate of 20 µl/min for a total volume of 20 µl. Attach a pulled glass needle to the end of the tubing. Move the pump close to the injection stage.

6. Injection of Microbubbles into Embryos

1. Randomly select and remove (with perforated spoon) one embryo from the chilled media dish. Place in dissection dish located on the ultrasound stage under the stereoscope and remove the yolk sac and amniotic membranes with the fine forceps, cutting/tearing from the side that appears least vascularized (the antimesometrial side). This is most easily achieved by piercing an area adjacent to the head region.

1. Cut enough to remove the embryo from within, but no more. Stabilize the dissection dishes using small pieces of plasticine.

2. Gently maneuver the sac from around the embryo. Position the embryo on its side, with the placenta and umbilical vessels in front. Using the insect pins, pin down the yolk sac (4 pins recommended) and edges of the placenta as necessary to affix in place. Avoid major vessels.
3. Wash the embryo with pre-warmed 45 °C PBS until the embryo revives. Identify the umbilical vein and its associated vascular network. Position the dish so that injection can be done comfortably.
   NOTE: Once warmed, blood in the embryo will begin to flow, visibly pumping in the umbilical artery. When flow first begins, the veins will be a bright red, with the blood in both vessels quickly appearing identical. The branches arising from the umbilical vein usually overlay those from the umbilical artery on the placental surface.
4. Once the embryo has revived, cover it (but not the placenta) with pre-warmed US gel, being sure to delicately remove any air bubbles (using the fine forceps) from around the embryo. Top up the dish with pre-warmed PBS.
   NOTE: Keep an eye on the level of solution in the PBS and gel syringes and add backup syringes to the heating beaker as needed.
5. Mount the glass needle on a large ball of plasticine at the edge of the dissection dish and insert the needle end into the PBS. Choosing a vein on the chorionic surface of the placental disc, as far from the main branch as reasonable, trim the tip to size using scissors. Injection is easiest if the tip is cut at a slight angle. Remove any jagged edges of the glass using the edge of the spring scissors.
6. Using the syringe pump, slowly inject the microbubble solution at 20 µl/min into the glass needle until all of the air is expelled from the needle tip and microbubbles can be seen to flow freely into the PBS. Stop the pump and reset for an injection volume of 20 µl. Do not allow air to enter the embryo's vascular system during injection.
7. Insert the glass needle tip gently into one of the veins in the placenta and ensure it is immobile. Swing away the stereoscope head and position the transducer above the embryo. Initiate imaging.

7. Ultrasound Molecular Imaging

NOTE: Using the contrast nonlinear imaging conditions set previously, position the transducer so that the embryo is situated evenly between the foci at 6 and 10 mm. Once positioned, start the bolus injection of the microbubbles. When the injection is complete, start the timer.

1. Perfusion Imaging
   1. Ensure that the maximum number of frames for nonlinear contrast mode is selected by pressing the '*study management*' button and clicking the '*Prefs*' tab at the top of the study browser. Check the appropriate box in the '*Preferences*' window.
   2. Adjust the imaging settings by initiating nonlinear imaging. Press the '*contrast mode*' button on the control panel. Refine the 2D gain by adjusting the '*gain*' dial to 30 dB, reduce the power to 4% using the '*power*' dial, decrease the frequency to 1 Hz using the '*frequency*' dial and set the beam width (bottom left corner) to wide using the roller ball/mouse.
      NOTE: All controls are located on the control panel of the ultrasound system.
   3. Capture the entire wash-in and dissipation of the microbubble bolus by recording a 20 min cine loop at a frame rate of 1 Hz. Press scan/freeze to start the data acquisition.
   4. Once the full sequence of images has been captured, press the '*cine store*' button located on the control panel to save the sequence of images in the system buffer. The system creates a date-stamped cine loop of the nonlinear contrast model data acquired.

2. Targeted Microbubble Imaging
   1. Under '*Prefs*', set the burst time to 0.1 sec. Set the appropriate imaging parameters using the controls described above (7.1.2.).
   2. Initiate nonlinear contrast imaging by pressing the '*contrast mode*' button on the control panel. Ensure the embryo is properly situated under the transducer and that the flow of microbubble contrast agent through the embryo is visible in the nonlinear contrast mode image.
   3. To assess targeted microbubble binding during embryonic molecular imaging studies, allow the microbubbles to circulate undisturbed for 3 min and 40 sec after completion of injection. This time is to allow retention of the circulating microbubbles. Press 'scan/freeze' to halt imaging during this time.
   4. At 3 min and 40 sec, resume imaging to confirm that the embryo is still visible, is adequately covered with PBS, and that microbubbles continue to circulate. Press 'scan/freeze' to initiate imaging.
   5. Acquire the disruption/replenishment sequence at 3 min and 50 sec post injection by recording a 'pre-destruction' acoustic response sequence at 29 Hz. Press 'scan/freeze' to start the data acquisition. At 4 min post injection[18,19], initiate a 0.1 sec high-frequency acoustic burst. Press the 'burst' button to implement a burst.
   6. Continue to record frames for the remainder of the sequence (until the buffer line is full) and save the cine loop by pressing 'cine store'.
   7. Collect an additional cine loop of circulating microbubbles. This subsequent 'post-destruction' imaging sequence is assumed to contain only circulating bubble signals that have replenished the beam. Press 'scan/freeze' to initiate imaging and 'cine store' to collect the images.
   8. Label the cine loops after imaging each embryo. Press 'study management', highlight the file using the roller ball and the 'select' button, press 'image label' and name appropriately.

8. Handling of Embryos after Injection

1. Post imaging, move the transducer, unpin the embryo and euthanize (via decapitation, cervical dislocation, carbon dioxide asphyxiation, or anesthesia followed by rapid freezing or fixation) as desired.
2. For each subsequent embryo injection a fresh dissection dish, needle, syringe and tubing segment must be used, with preparation of the next batch of microbubble injection solution taking place during dissection and revival.
3. Save tissue samples (*e.g.*, tail, brain) for additional analyses (*e.g.*, genotyping determined by PCR analysis of isolated tail DNA, lacz staining, or semi-quantitative measures of biomarker expression using western blots[21]).

Representative Results

The injection of ultrasound contrast agents into *ex utero* mouse embryos is dependent on the successful isolation of living, late-gestational stage embryos from the uterus and maintenance of viability over the course of the injection and related ultrasound imaging. Once the embryo has been exteriorized and positioned, as shown in **Figure 1**, careful injection of contrast agent into the embryonic vasculature is possible. A typical B-mode ultrasound image of an E17.5 mouse embryo is shown in **Figure 2A**. The wash-in of microbubbles and corresponding enhancement in the inferior vena cava, the heart, the brain, and in the entire animal can be captured using nonlinear contrast imaging methods, thereby demonstrating the feasibility of this technique. Individual time points are depicted in **Figure 2B** and show the change in contrast over time.

By recording the entire wash-in and wash-out of the microbubble bolus, it is possible to measure various perfusion parameters. In an offline analysis, the contrast region trace tool was used to create 1.5 mm^2 regions of interest (ROI) in the embryonic left and right brain hemispheres. The average signal intensity within these ROIs was then plotted as a function of time and smoothed using a seven point median filter. From the resulting contrast intensity curve (shown in **Figure 2C**), the brain peak enhancement was determined. The wash-in rate, defined as the slope between 10% and 50% of the maximum peak enhancement (PE), was also calculated. Finally, time to peak (TTP) was computed from the onset of the signal enhancement to the peak time. Differences in mean perfusion parameters across different bubble types were tested using separate t-tests[12]. These results, summarized in **Table 1**, demonstrate that the injection of microbubbles provides a valuable method for ascertaining important perfusion parameters in the developmental mouse.

Introduction of microbubbles into the embryonic vasculature also enables the use of mouse embryos as model systems to define and characterize the quantitative capacities of molecular ultrasound imaging. In the following example, a loss of function model, where genetic manipulation generated heterozygous and homozygous expression patterns of endoglin in embryonic mice, was used to test the ability of molecular ultrasound imaging to differentiate between genotypes. After microbubble injection and implementation of destruction/replenishment imaging, the ratio of the average signal intensity of the 'pre-destruction' to 'post-destruction' sequences was used to produce a measure of the molecular signal called the contrast mean power ratio (CMPR). A linear mixed model (with Bonferroni adjustments made for multiple comparisons) was performed to ascertain whether there was any significant difference between endoglin targeted, control and untargeted microbubble binding to $Eng^{+/+}$ or $Eng^{+/-}$ embryos (identified post injection via polymerase chain reaction (PCR) of tail samples). The estimated CMPR means (mean ± 95% Confidence Interval (CI)) are presented for each microbubble and embryo type in **Figure 3** and are summarized in **Table 2**.

These findings provide a concrete demonstration that injection of ultrasound contrast agents in isolated living embryos can be achieved. Furthermore, this protocol facilitates the assessment of perfusion parameters and may be used to compare targeted microbubble binding in embryo models of vascular disease in an effort to elucidate the capacities of molecular ultrasound imaging.

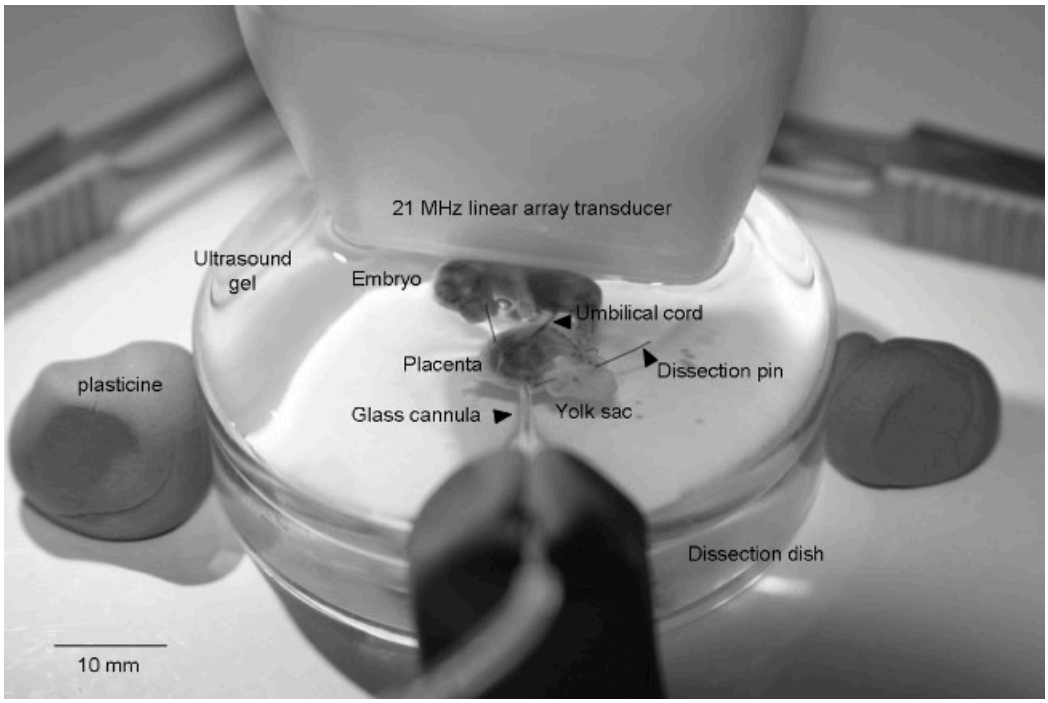

Figure 1. Experimental set-up for the injection of microbubbles into isolated embryos. A 21 MHz linear array transducer is positioned above an exteriorized living E16.5 embryo as a 20 µl microbubble solution is injected into a placental vein using a glass cannula. Scale bar = 10 mm. From Denbeigh, J.M. *et al.*[12] with permission. Please click here to view a larger version of this figure.

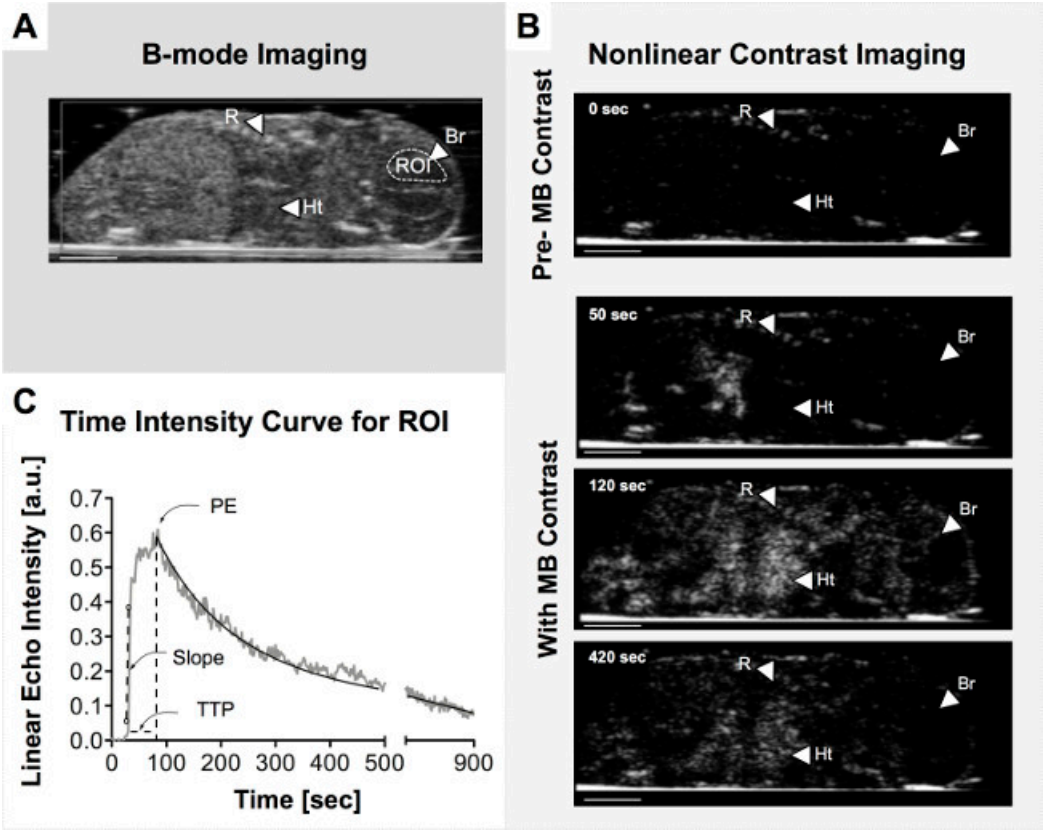

Figure 2. Ultrasound Imaging of Exteriorized Living E17.5 Embryos. (A) B-mode ultrasound image of an embryo. Prior to injection of targeted microbubbles (t= 0 sec). **(B)** Non-linear contrast image of the embryo before and after injection of microbubbles. Nonlinear contrast image prior to microbubble injection (t = 0 sec). Only the strongly reflecting interfaces (*e.g.,* bone) are visible, with minimal signal from the soft tissues. Below, nonlinear contrast image of microbubbles within the embryo at t= 50 sec, t = 120 sec and t = 420 sec after a bolus injection. Contrast is detected throughout the animal, including the heart and brain. **(C)** Perfusion parameters derived from time intensity curves. Intensity plot of microbubbles as a function of time within a single ROI in the embryonic brain. Perfusion parameters are identified on the graph, including peak enhancement (PE), wash-in rate (slope), and time to peak (TTP). Arrowheads: R = ribs, Ht = heart, Br = brain. a.u., arbitrary units; ROI, region of interest; sec, seconds. Scale bar = 3 mm. This figure has been modified from Denbeigh, J.M. *et al.*[12]. Please click here to view a larger version of this figure.

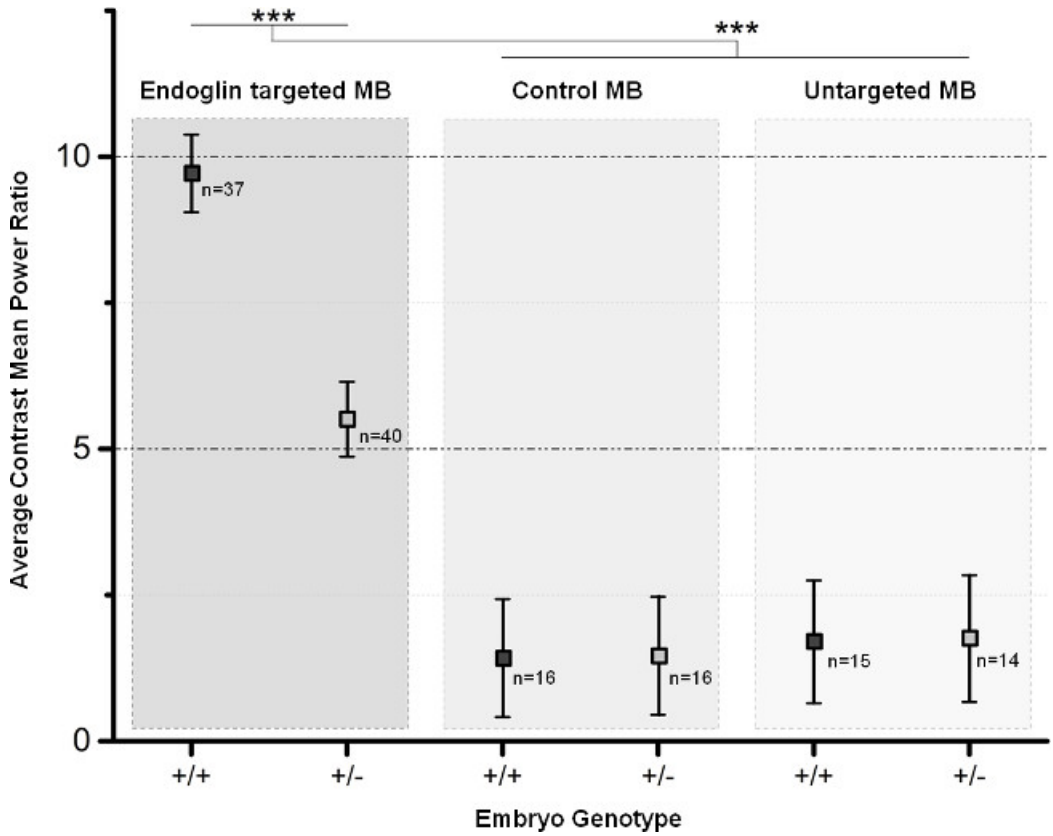

Figure 3. Summary of the average contrast mean power ratios (CMPR) for endoglin targeted (MB_E), control (MB_C) and untargeted microbubbles (MB_U) in *Eng*$^{+/+}$ and *Eng*$^{+/-}$ embryos. CMPRs from endoglin targeted microbubbles were significantly higher (***, p <0.001) than those collected for MB_C and MB_U, (not significantly different from each other, regardless of genotype). MB_E binding was found to be significantly higher in *Eng*$^{+/+}$ embryos (dark markers) compared to *Eng*$^{+/-}$ embryos (light markers). Results presented as mean ± 95% confidence interval. n indicates the number of unique embryos in each category. From Denbeigh, J.M. *et al.*[22] with permission. Please click here to view a larger version of this figure.

	Microbubble Type			**T-tests**		
				p-value		
Perfusion Parameter	**MB_U**	**MB_C**	**MB_V**	**MB_U vs MB_C**	**MB_U vs MB_V**	**MB_C vs MB_V**
Peak Enhancement, **PE** (a.u.)	.427 ± .063	.490 ± .079	.499 ± .064	0.19	0.06	0.81
Wash-in rate (a.u./ sec)	.008 ± .002	.009 ± .002	.011 ± .002	0.18	0.01	0.09
Time to Peak, **TTP** (sec)	53.75 ± 7.96	51.75 ± 4.46	45.00 ± 5.33	0.55	0.03	0.03
# embryos injected	4	4	3			

Table 1. Summary of E17.5 embryonic perfusion parameters. Mean ± standard deviations derived from time intensity plots for all embryo ROIs (a.u. = arbitrary units). The table includes measurements for untargeted (MB_U), IgG_2 control targeted (MB_C) and VEGFR2 targeted (MB_V) microbubbles. Separate t-tests were performed to compare groups. This table has been modified from Denbeigh, J.M. *et al.*[12].

Microbubble type	**Genotype**	**CMPR Mean**	**95% Confidence Interval**
MB_E	$Eng^{+/+}$	9.71	9.05, 10.38
	$Eng^{+/-}$	5.51	4.87, 6.15
MB_C	$Eng^{+/+}$	1.42	0.41, 2.43
	$Eng^{+/-}$	1.46	0.45, 2.47
MB_U	$Eng^{+/+}$	1.7	0.65, 2.75

	$Eng^{+/-}$	1.76	0.67, 2.84
	Linear Mixed Model: Between-subject Effects		
	df	**F**	**p value**
Genotype	1	12.75	<0.001
Microbubble type	2	147.65	<0.001
Genotype * Microbubble type	2	18.29	<0.001

Table 2. Summary of linear mixed model analysis for microbubble binding in embryos, with a Bonferroni correction for multiple comparisons. Genotype, microbubble type and the combined interaction (genotype*microbubble type) were found to be significant factors in determining CMPR. df = degrees of freedom. From Denbeigh, J.M. *et al.*[22] with permission.

Discussion

Ultrasound contrast agents were injected into late-stage gestation mouse embryos and nonlinear contrast images were acquired to measure perfusion parameters and targeted microbubble binding. Successful imaging of microbubbles within embryonic vasculature was dependent on a number of factors, the first being embryo viability. All equipment and apparatus were prepared in advance in order to minimize the time required for isolation of embryos from the uterus to the start of injection. Since the effects of single or repeated exposure to anesthesia on embryonic mice have not been studied in detail, the use of anesthesia in the mother was avoided before sacrifice[16]. During embryo isolation, it was important to prevent damage to the yolk sac and to ensure embryos were kept chilled at all times, in frequently refreshed embryo media. When exteriorizing the embryo prior to injection and during pinning, major vitelline vessels were avoided to limit the likelihood of bleeding, which could be lethal. We found that when reviving the embryo, it was best to first cover the placenta and then the embryo with PBS, before applying ultrasound gel to the embryo in a back and forth motion and removing any large bubbles with forceps. The remainder of the Petri dish was then filled with PBS. The use of gel and PBS limited the possibility of air pockets between the embryo and the transducer, which could impact image quality. As the embryo revived, blood began to flow, visibly pumping in the umbilical artery while the umbilical veins appeared bright red[23]. For reference, the branches arising from the umbilical vein usually overlay those from the umbilical artery on the placental surface. Although it was possible to inject into the placental labyrinth arterioles, injecting the microbubbles in the direction of flow reduced placental bleeding and venule injections also allowed the microbubbles to pass directly through the embryo before circulating through the placenta.

Due to their fragility, microbubble preparation and handling also required care. Ultrasound microbubbles can be destroyed under high-pressure environments. Therefore, the exact same procedure was repeated during each reconstitution and bubble concentration was measured to ensure experimental consistency. A single vial of microbubbles was typically enough for about 5-7 embryos. Since the microbubbles are stable within the vial for up to 3 hr, multiple vials were often required for a single experiment. To avoid tearing the tissue, it was best for the glass tip of the injection needle to be sharp and cut on a slight angle while approaching the vessel at as small an angle as possible. Once trimmed, the tip of the needle had to be large enough for the bubbles to flow easily through the end without clumping, but small enough to fit easily within the vessel. The ideal size was generally <100 µm. Some practice at judging the appropriate size was necessary. In the event that the tip was cut too large, or became blocked, a new glass needle was applied. It some cases, it was possible to trim the needle slightly above the blockage. It was critical that air not be allowed to enter the embryo's vascular system during injection, as this could result in the death of the animal and was undesirable during imaging (air bubbles could lodge in the vasculature of the embryo and be detectable in the ultrasound image, artificially increasing any measured signal). Nonlinear contrast imaging performed in this protocol used a state of the art high frequency (21 MHz) micro-ultrasound system (Vevo2100) paired with linear arrays specifically designed for small animal imaging (MS250). Micro- or high frequency -ultrasound is one of the few modalities able, at this time, to provide high resolution images of living murine embryos and newborns[16]. This system can achieve axial and lateral resolutions of 75 µm and 165 µm respectively at depths as great as 15 mm. It was therefore possible to image the entire embryo at much higher resolutions than typically achieved using clinical instruments and the acoustic signal from microbubbles could be distinguished from tissue using nonlinear contrast imaging methods (including pulse inversion and amplitude modulation techniques[24]). Although we had the best success with the ultrasound settings described here, it is possible that some adjustment to these parameters will also produce satisfactory results. In all cases, a low power is required for microbubble imaging to avoid undesirable destruction of the microbubbles prior to initiation of a burst, while the short burst eliminates only a small fraction of the microbubble population. With practice, the entire procedure for a single embryo, from positioning to the completion of molecular ultrasound imaging, took approximately 15 min. We have successfully injected as many as 12 embryos from one litter in a single session.

Injection experiments were restricted to a 4 hr window due to the limited viability of the embryos. Since the distribution of microbubbles was dependent upon the circulation of the embryo itself, injections could not take place before the heart beat (E8.5) and detectable flow in the vitelline and umbilical circulation (E9.5) was established[25]. With maturation of the placenta not complete until E14.5, the injection of contrast agents through the placental labyrinth was limited to embryos of mid to late gestational age. Based on observations, embryos nearer to term were hardier and could withstand increases in their vascular volume better than their younger counterparts. Combined, these factors restricted contrast enhanced ultrasound imaging of the embryonic vasculature to later stages of development and angiogenic growth. This may impact the availability of transgenic mouse models, since manipulation of receptors involved in vascular development and maintenance (*e.g.,* VEGFR2, VCAM-1) may lead to embryonic lethality or defects in the development and regulation of embryonic circulatory systems[26,27]. Nevertheless, a number of relevant model systems are available for vascular biomarkers of interest, including $\alpha_2\beta_1$[28], $\alpha_v\beta_3$[29], platelet endothelial cell adhesion molecule-1[30], and vascular cell adhesion molecule-1[31], intercellular adhesion molecule-1[32] and -2[33] and P-selectin[34]. What is more, brain histogenesis begins after mid-gestation[11], making the expression of angiogenic markers in this tissue likely and thereby providing an excellent alternative to the traditional tumor model for studies assessing the quantitative aspects of molecular imaging.

The *ex vivo* nature of the injections does present some limitations. It is important to note that after the embryos were removed from the mother and the placental labyrinth punctured, it was generally not possible (nor desirable) to do repeat injections. In addition, isolating the embryo from

maternal circulation limits the supply of nutrients and O_2, and embryonic health is expected to deteriorate with time. While chilling and revival prolongs the viability of the embryos, it may also impact cardiac function. For example, we observed that isolated embryos had a reduced heart rate (data not included, refer to Denbeigh *et al.*[12]) compared to those previously observed *in vivo*[35]. It is therefore likely that studies assessing cardiovascular physiology will not accurately reflect *in vivo* conditions. With few studies characterizing circulatory hemodynamics in the living late stage mouse embryo, however, it is difficult to say with certainty the extent to which these experimental conditions may alter physiological function. One alternative is to conduct injections *in utero*, either through a laparoscopic incision[36] or through the belly of the pregnant mother directly[37], although this approach may present its own set of challenges[11]. In some instances, the isolation and exteriorization of the embryo may also result in significant bleeding from the yolk sac. Although we found that we could inhibit the blood flow with ultrasound gel, any significant loss of blood could impact the delivery and concentration of microbubbles, and these animals were excluded from analysis. Finally, while isolation does limit maternal and embryonic sources of motion, periodic motion related to cardiac activity is unavoidable. We elected to image the static brain in order to directly examine the effect of receptor expression on targeted microbubble signal, however implementation of real-time motion compensated contrast-enhanced ultrasound imaging[38] may extend these studies to other organs.

There are few imaging applications that are currently able to assess the vascular landscape of the living developing mouse embryo. Some studies have successfully performed live imaging of blood flow in *ex vivo* murine embryos using Doppler swept-source optical coherence tomography[39,40], while magnetic resonance imaging, vascular corrosion casts, microcomputed tomography and optical projection tomography have been implemented postmortem[16]. This is unfortunate, since this period of embryonic growth can reveal crucial information regarding early vascular development and function. Microbubble imaging within living embryos could therefore contribute to our understanding of developmental physiology. It might also be used to visualize biomarker distribution in the whole body of the living mouse fetus in real-time, although this technique is unlikely to replace existing methods (including histology and fluorescent imaging) for the measurement of molecular signal in embryonic tissue. The rapidly developing mass of vessels in the embryos, however, closely resembles tumor growth, with expression of many of the same key receptors identified in tumor angiogenesis[41,42] and may therefore serve as an excellent tumor surrogate for studies examining the quantitative abilities of molecular ultrasound. We therefore anticipate that the described methods will be useful not only for assessing and characterizing embryonic models of vascular health and disease through functional imaging, but offers an avenue for exploring the strengths and limitations of molecular imaging as well. Its application might also extend to other interesting areas of investigation, including *in utero* gene transfer therapy where microbubbles have been tested as a means of delivering naked DNA to fetal mouse tissue[10]. Alternatively, 3D vascular mapping of the living embryo is also possible, which may provide invaluable information as to the morphological abnormalities in genetically modified mice. Introduction of ultrasound contrast agents into isolated living embryos could therefore be another tool for increasing our understanding of vascular disease and translating contrast-enhanced ultrasound applications to the clinic.

Disclosures

F.S. Foster acknowledges his role as consultant to VisualSonics Inc.

Acknowledgements

This work was supported by the Terry Fox Program of the National Cancer Institute of Canada.

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