Journal of Visualized Experiments

High-Resolution Three-Dimensional Imaging of the Footpad Vasculature in a Murine Hindlimb Gangrene Model

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63284R1
Full Title:	High-Resolution Three-Dimensional Imaging of the Footpad Vasculature in a Murine Hindlimb Gangrene Model
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Medicine
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Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Miami, Florida, United States
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TITLE:

High-Resolution Three-Dimensional Imaging of the Footpad Vasculature in a Murine Hindlimb Gangrene Model

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KEYWORDS:

Hindlimb ischemia, gangrene, femoral artery ligation, L-NAME, DiI perfusion, peripheral arterial disease, neovascularization, angiogenesis

SUMMARY:

The present protocol describes a unique, clinically relevant model of peripheral arterial disease that combines femoral artery and vein electrocoagulation with the administration of a nitric oxide synthase inhibitor to induce hindlimb gangrene in FVB mice. Intracardiac DiI perfusion is then used for high-resolution, three-dimensional imaging of the footpad vasculature.

ABSTRACT:

Peripheral arterial disease (PAD) is a significant cause of morbidity resulting from chronic exposure to atherosclerotic risk factors. Patients suffering from its most severe form, chronic limb-threatening ischemia (CLTI), face substantial impairments to daily living, including chronic pain, limited walking distance without pain, and nonhealing wounds. Preclinical models have

been developed in various animals to study PAD, but mouse hindlimb ischemia remains the most widely used. There can be significant variation in response to ischemic insult in these models depending on the mouse strain used and the site, number, and means of arterial disruption. This protocol describes a unique method combining femoral artery and vein electrocoagulation with the administration of a nitric oxide synthase (NOS) inhibitor to reliably induce footpad gangrene in Friend Virus B (FVB) mice that resembles the tissue loss of CLTI. While traditional means of assessing reperfusion such as laser Doppler perfusion imaging (LDPI) are still recommended, intracardiac perfusion of the lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) is used to label the vasculature. Subsequent whole-mount confocal laser scanning microscopy allows for high-resolution, three-dimensional (3D) reconstruction of footpad vascular networks that complements traditional means of assessing reperfusion in hindlimb ischemia models.

INTRODUCTION:

Peripheral arterial disease (PAD), characterized by reduced blood flow to the extremities due to atherosclerosis, affects 6.5 million people in the United States and 200 million people worldwide[1]. Patients with PAD experience reduced limb function and quality of life, and those with CLTI, the most severe form of PAD, are at increased risk for amputation and death with a 5-year mortality rate nearing 50%[2]. In clinical practice, patients with ankle-brachial indices (ABI) <0.9 are considered to have PAD, and those with ABI <0.4 are associated with either rest pain or tissue loss as having CLTI[3]. Symptoms vary among patients with similar ABIs depending on daily activity, muscle tolerance to ischemia, anatomic variations, and differences in collateral development[4]. Digit and limb gangrene is the most severe manifestation of all vascular occlusive diseases that result in CLTI. It is a form of dry necrosis that mummifies the soft tissues. In addition to atherosclerotic PAD, it can also be observed in patients with diabetes, vasculitides such as Buerger's disease and Raynaud's phenomenon, or calciphylaxis in the setting of end-stage renal disease[5,6].

Several preclinical models have been developed to study the pathogenesis of PAD/CLTI and test the efficacy of potential treatments, the most common of which remains mouse hindlimb ischemia. Inducing hindlimb ischemia in mice is typically accomplished by the obstruction of blood flow from the iliac or femoral arteries, either by suture ligation, electrocoagulation, or other means of constricting the desired vessel[7]. These techniques drastically reduce perfusion to the hindlimb and stimulate neovascularization in the thigh and calf muscles. However, there are essential murine strain-dependent differences in sensitivity to ischemic insult partially owing to anatomical differences in collateral distribution[8,9]. For example, C57BL/6 mice are relatively resistant to hindlimb ischemia, demonstrating reduced limb function but generally no evidence of gangrene in the footpad. On the other hand, BALB/c mice have an inherently poor capacity to recover from ischemia and typically develop auto-amputation of the foot or lower leg following femoral artery ligation alone. This severe response to ischemia narrows the therapeutic window and can preclude longitudinal assessment of limb reperfusion and function. Interestingly, genetic differences in a single quantitative trait locus located on murine chromosome 7 have been implicated in these differential susceptibilities of C57BL/6 and BALB/c mice to tissue necrosis and limb reperfusion[10].

Compared to C57BL/6 and BALB/c strains, FVB mice demonstrate an intermediate but inconsistent response to femoral artery ligation alone. Some animals develop footpad gangrene in the form of black ischemic nails or mummified digits, yet others without any overt signs of ischemia[11]. Concomitant administration of N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), a nitric oxide synthase (NOS) inhibitor[12], prevents compensatory vasodilatory mechanisms and further increases oxidative stress in hindlimb tissue. In combination with femoral artery ligation or coagulation, this approach consistently produces footpad tissue loss in FVB mice that resembles the atrophic changes of CLTI but rarely progresses to limb auto-amputation[11]. Oxidative stress is one of the hallmarks of PAD/CLTI and is propagated by endothelial dysfunction and diminished bioavailability of nitric oxide (NO)[13,14]. NO is a pluripotent molecule that usually exerts beneficial effects on arterial and capillary blood flow and platelet adhesion and aggregation, and leukocyte recruitment and activation[13]. Reduced levels of NOS have also been shown to activate the angiotensin-converting enzyme, which induces oxidative stress and accelerates the progression of atherosclerosis[15].

Once a model of hindlimb ischemia is established, monitoring subsequent limb reperfusion and the therapeutic effect of any potential treatments are also needed. In the proposed murine gangrene model, the degree of tissue loss can first be quantified using the Faber score to assess the gross appearance of the foot (0: normal, 1-5: loss of nails where score represents the number of nails affected, 6-10: atrophy of digits where score represents the number of digits affected, 11-12: partial and complete foot atrophy, respectively)[9]. Quantitative measurements of hindlimb perfusion are then typically made using LDPI, which relies on Doppler interactions between laser light and red blood cells to indicate pixel-level perfusion in a region of interest (ROI)[16]. While this technique is quantitative, non-invasive, and ideal for repeated measurements, it does not provide granular anatomical detail of the hindlimb vasculature[16]. Other imaging modalities, such as micro-computed tomography (micro-CT), magnetic resonance angiography (MRA), and X-ray microangiography, prove either costly, requiring sophisticated instrumentation, or otherwise technically challenging[16]. In 2008, Li et al. described a technique for labeling blood vessels within the retina with the lipophilic carbocyanine dye DiI[17]. DiI incorporates into endothelial cells and, by direct diffusion, stains vascular membrane structures such as angiogenic sprouts and pseudopodal processes[17,18]. Due to its direct delivery into endothelial cells and the highly fluorescent nature of the dye, this procedure provides intense and long-lasting labeling of blood vessels. In 2012, Boden et al. adapted the technique of DiI perfusion to the murine hindlimb ischemia model *via* whole-mount imaging of harvested thigh adductor muscles following femoral artery ligation[19].

The current method provides a relatively inexpensive and technically feasible way for assessing neovascularization in response to hindlimb ischemia and gene or cell-based therapeutics. In a further adaptation, this protocol describes the application of DiI perfusion to image the footpad vasculature in high resolution and 3D in a murine model of hindlimb gangrene.

PROTOCOL:

All animal experiments described in the protocol were approved by the University of Miami

Institutional Animal Care and Use Committee (IACUC). FVB mice, both male and female, aged 8-12 weeks, were used for the study.

1. Preparation of L-NAME solution

1.1. Under sterile conditions in a laminar flow hood, prepare an L-NAME stock solution by dissolving 1g of L-NAME powder (see **Table of Materials**) with 20 mL of sterile water to make a 50 mg/mL of solution. Store the stock solution in 300-500 µL aliquots at -80 °C for up to 3 months.

1.2. To make a working L-NAME solution, thaw an aliquot of L-NAME stock solution and dilute with PBS (1:4) under the sterile condition to obtain a final 10 mg/mL concentration.

1.2.1. To prepare PBS (pH 7.4), dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.23 g of NaH_2PO_4 in 800 mL of distilled water. Adjust pH to 7.4 with HCl. Add water to a total volume of 1,000 mL and filter through a 0.22 µm bottle top filter.

NOTE: Intraperitoneal (IP) injection of 4 µL/g of the mouse of L-NAME working dilution is equivalent to the desired 40 mg/kg dose of L-NAME. L-NAME working solution should be kept on ice during use, and new dilutions should be made daily using freshly thawed aliquots of stock solution.

2. Chemical and surgical induction of hindlimb gangrene

2.1. Obtain FVB mice, aged 8-12 weeks, either from a breeder or bred in-facility (see **Table of Materials**). 2 h before surgery, administer a 40 mg/kg of IP dose of L-NAME.

2.2. Anesthetize mice with IP injection of 100 mg/kg of ketamine and 10 mg/kg of xylazine (see **Table of Materials**) diluted in PBS. Confirm adequate sedation by the absence of toe-pinch reflex and continue monitoring respiratory rate during the procedure.

2.2.1. Remove hair from bilateral hindlimbs and groins using shears and/or a depilatory cream. Position the animal under a surgical microscope supine; extend and tape the extremities in place. Sterilize the surgical field by circumferentially applying the povidone-iodine solution to the surgical site.

2.3. Under 10-20x magnification, use scissors or a scalpel to make a 1 cm incision along the groin crease ju'st inferior to the inguinal ligament. Use fine forceps and a sterile cotton tip applicator to bluntly dissect the inguinal fat pad laterally from the inguinal ligament and expose the underlying femoral sheath so that the femoral artery, vein, and nerve are clearly identified (**Figure 1**).

2.4. Using fine forceps, pierce the femoral sheath. Carefully brush the femoral nerve away from the femoral artery. Identify the take-off of the lateral circumflex branch of the femoral artery (LCFA) deep to the femoral nerve (**Figure 1**).

2.4.1. Proceed with electrocoagulation of the femoral artery and vein just proximal to the LCFA by activating the cautery device (see **Table of Materials**) and gently contacting the vessels with a side-to-side motion, ensuring that the femoral nerve is well-isolated and remains protected from thermal injury. Divide the coagulated vessel segment with scissors.

2.5. Proceed with the exposure of the distal femoral artery and vein by mobilizing the inguinal fat pad medially. Identify the superficial epigastric artery and saphenopopliteal junction more distally.

2.5.1. Pierce the femoral sheath between these two locations and carefully dissect the femoral nerve away from the femoral vessels. Proceed with coagulation and transection of the femoral artery and vein as described in step 2.4.1.

2.6. Irrigate the surgical field using a syringe filled with sterile PBS. Obtain hemostasis by applying gentle pressure with a cotton tip applicator for 3-5 min to any areas of bleeding.

2.6.1. Proceed with the closure of the incision using absorbable 5-0 suture in a simple continuous fashion. Administer a 1 mg/kg dose of sustained-release buprenorphine (see **Table of Materials**) for postoperative pain relief.

2.7. Confirm loss of footpad perfusion in the ligated hindlimb by LDPI (see **Table of Materials**). While still anesthetized, place the animal on a dark foam pad in a prone position underneath the LDPI machine and use loops of electrical tape to secure the feet in place.

2.7.1. Proceed with LDPI of bilateral feet. Once the scanning is complete, draw an ROI around each footpad and obtain the mean flux values.

2.7.2. Calculate the perfusion index as the ratio of mean flux values from the ligated to non-ligated footpad. Ensure that the perfusion index is less than 0.1.

2.8. Transfer the animal back to a clean cage with a heating pad or overhead lamp to maintain core body temperature. Ensure complete recovery from anesthesia before transferring mice back to the animal facility.

3. Postoperative administration of L-NAME and monitoring of hindlimb gangrene

3.1. On postoperative days 1-3, administer an additional 40 mg/kg IP dose of L-NAME to each animal. At the same time, carefully evaluate the foot from the ischemic limb.

3.2. Quantify the degree of hindlimb ischemia and gangrene using the Faber hindlimb ischemia score[9]. Scores 1-5: number of ischemic nails; scores 6-10: 1-5 ischemic digits; scores 11 and 12: partial and complete foot atrophy. Record Faber scores on postoperative days 1-3 and then weekly.

4. Preparation of DiI and working solutions for animal perfusion

4.1. To prepare DiI stock solution, dissolve 100 mg of DiI crystals (see **Table of Materials**) in 16.7 mL of 100% ethanol. Cover in aluminum foil and leave on a rocking platform overnight in the dark at room temperature.

4.2. To prepare the diluent, dissolve 50 g of glucose in 1,000 mL of distilled water to yield a 5% glucose solution. Filter through a 0.22 µm bottle top filter. Mix PBS and 5% glucose solutions in a 1:4 ratio to prepare a working diluent solution.

5. Equipment setup and DiI perfusion

5.1. Make DiI working solution by adding 200 µL of DiI stock solution to 10 mL of the working diluent solution (prepared in step 4.2) immediately before use. Shake by hand to mix well.

5.2. Connect two or three 3-way stopcocks and a 25 G butterfly needle in series. Prepare 10 mL syringes with 4 mL of PBS, 10 mL of DiI solution, and 10 mL of 10% neutral-buffered formalin (see **Table of Materials**).

5.3. Connect the syringe with formalin to the proximal inflow port and inject the solution to flush air from the line; turn the stopcock to close the port. Repeat the same procedure sequentially, connecting the syringes with DiI and then PBS to the middle and distal inflow ports, respectively, taking care to flush all air bubbles through the stopcock assembly.

NOTE: Ensure that there are no air bubbles in any portion of the stopcock assembly or tubing. Air bubbles can occlude small arteries during perfusion resulting in poor intravascular DiI distribution and compromised imaging results.

5.4. Once the setup is complete, euthanize the animal by CO_2 overdose in an induction chamber.

5.5. Place the animal to be perfused in a supine position on an absorbent pad and secure axillae and lower extremities with needles.

5.6. Using scissors, make a transverse incision to open the abdominal cavity. Expose and then divide the left and right diaphragm to access the thoracic cavity.

5.6.1. Cut the chest wall on either side of the sternum from the lower ribs to the first or second ribs, avoiding the internal thoracic (mammary) arteries medially. Use a hemostat (see **Table of Materials**) to grasp the lower end of the sternum and reflect it towards the animal's head to expose the thoracic cavity.

5.7. Identify the left ventricle, which appears lighter in color than the right ventricle. Gently

grasp the heart with blunt forceps and insert the butterfly needle into the left ventricle.

5.7.1. Use scissors or an 18 G needle to puncture the right atrium, allowing blood and perfusion solutions to return to the heart to drain. Stabilize the needle with one or two hands, taking care not to inadvertently puncture the right ventricle and perfuse the pulmonary rather than systemic circulation.

5.8. Open the port of the syringe with PBS and manually inject 2-4 mL at a rate of 1-2 mL/min for 1-2 min to flush blood from the vascular system. Ensure successful perfusion by observing bleeding from the right atrium. After injection, close the port of the PBS syringe.

5.9. Open the port of the syringe with DiI and inject 5-10 mL at a rate of 1-2 mL/min for 5 min. Monitor the ears, nose, and palms which should turn slightly pink with the injection of DiI solution. After injection, close the port of the DiI syringe and wait for 2 min to allow incorporation of the dye before injection of fixative.

5.10. Open the port to the syringe with formalin and inject 5-10 mL at a rate of 1-2 mL/min for 5 min. After injection, remove the needle from the left ventricle and proceed to harvest the tissues of interest.

5.11. Using heavy scissors, dislocate the tibia at the ankle, completely separating the left and right feet from the lower legs. Place harvested feet in a 6- or 12-well plate with 1-2 mL of 10% formalin solution. Wrap the plate with foil and store at 4 °C overnight.

6. Preparation of footpad tissue for confocal laser scanning microscopy

6.1. The next day, replace the fixative solution in 6- or 12-well plate with 1-2 mL of PBS per well.

6.2. To skin the foot, first, make a longitudinal incision with a scalpel on the plantar and dorsal aspects of the foot. Then, using toothed forceps and a small hemostat, carefully remove all skin from the foot and digits, not damaging the underlying soft tissues.

6.3. Proceed to mounting and imaging of tissues, preferably within 1-2 days of perfusion and harvest. Alternatively, return footpads to 6- or 12-well plates with 1-2 mL of PBS; cover with foil and store at 4 °C to maintain fluorescence for up to 1 month.

6.4. To mount tissues, individually place feet between two glass microscope slides with a foam biopsy pad folded over itself (once or twice depending on tissue thickness) at each end (see **Table of Materials**). Use two small binder clips to compress the glass slides together at each end (final thickness approximately 1 mm).

NOTE: Thicker tissues will require longer scanning times. The skinned footpad can be compressed between glass slides one day before imaging to reduce tissue thickness.

7. Confocal laser scanning microscopy

7.1. Prepare for the imaging session: turn on the imaging system and start the acquisition software (see **Table of Materials**). Use a low magnification/low numeric aperture objective (e.g., x5/0.15) to capture images as lower magnification lenses typically have longer working distances required for this experiment.

7.2. Click on **Yes** to the Activate Stage dialog box. Activate the 561 nm laser in the Configuration tab. On the main screen, activate a visible beam path by clicking on the **Visible** button. Set a detector to the 570-600 nm range by clicking on the corresponding **Active** checkbox.

7.3. Select the **Tile Scan** icon in the **Acquire** > **Acquisition** tab and set the desired resolution (512 x 512 or 1024 x 1024).

7.4. Position dry-mounted (no water or PBS added) tissue sample compressed between glass slides on the microscope stage and bring tissue into focus.

7.5. To set the scanning boundaries, navigate to the upper left or right corner of the sample. In the Acquisition tab, under the Tile Scan menu, click on the **Mark Position** button. Navigate to the opposite corner (lower right or left, respectively) and click on the **Mark Position** button once again.

7.6. To set the depth of the Z-stack, click on the **Live** button at the lower-left corner of the screen and navigate to the center of the sample. Use the z-axis knob to scroll through to the bottom of the sample.

7.7. In the Acquisition tab, under the Z-Stack menu, click on the **Begin** button. Scroll through to the top of the sample and click on the **End** button. Click on **Z-step Size** and set to the desired value (e.g., 50 µM).

7.8. In the lower right corner of the screen, click on **Start** to begin image acquisition.

8. Quantitative analysis and 3D reconstruction of footpad vascularity

8.1. Download and install the latest version of Fiji (ImageJ) as well as the Vessel Analysis plugin[20]. Open microscopy image files in Fiji, which will combine individual Z-series into Z-stacks that can be viewed in the z-axis using the slider at the bottom of the image.

8.2. Select the composite Z-stack image and then under the menu Image, choose **Stacks** > **Z Project** to create a two-dimensional projection. Next, convert the Z-projection to binary under **Process** > **Binary** > **Make Binary**.

8.3. Run the Vascular Density plugin under **Plugins** > **Vascular Density**. When prompted, use

the cursor to trace an ROI around the perimeter of the footpad and digits. Take note of the vessel density that is reported, which is expressed as a percentage of the ROI (vascular area fraction).

8.4. To create 3D reconstructions in Fiji, select **Stacks** > **3D Project** and set the desired rotation axis, angle, and speed under the menu Image. Alternatively, select **Volume Viewer** under the **Plugins** menu to visualize images as slices or manipulate the reconstruction in the desired axes.

8.5. For more involved 3D rendering, use alternative image analysis and processing software (see **Table of Materials**). Convert files to the desired software's format and stitch individual tile scans using the tile stitching functionality.

8.6. After stitching individual tile scans together, open the composite file and proceed with volume surface rendering. Click on **Add New Surface** to open the Surface Creation Wizard and use the arrows to toggle through menus, notably setting the ROI and threshold intensity. Once satisfied with the surface rendering, utilize the Animation to create videos of the processed image.

REPRESENTATIVE RESULTS:

This protocol details a reliable means of inducing ischemia and tissue loss in the murine footpad using a combination of femoral artery and vein coagulation with L-NAME administration, a nitric oxide synthase inhibitor, in susceptible FVB mice. **Figure 1** details the anatomy of the murine hindlimb vasculature and indicates the sites of the femoral artery and vein coagulation (yellow X), just proximal to the lateral circumflex femoral artery (LCFA) and proximal to the saphenopopliteal junction. The LCFA needs to be identified, and the coagulation sites respective to this structure are kept consistent throughout all surgical procedures. As described, 2 h before surgical procedures and on postoperative days 1-3, mice were also administered 40 mg/kg IP of L-NAME to maintain elevated tissue levels of oxidative stress. **Figure 2** shows the variation in tissue loss that can be expected from this model one week after surgery, with Faber scores[9] recorded in the bottom right-hand corner of each image.

DiI perfusion was performed in FVB mice at 5 and 20 days after femoral artery and vein coagulation to assess hindlimb reperfusion following induction of ischemia. **Figure 3A** illustrates the murine anatomy after dissection to expose the thoracic cavity. A butterfly needle is inserted into the left ventricle to begin cardiac perfusion. Note that the left ventricle appears slightly paler in color than the right ventricle. **Figure 3B** depicts the equipment set up with stopcocks connected in series and three syringes filled with PBS, DiI solution, and fixative. Following DiI perfusion, feet were harvested, skinned, and compressed between microscope slides as shown in **Figure 3C,D** before imaging with a confocal laser scanning microscope under 5x magnification. Reconstruction microscopy images revealed normal vascular anatomy in non-ligated control footpad (**Figure 4A**) compared with severely diminished perfusion to the footpad of ligated hindlimb 5 days after surgery (**Figure 4B**). Twenty days after surgery, perfusion to the footpad significantly improved (**Figure 4C,D,** and **Figure 5B**), although not to the extent of non-ligated control (**Figure 4A** and **Figure 5A)**. Vascularity was quantified as described above using the Vessel Density plugin in Fiji. The vascular fraction for the control footpad was 28%. Five days after

surgery, footpad vascular fraction was severely reduced to 2% but gradually recovered to 15% and 18% in two separate mice by 20 days postoperatively. To visualize the footpad vascular anatomy in 3D, we imported a stitched microscopy image into alternate image analysis and processing software to create a surface rendering as described previously (**Supplementary Figure 1**). A video of the surface rendering was then created using the animation functionality (**Video 1**).

FIGURE LEGENDS:

Figure 1: Anatomy of the murine hindlimb vasculature and sites of the femoral artery and vein coagulation. The external iliac artery continues as the femoral artery (FA) distal to the inguinal ligament. The first branches of the femoral artery include the lateral circumflex (LCFA) and deep femoral arteries (not pictured). More distally, the proximal caudal femoral (PCFA) and superficial caudal epigastric arteries (SCEA) branch from the FA proximal to the bifurcation of the saphenous (SA) and popliteal arteries (PA). The femoral nerve (FN) courses alongside the femoral vessels and should be gently isolated before coagulation of the femoral vessels. FA and femoral vein (FV) coagulation sites are also indicated (X).

Figure 2: Representative images of hindlimb gangrene in FVB mice with corresponding Faber scores. The degree of ischemic changes induced by this model varies from one or more ischemic nails (Faber scores 1-5) to gangrenous digits (Faber scores 6-10) and partial or complete foot atrophy.

Figure 3: Animal dissection and equipment setup for DiI perfusion and mounting of mouse foot for imaging. (**A**) Anatomical photograph of the murine anatomy during DiI perfusion. The abdominal and the thoracic cavities are opened, the sternum is reflected, and the ribs are cut on either side of the sternum. A 25 G butterfly needle connected to the stopcock assembly is inserted into the left ventricle. (**B**) Three 3-way stopcocks are connected in series. Three 10 mL syringes are filled with fixative, DiI, and PBS and connected to the stopcock assembly. A 25 G butterfly needle is connected to the outflow port of the proximal stopcock. (**C**) Mounting skinned foot between two microscope slides with a folded foam biopsy pad and binder clip at each end to compress the slides together. (**D**) An alternative view of the skinned foot compressed between microscope slides.

Figure 4: Representative 5x images obtained by confocal laser scanning microscopy of the mouse footpad following DiI perfusion with quantified vessel density expressed as a percent of ROI. (**A**) Normal footpad vasculature. (**B**) Footpad vasculature 5 days after femoral artery and vein coagulation shows severely reduced perfusion with minimal vessel opacification. (**C**) Footpad vasculature 20 days after femoral artery and vein coagulation demonstrates some reconstitution of distal flow to the metatarsal and digital arteries. (**D**) Image of an additional mouse footpad obtained 20 days after femoral artery and vein coagulation showing minimal large vessel compared to microvascular opacification.

Figure 5: Magnified images of the footpad vasculature. (**A**) 5x and 20x images of control footpad

vasculature demonstrating intact perfusion *via* the metatarsal and digital arteries. (**B**) 5x and 20x images of footpad from ligated hindlimb 20 days postoperatively showing reduced perfusion *via* larger metatarsal arterial branches but the development of an extensive, plush capillary network.

Video 1: Animation of the 3D surface rendering of the footpad vasculature. Video displaying a surface rendering of the footpad vasculature illustrates the 3D resolution achievable with the described protocol.

Supplementary Figure 1: Steps in the surface rendering of DiI perfusion images. (**A**) Original DiI perfusion image imported into image analysis and processing software. (**B**) Surface rendering overlaid onto DiI perfusion image during setting of the threshold intensity. (**C**) Final 3D surface rendering of DiI perfusion microscopy image.

DISCUSSION:

While mouse hindlimb ischemia is the most widely used preclinical model to study neovascularization in PAD and CLTI, there is significant variation in ischemia severity and recovery depending on the specific mouse strain used and the site, number, and method of arterial disruption. The combination of femoral artery ligation and IP administration of L-NAME can reliably induce hindlimb gangrene in FVB mice[11]. The same treatment results in hindlimb ischemia without tissue loss in C57BL/6 mice, whereas in BALB/c mice, auto-amputation of the foot or leg can be induced by femoral artery ligation alone. As such, the above-described technique of femoral artery coagulation with concurrent L-NAME administration in FVB mice, which have an intermediate response to ischemia insult, provides a unique and reproducible model of footpad gangrene akin to that seen in the most severe manifestation of diseases that lead to CLTI. The degree of tissue loss observed with this model can vary from a few ischemic nails to multiple gangrenous digits but rarely progresses to auto-amputation of the foot or leg, which allows for longitudinal assessment of limb reperfusion and function. Unlike BALB/c mice, in which the onset of gangrene is rapid with limb auto-amputation typically occurring in less than one week, there is delayed onset of tissue loss in this FVB mouse gangrene model. Femoral artery coagulation acutely restricts blood flow to the hindlimb. Still, accumulation of oxidative stress due to L-NAME administration on postoperative days 0-3 is more gradual, with peak atrophic changes observed between 7-14 days. Therefore, this model offers an improved therapeutic window to evaluate the effects of a particular intervention on gross tissue appearance and rescue of tissue loss in addition to quantifying reperfusion and assessing limb function.

Regarding surgical technique, coagulation or ligation of both the femoral artery and vein is favored due to the relative ease of this operation compared to the isolation of the femoral artery. While this technique can lead to venous thrombosis and insufficiency, it compounds the ischemic insult and helps to induce gangrenous changes more reliably. Additionally, chronic venous insufficiency (CVI) is highly prevalent in the general population, with 10%-30% of adults affected. Consistently, approximately 20% of patients with PAD, especially those with severe arterial insufficiency, also have comorbid CVI[21,22]. Regardless of whether one decides to ligate or coagulate the femoral vein, it is critically important to maintain the specific site(s) of femoral artery disruption constant across experimental groups. More proximal ligations, such as that of

the iliac artery, lead to occlusion of additional downstream collaterals and limit the possibility for arteriogenesis[8,16]. However, angiogenesis in the distal part of the limb, especially the gastrocnemius muscle, should still be triggered. In FVB mice, double ligation or coagulation of the femoral artery just proximal to the lateral circumflex femoral artery and proximal to the saphenopopliteal bifurcation more consistently induces gangrene than a single ligation or coagulation site.

It should be noted that in PAD and CLTI patients, limb ischemia is caused by atherosclerotic obstruction (a chronic process). In contrast, in mouse models, limb ischemia is induced surgically (an acute process). Although this FVB mouse hindlimb gangrene model has a relatively slower onset of gangrene with delayed peak severity of tissue loss, it is not directly comparable with the chronic, progressive arterial stenosis characteristic of PAD and CLTI. Other groups have developed subacute femoral artery occlusion techniques using ameroid constrictors comprised of an outer metal sleeve and an inner layer of moisture-absorbing material that gradually self-expands. This technique has been shown to result in decreased expression of inflammatory markers, lower blood flow recovery at 4-5 weeks, and reduced muscle necrosis[23,24]. Other than differences in ischemia acuity, preclinical models using young, healthy animals also fail to replicate risk factors such as diabetes, hypertension, obesity, hyperlipidemia, smoking, and infection that contribute to major adverse limb events and the burden of vascular disease.

In most studies of murine hindlimb ischemia, restoration of blood flow to the ischemic hindlimb is typically assessed *via* LDPI[24,25]. This method is non-invasive and repeatable but can be influenced by core body temperature, anesthetic use, hair presence, and positioning of the hindlimbs[26]. Standardization of these procedures and using the non-ligated hindlimb as an internal control can help mitigate any variations. In contrast to LDPI, micro-CT and MRA provide high-resolution, 3D anatomical information but traditionally require the injection of contrast agent[16]. X-ray microangiography is also invasive and technically challenging[16,27]. Like DiI, perfusion with radiopaque silicone casting agents allows for post-mortem 3D reconstruction of the peripheral vasculature[28]. Intracardiac or tail vein injection of fluorescent lectin has also been described for labeling of tissue vasculature[29]. Following harvest of tissues of interest, immunohistochemical staining endothelial-specific markers, such as CD31 and von Willebrand factor, are often used to quantify capillary density[30].

Compared to the techniques mentioned above, DiI perfusion provides several advantages. Firstly, the reagents and materials required are relatively inexpensive, provided access to a confocal laser scanning microscope is available. This method allows for 3D reconstruction of the vasculature, which can be quantified using image analysis software. While this protocol focuses on the footpad vasculature, whole-mount imaging of other murine hindlimb tissues, notably the gastrocnemius and adductor muscles, is also feasible and relevant to angiogenesis and arteriogenesis[19] studies. This technique can be modified for larger animal models, including rats and rabbits, by increasing the volume of perfusion solutions. However, imaging constraints regarding tissue size are described below.

Critical portions of DiI perfusion are as follows. Air bubbles in the apparatus may occlude small

vessels and hinder the distribution of DiI throughout the vasculature, thereby influencing imaging results. As such, care must be taken to remove any air bubbles in the stopcock apparatus and tubing before perfusion. Filtering all solutions except DiI through a 0.22 µm bottle top filter is also recommended to remove any microparticles. During intracardiac perfusion, carefully monitor the lungs. If they become enlarged and turn pink in color, this is a sign that the butterfly needle has penetrated through to the right ventricle and needs to be retracted slightly.

An important limitation of DiI perfusion is the procedure's terminal nature, which does not allow for repeated measurements. Because poor perfusion results may reflect underlying arterial insufficiency or technical error, harvesting and imaging the non-ligated hindlimb as an internal control is recommended. With regards to imaging, optimal tissue thickness for laser penetration is ~1 mm after compression. Consequently, larger tissues require sectioning into smaller pieces to be mounted on slides and accommodated on the microscope stage and proportionally longer image acquisition times.

In summary, this protocol outlines a unique preclinical model for studying PAD and CLTI. Specifically, femoral artery and vein coagulation with concurrent administration of L-NAME, a NOS inhibitor, reliably induces tissue loss in the footpads of FVB mice. Post-mortem, intracardiac DiI perfusion is then used to label the vasculature fluorescently. Subsequent whole-mount imaging of the harvested feet with confocal laser scanning microscopy allows for high-resolution, 3D reconstruction of the footpad vasculature and visualization of arterial and capillary networks that complements traditional means of assessing reperfusion in hindlimb ischemia models.

ACKNOWLEDGMENTS:

This work was supported by grants to Z-J L and OC V from the National Institutes of Health [R01HL149452 and VITA (NHLBI-CSB-HV-2017-01-JS)]. We also thank the Microscopy and Imaging Facility of the Miami Project to Cure Paralysis at the University of Miami School of Medicine for providing access to their image analysis and processing software.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1.pdf

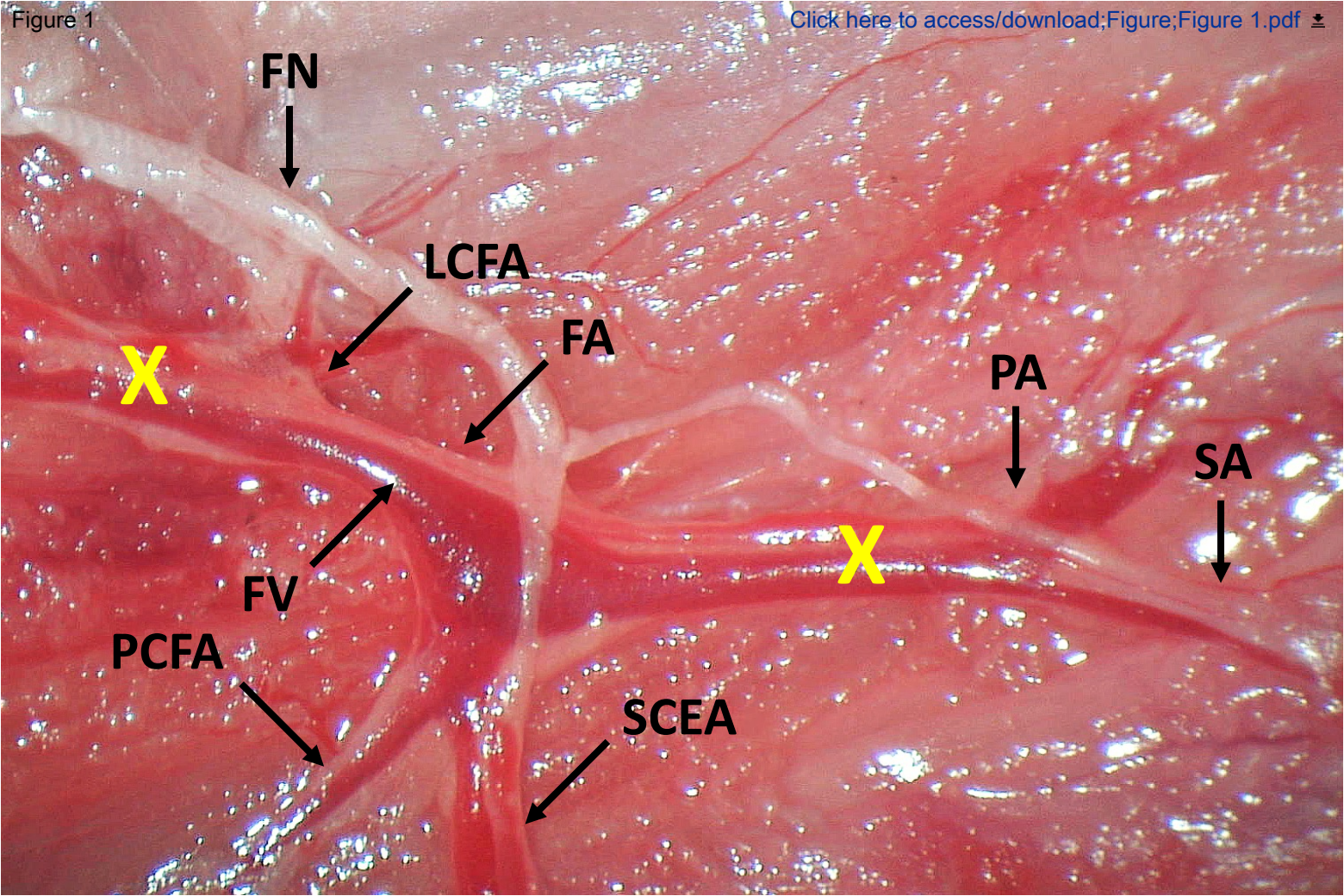

Figure 2

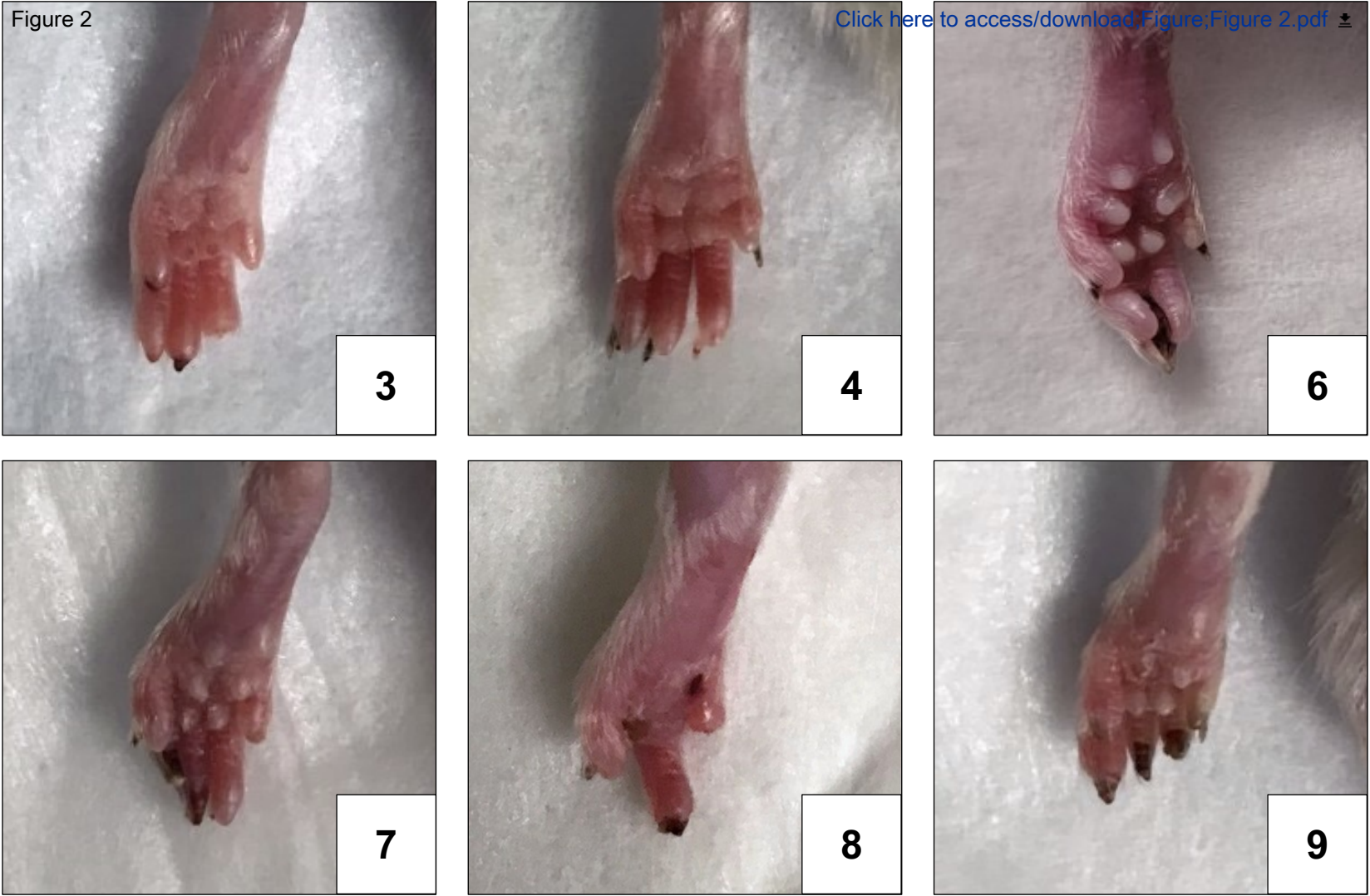

Figure 3

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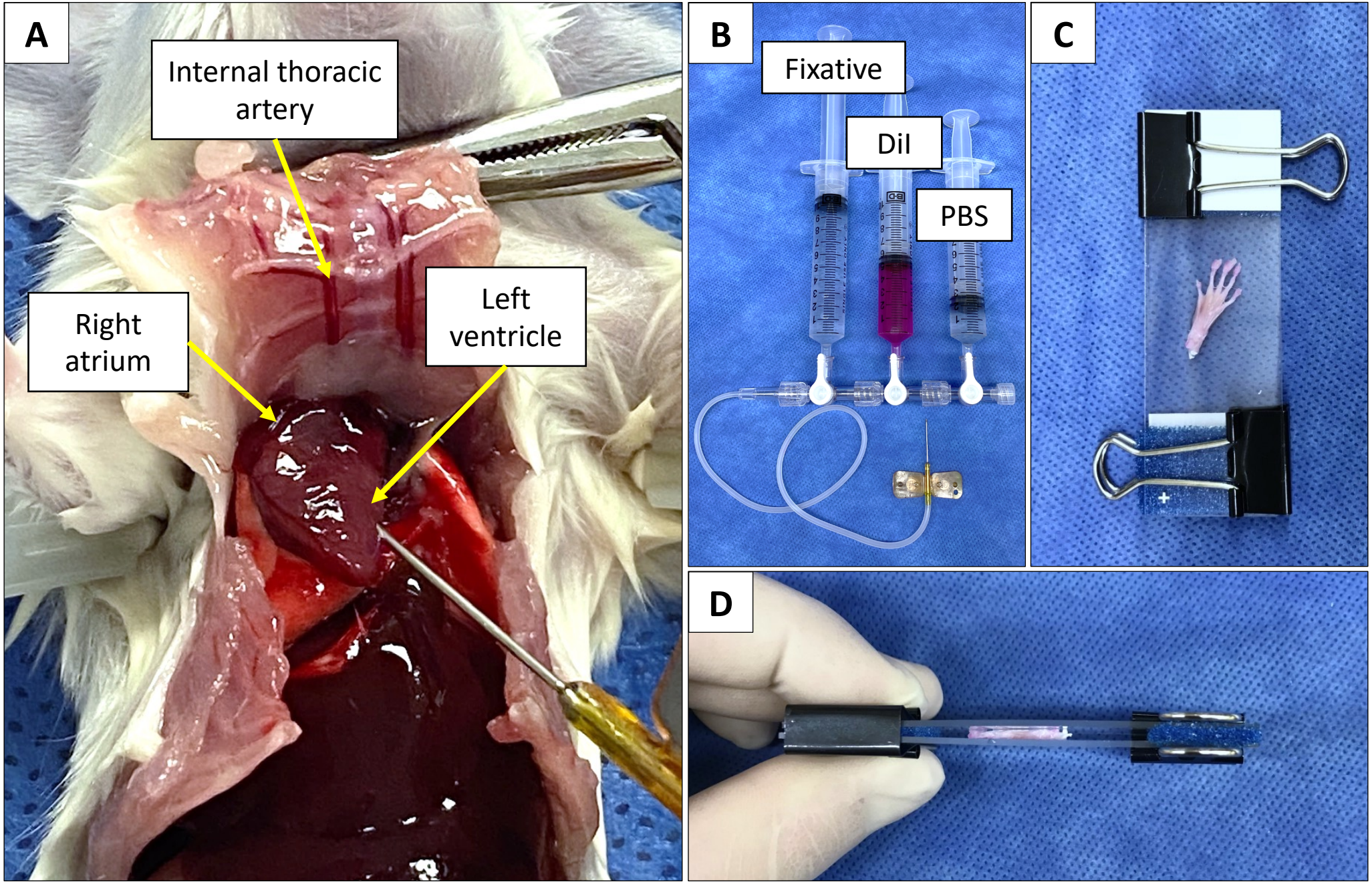
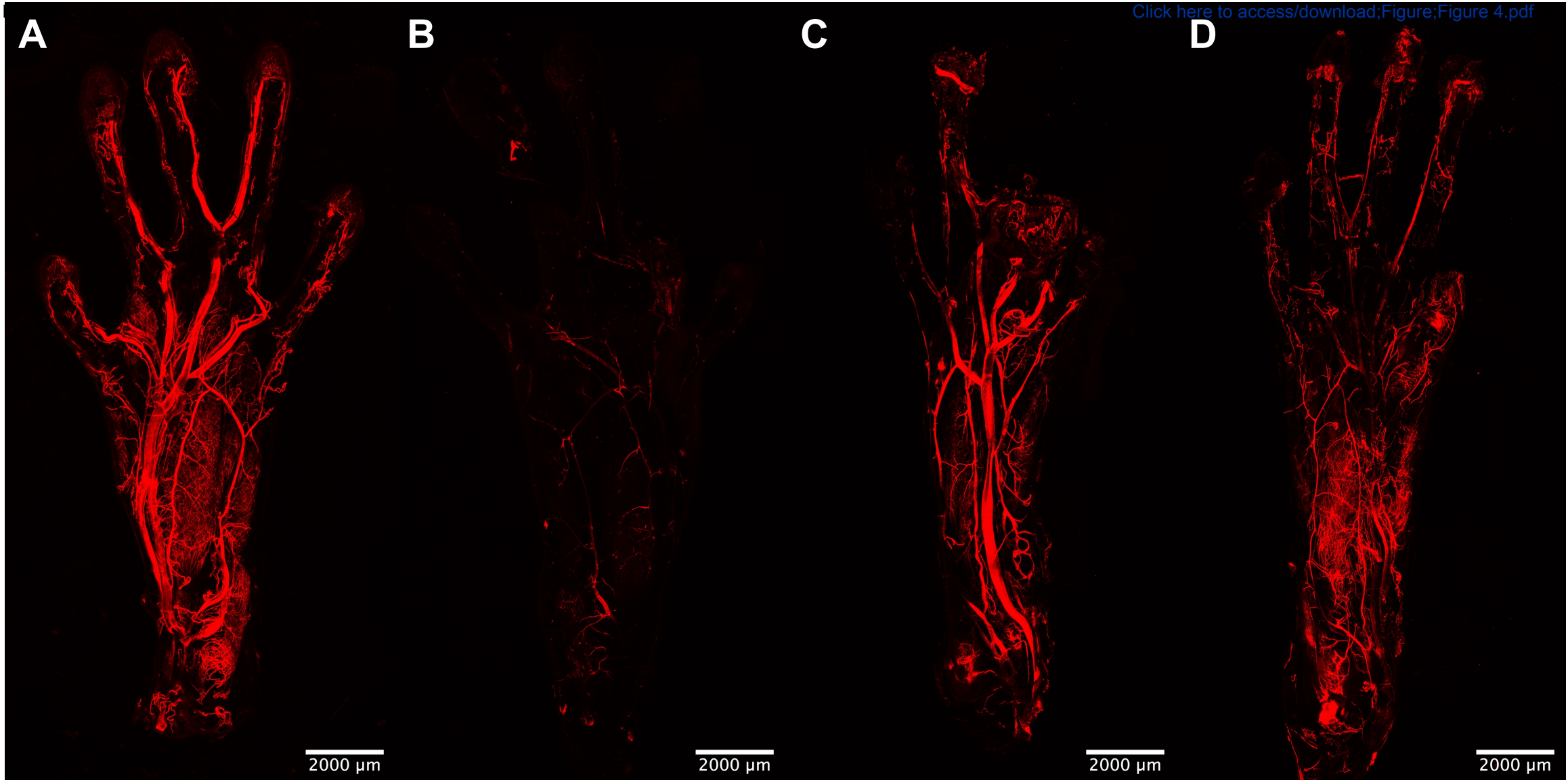
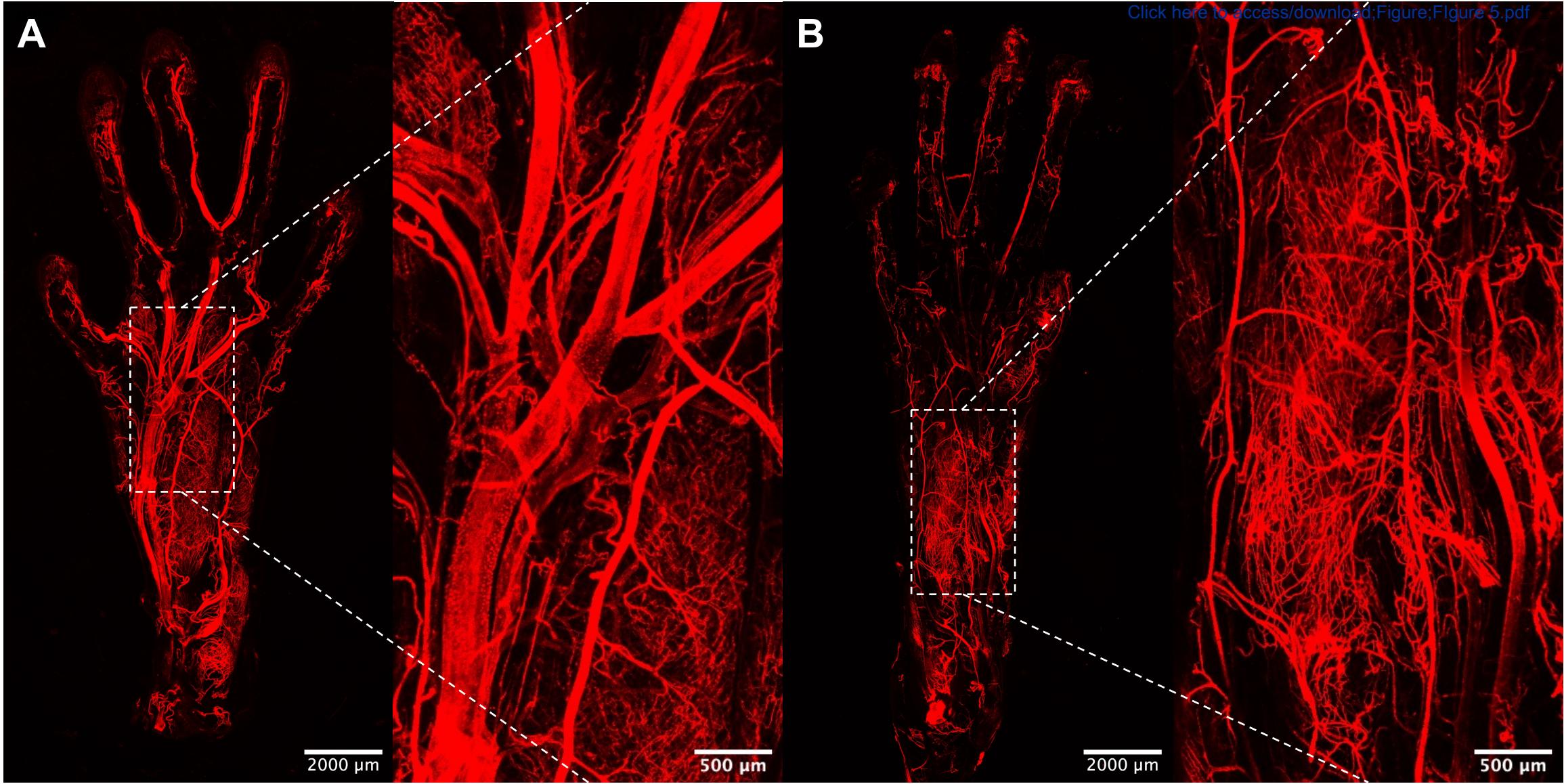




Non-ligated (28%)	Ligated, Day 5 (2%)	Ligated, Day 20 (15%)	Ligated, Day 20 (18%)



Video 1

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Video or Animated Figure

Video 1.mov

Editorial comments:

Editorial Changes

Changes to be made by the Author(s):

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

We have proofread the manuscript for spelling and grammar issues.

2. *Please revise the following lines to avoid previously published work: 217-218, 239-240, 244-245, 251-252 (see attached file).*

We have revised the above lines as follows:

217-218 (now 215-218): "5.3. Connect the syringe with formalin to the proximal inflow port and inject solution to flush air from the line, turn the stopcock to close the port. Repeat the same procedure sequentially connecting the syringes with DiI and then PBS to the middle and distal inflow ports, respectively, taking care to flush all air bubbles through the apparatus."

239-240 (now 234-238): "5.6. Identify the left ventricle, which appears lighter in color than the right ventricle. Gently grasp the heart with blunt forceps and insert the butterfly needle into the left ventricle. Use scissors or an 18-gauge needle to puncture the right atrium to allow blood and perfusion solutions returning to the heart to drain. Stabilize the needle with one or two hands, taking care not to inadvertently puncture the right ventricle and perfuse the pulmonary rather than systemic circulation."

244-245 (now 240-242): "5.7. Open the port to the syringe with PBS and inject 2-4 mL at a rate of 1-2 mL/min for 1-2 min to flush blood from the vascular system. Ensure successful perfusion by observing bleeding from the right atrium. After injection, close the port to the PBS syringe."

251-252 (now 249-251): "5.9. Open the port to the syringe with formalin and inject 5-10 ml at a rate of 1-2 mL/min for 5 min. After injection, remove the needle from the left ventricle and proceed to harvest the tissues of interest."

3. *Please define all abbreviations before use. For example, FVB, Dil, etc.*

We have defined abbreviations with their first use, including PAD, CLTI, FVB, DiI, LDPI, NOS, and 3D in the Abstract and ABI, L-NAME, NO, ROI, micro-CT, and MRA in the Introduction.

4. *Please provide all in-text citations as superscript numbers before the punctuation.*

We have changed all citations to this format.

5. *Please provide citations for the following lines*:

We have provided citations for the following lines:

84-85: lines deleted
86-91 (now 93-96): Parikh et al. (2018)
95-97 (now 96-97): Soiza et al. (2020), McDermott et al. (2020)
104-108 (now 104-108): Chalothorn et al. (2007)
111-115 (now 111-115): Aref et al. (2019)
118-122 (now 116-118): Honig et al. (1989), Li et al. (2008)
415-418 (now 62-63): Mills et al. (2014)
418-420 (now 64-65): Conte et al. (2019)
453-454 (now 466-467): Padgett (2016), Nowak-Sliwinska et al. (2018)
454-457 (now 467-468): Greco et al. (2013)
457-459 (now 470-471): Aref et al. (2019)

6. *Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.*

We have revised the text accordingly.

7. *JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.*
For example: Leica #506224, Imaris (version 9.6.0, Oxford Instruments, UK), etc.

We have removed all commercial language and replaced with generic terms as requested.

8. *Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?*
Step 2.2: How was proper sedation confirmed? Please mention. Was the surgical site sterilized before making the incision? If yes, please mention how was it done. How was the surgical site prepared, please describe in brief? Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

We have edited this step to: "2.2. Anesthetize mice with IP injection of 100 mg/kg ketamine and 10 mg/kg xylazine diluted in PBS. Confirm adequate sedation by absence of toe-pinch reflex and continue monitoring respiratory rate during the procedure. Remove hair from bilateral hindlimbs and groins using shears and/or a depilatory cream. Position the animal under a surgical microscope in supine position; extend and tape the extremities in place. Sterilize the surgical field by circumferentially applying chlorhexidine or povidone iodine solution to the surgical site." **(pg. 4, line 146-151).**

Step 2.4: How was the cautery device coagulation done? Please describe in brief.

We have edited this step to: "Proceed with electrocoagulation of the femoral artery and vein just proximal to this branch by activating the cautery device and gently contacting the vessels with a side-to-side motion, ensuring that the femoral nerve is well-isolated and remains protected from thermal injury." **(pg. 4, line 160-163).**

Step 2.6: How was PBS injected? How was hemostasis maintained? Please describe.

We have edited this step to: "2.6. Irrigate the surgical field using a syringe filled with sterile PBS. Obtain hemostasis by applying gentle pressure with a cotton tip applicator for 3-5 min to any areas of bleeding." **(pg. 4-5, line 171-172).**

Step 2.7: How was the mean perfusion index calculated? Please describe. Alternatively, add references to published material specifying how this can be calculated.

We have edited this step to: "Proceed with LDPI of bilateral feet. Once scanning is complete, draw a ROI around each footpad and obtain the mean flux values. Calculate the perfusion index as the ratio of mean flux values from the ligated to non-ligated footpad. Ensure that the perfusion index is less than 0.1." (pg. 5, line 178-181)**. In the Discussion, we also include a reference from *JoVE* that details how to obtain and analyze LDPI images (Padgett et al. 2016) (pg. 11, line 466-467).**

Step 3.2: Please provide references for the Faber hindlimb ischemia score.

We have added the following reference: Chalothorn et al. (2007).

Step 7.3: Please elaborate the steps for acquiring images. Please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. Please keep in mind that software steps without a graphical user interface (GUI) cannot be filmed.

We have edited this step to:
"7.1. Prepare for the imaging session: turn on the imaging system and start the acquisition software. Use a low magnification/low numeric aperture objective (e.g., x5/0.15) to capture images as lower magnification lenses typically have longer working distances required for this experiment.

7.2. Click **Yes** to the **Activate Stage** dialog box. Activate the 561 nm laser in the **Configuration** tab. In the main screen, activate a visible beam path by clicking on the **Visible** button. Set a detector to the 570-600 nm range by clicking the corresponding **Active** checkbox.

7.3. In the **Acquire** > **Acquisition** tab, select the **Tile Scan** icon and set the desired resolution (512x512 or 1024x1024).

7.4. Position dry-mounted (no water or PBS added) tissue sample compressed between glass slides on the microscope stage and bring tissue into focus.

7.5. To set the scanning boundaries, navigate to the upper left or right corner of the sample. In the **Acquisition** tab, under the **Tile Scan** menu, click the **Mark Position** button. Navigate to the opposite corner (lower right or left, respectively) and click the **Mark Position** button once again.

7.6. To set the depth of the Z-stack, click the **Live** button at the lower left corner of the screen and navigate to the center of the sample. Use the z-axis knob to scroll through to the bottom of the sample. In the **Acquisition** tab, under the **Z-Stack** menu, click the **Begin** button. Scroll through to the top of the sample and click the **End** button. Click **Z-step Size** and set to desired value (e.g., 50 µM).

7.7. In the lower right corner of the screen, click **Start** to begin image acquisition." **(pg. 7, line 277-303)**

Step 8: Please bold letters with the initial letter capitalized of all the commands/options.

We have revised the text in this format.

9. Please also include in the Discussion the following in detail along with citations:
a) Critical steps within the protocol

We have added the following lines regarding critical portions of the FVB gangrene model: "Regardless of whether one decides to also ligate or coagulate the femoral vein, it is critically important to maintain the specific site(s) of femoral artery disruption constant across experimental groups." **(pg. 11, line 443-445) and DiI perfusion:** "Critical portions of DiI perfusion are as follows. Air bubbles in the apparatus may occlude small vessels and hinder the distribution of DiI throughout the vasculature, thereby influencing imaging results. As such, care must be taken to remove any air bubbles in the stopcock apparatus and tubing prior to perfusion. Filtering all solutions except DiI through a 0.22 µm bottle top filter is also recommended to remove any microparticles. During intracardiac perfusion, carefully monitor the lungs. If they become enlarged and turn pink in color, this is a sign that the butterfly needle has penetrated through to the right ventricle and needs to be retracted slightly." **(pg. 12, line 489-495).**

b) Any limitations of the technique

We have added the following lines regarding limitations of this FVB gangrene model: "It should be noted that in PAD and CLTI patients, limb ischemia is caused by atherosclerotic obstruction (a chronic process), whereas in mouse models, limb ischemia is induced surgically (an acute process). Although this FVB mouse hindlimb gangrene model has a relatively slower onset of gangrene with delayed peak severity of tissue loss, it is not directly comparable with

the chronic, progressive arterial narrowing characteristic of PAD and CLTI." **(pg. 11, line 453-457) and** "Other than differences in ischemia acuity, preclinical models using young, healthy animals also fail to replicate risk factors such as diabetes, hypertension, obesity, hyperlipidemia, smoking, and infection that contribute to major adverse limb events and the burden of vascular disease." **(pg. 11, line 462-464), as well as limitations of DiI perfusion:** "An important limitation of DiI perfusion is the terminal nature of the procedure which does not allow for repeated measurements. Because poor perfusion results may reflect underlying arterial insufficiency or technical error, harvesting and imaging the non-ligated hindlimb as an internal control is recommended. With regards to imaging, optimal tissue thickness for laser penetration is approximately 1 mm after compression. Consequently, larger tissues require sectioning into smaller pieces to be mounted on slides and accommodated on the microscope stage, as well as proportionally longer image acquisition times." **(pg. 12, line 497-503).**

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes a surgical model of limb ischemia in FVB mice that produces digit necrosis. Additionally, the authors describe a method for fixations and perfusion of DiI compound which allows 3D reconstruction of the vascular beds in the paw. Overall, the authors describe a solid surgical approach of coagulation of the femoral artery and vein are two locations that when combined with L-NAME produce digit necrosis in FVB mice. Overall, the methods are of interest to the field and describe a reasonably simple means of evaluating 3D vascular structure in hindlimb ischemia. Below are a few comments that I think are worthy of clarification:

Major Concerns:

1) *It is not clear to this reviewer whether delivery of L-NAME is required to produce the CLI-like phenotype. The description of the surgery as provided would consistently produce digit necrosis in BALB/c mice without L-NAME.*

We thank the Reviewer for the opportunity to clarify. "There are important murine strain-dependent differences in sensitivity to ischemic insult partially owing to anatomical differences in collateral distribution. C57BL/6 mice are relatively resistant to hindlimb ischemia, demonstrating reduced limb function but generally no evidence of gangrene in the footpad. On the other hand, BALB/c mice have an inherently poor capacity to recover from ischemia and typically develop auto-amputation of the foot or lower leg following femoral artery ligation alone." **(pg. 2, line 126-132). The Reviewer is, thus, correct in that this surgery would produce digit or limb necrosis in BALB/c mice without L-NAME. However, the degree of tissue loss is often severe with auto-amputation of the lower limb, which prevents longer-term assessment of footpad reperfusion and functional recovery. However, in the FVB model described, L-NAME is required to produce atrophic changes of the digits and footpad, but it rarely progresses to auto-amputation of the leg as observed in BALB/c mice offering an**

improved therapeutic window to study tissue loss. We have clarified this in the Introduction: "Compared to C57BL/6 and BALB/c strains, FVB mice demonstrate an intermediate but inconsistent response to femoral artery ligation alone, with some animals developing footpad gangrene in the form of black ischemic nails or mummified digits, yet others without any overt signs of ischemia. Concomitant administration of N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), a reversible inhibitor of NOS, prevents compensatory vasodilatory mechanisms and further increases oxidative stress in hindlimb tissue. In combination with femoral artery ligation or coagulation, this approach consistently produces footpad tissue loss in FVB mice that resembles the atrophic changes of CLTI but rarely progresses to limb auto-amputation." **(pg. 3, line 88-96)**, **and in the Discussion:** "the above-described technique of femoral artery coagulation with concurrent L-NAME administration in FVB mice, which have an intermediate response to ischemia insult, provides a unique and reproducible model of footpad gangrene akin to that seen in the most severe manifestation of diseases that lead to CLTI. The degree of tissue loss observed with this model can vary from a few ischemic nails to multiple gangrenous digits, but rarely progresses to auto-amputation of the foot or leg which allows for longitudinal assessment of limb reperfusion and function." **(pg. 10, line 422-428).**

2) *Can the authors expand the discussion on why the current FVB coagulation/L-NAME model produces a delayed appearance of necrosis? Is there no recovery of limb/paw perfusion across 7-14 days as we typically see in femoral artery ligation? It would be useful to have LDPI recovery data to complement the imaging. If paw perfusion is increasing similar to FAL models, then it is not clear what is causing delayed toe necrosis.*

We thank the Reviewer for this suggestion. In contrast to the acute ischemic insult caused by femoral artery ligation or coagulation, oxidative stress generated by L-NAME administration requires a more gradual accumulation and we believe this explains the delayed peak severity of tissue loss despite some recovery of blood flow (mean perfusion index 10% on day 7 compared to <5% post-operatively). Because this paper focuses on the protocol for inducing gangrene and then DiI perfusion, we did not include LDPI data but have previously published this in Parikh et al. (2018). This is now clarified in the Discussion as follows: "Unlike BALB/c mice, in which the onset of gangrene is rapid with limb auto-amputation typically occurring in less than one week, there is delayed onset of tissue loss in this FVB mouse gangrene model. Femoral artery coagulation acutely restricts blood flow to the hindlimb, but accumulation of oxidative stress due to L-NAME administration on postoperative days 0-3 is more gradual with peak atrophic changes observed between 7-14 days." **(pg. 10, line 428-433).**

Minor Concerns:
1) *Can the authors comment on how compression of the footpad between slides might alter the 3D reconstruction of the vasculature?*

Compression of the footpad is limited by bony structures and does not appreciably affect 3D structure and reconstruction of the vasculature.

2) *In the discussion, the authors focus the model relevance solely on tissue loss/necrosis as*

means of highlighting the relevance of the current model. These authors should be encouraged to acknowledge that ischemic burden is not the sole predictor or reason for tissue loss in CLI patients. There are many other reasons that patients develop necrosis and non-healing wounds that lead to major adverse limb events. These other features of the CLI patient population should at least be acknowledged, as the current model does not replicate those factors.

We thank the Reviewer for this suggestion and agree that there are many other factors that contribute to PAD/CLI. We have added to the Discussion: "It should be noted that in PAD and CLTI patients, limb ischemia is caused by atherosclerotic obstruction (a chronic process), whereas in mouse models, limb ischemia is induced surgically (an acute process). Although this FVB mouse hindlimb gangrene model has a relatively slower onset of gangrene with delayed peak severity of tissue loss, it is not directly comparable with the chronic, progressive arterial narrowing characteristic of PAD and CLTI… Other than differences in ischemia acuity, preclinical models using young, healthy animals also fail to replicate risk factors such as diabetes, hypertension, hyperlipidemia, smoking, and infection that contribute to major adverse limb events and the burden of vascular disease." **(pg. 11, line 453-464).**

3) *Can the authors comment on the ability to use this approach on other hindlimb tissues (i.e. smaller muscles) that might be relevant to studying angiogenesis? It would appear that the model and the imaging capabilities would be amenable to several other tissues, but this is not well discussed.*

We thank the Reviewer for this suggestion since this approach is readily adapted to imaging of other hindlimb tissues such as the adductor and gastrocnemius muscles. We have revised the Discussion to better reflect this: "While this protocol focuses on the footpad vasculature, whole-mount imaging of other murine hindlimb tissues, notably the gastrocnemius and adductor muscles, is also feasible and relevant to studies of angiogenesis and arteriogenesis." **(pg. 12, line 482-485).**

Reviewer #2:

Manuscript Summary:

The submitted manuscript describes the derivation of a rodent model of hindlimb ischemia utilizing FVB mouse strain and early NOS inhibition. The protocol is detailed and well written, and the authors are experts in the field with prior publications using similar methods. Many interested readers may be familiar with the femoral artery ligation model from which this protocol is derived, and will be familiar with the result of femoral artery ligation on some strains of mice. The authors have previously published their data subjecting C57Bl6, Balb/C, and the FVB strains using this technique. Because the FVB strain mice consistently develop ischemic gangrene when NOS inhibition is administered, this strain is proposed to be used for the technique. The protocol for imaging the microcirculation of the hind paw is interesting and may be of interest to readers in the field. I have only some minor concerns.

Major Concerns:
None.

Minor Concerns:
** 6.1 - How long should the tissue stay in fixative? Is there a preferred time frame from the harvest of tissue to imaging?*

Tissue should be kept in fixative overnight and then the bathing solution changed to PBS for longer-term storage. Preferred time frame from harvest to imaging is 1-2 days, although tissues stored in PBS at 4 °C in the dark will maintain fluorescence for up to 1 month. This is now clarified in the Protocol: "6.3. Proceed to mounting and imaging of tissues, preferably within 1-2 days of perfusion and harvest. Alternatively, return footpads to 6- or 12-well plates with 1-2 mL PBS; cover with foil and store at 4 °C to maintain fluorescence for up to 1 month." **(pg. 7, line 264-266).**

** 6.3 - Can the foam spacers be described more precisely in terms of dimensions and material?*
** Do the skeletal structures interfere with confocal imaging?*

We thank the Reviewer for this suggestion. We have recently started using histology cassette foam biopsy pads which will be more reproducible for readers. The text, Figures 3C and 3D, and Table of Materials are now revised accordingly. In our experience, skeletal structures do not significantly impede confocal imaging, but they do limit compression of the microscope slides which in turn affects tissue thickness and image acquisition.

** What is your assessment of the maximal effective thickness for tissues analyzed by this method?*

Confocal laser scanning microscopy is limited by laser penetration depth of 400-500 µm so we aim for a final tissue thickness of 1 mm after compression between microscope slides. Compression of mouse feet is limited by bony structures, but if imaging muscle only, samples of approximately 3-4 mm thickness can be compressed to 1 mm with good imaging results. Imaging constraints are outlined in the Discussion: "With regards to imaging, final tissue thickness after compression should be approximately 1 mm. Consequently, larger tissues require sectioning into smaller pieces to be mounted on slides and accommodated on the microscope stage, as well as proportionally longer image acquisition times." **(pg. 12, line 500-503).**

** Line 471 - It is stated that the protocol is easily modified for larger animals, but this would likely produce difficulty with using the described mounting and imaging methods. Have the authors experience in analyzing whole mounted tissues (paw) from rats and rabbits with these methods via confocal microscopy? Are any additional modifications necessary to accommodate larger size tissues?*

We thank the Reviewer for this suggestion. Our experience has been in mice only so have revised the text accordingly: "This technique can be modified for larger animal models, including rats and rabbits, by increasing the volume of perfusion solutions. Imaging constraints regarding tissue size are described below." **(pg. 12, line 485-487) and** "Consequently, larger tissues require sectioning into smaller pieces to be mounted on slides and accommodated on the microscope stage, as well as proportionally longer image acquisition times." **(pg. 12, line 501-503).**

Reviewer #3:
Manuscript summary:
Abstract
- Not critical limb ischemia - New nomenclature - chronic limb-threatening ischemia (CLTI)
- limited walking capacity - walking distance without pain

We have made these recommended changes.

Introduction
-Line 61 Chronic limb threatening ischemia

We have changed this and all other instances of critical limb ischemia (CLI) to chronic limb threatening ischemia (CLTI)

-Lines 76-78: Explain - Collateral development and anatomic variations are two factors that are responsible for different ABI in patients with similar topographic disease distribution.

We have edited this to: "In clinical practice, patients with ankle-brachial indices (ABI) <0.9 are considered to have PAD and those with ABI <0.4 associated with either rest pain or tissue loss as having CLTI. Symptoms vary among patients with similar ABIs depending on daily activity, muscle tolerance to ischemia, anatomic variations, and differences in collateral development." **(pg. 2, line 62-65), which is also relevant for the Reviewer's comment regarding line 418.**

Discussion
-Line 417 - This is not exactly correct. The diagnosis of CLTI implies that the clinical manifestation is the result of hemodynamic compromise, that is, that the pain at rest or the presence of trophic lesions in the limbs must be associated with an ABI lower than 0.4. Clinically each factor isolated don´t mean the presence of CLTI.

We thank the Reviewer for pointing this out and have clarified accordingly: "In clinical practice, patients with ankle-brachial indices (ABI) <0.9 are considered to have PAD and those with ABI <0.4 associated with either rest pain or tissue loss as having CLTI." **(pg. 2, line 62-63).**

-Line 418 - Symptoms depend on patient daily activity, muscle tolerance to ischemia, anatomic variations, collateral development.

We thank the Reviewer for this suggestion. We have added the above factors affecting patient symptoms and moved this sentence to the Introduction: "In clinical practice, patients with ankle-brachial indices (ABI) <0.9 are considered to have PAD and those with ABI <0.4 associated with either rest pain or tissue loss as having CLTI. Symptoms vary among patients with similar ABIs depending on daily activity, muscle tolerance to ischemia, anatomic variations, and differences in collateral development." **(pg. 2, line 62-65).**

-Line 420-422: This is not correct or translational. Please remove.

We have removed this sentence.

-Line 431: Lead to venous thrombosis and insufficiency

We have made the recommended change.