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# Establishing in situ closed circuit perfusion of lower abdominal organs and hind limbs in mice

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**TITLE:**

# Establishing In situ Closed Circuit Perfusion of Lower Abdominal Organs and Hind Limbs in Mice

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**KEYWORDS:**

In situ, mice, perfusion, bone marrow, prostate, iliac, tissue distribution, vasculature, dextran, lectin

**SUMMARY:**

A protocol is described for in situ perfusion of the mouse lower body, including the bladder, the prostate, sex organs, bone, muscle and foot skin.

**ABSTRACT:**

Ex vivo perfusion is an important physiological tool to study the function of isolated organs (e.g. liver, kidneys). At the same time, due to the small size of mouse organs, ex vivo perfusion of bone, bladder, skin, prostate, and reproductive organs is challenging or not feasible. Here, we report for the first time an in situ lower body perfusion circuit in mice that includes the above tissues, but bypasses the main clearance organs (kidney, liver, and spleen). The circuit is established by cannulating the abdominal aorta and inferior vena cava above the iliac artery and vein and cauterizing peripheral blood vessels. Perfusion is performed via a peristaltic pump with perfusate flow maintained for up to 2 h. In situ staining with fluorescent lectin and Hoechst solution confirmed that the microvasculature was successfully perfused. This mouse

model can be a very useful tool for studying pathological processes as well as mechanisms of drug delivery, migration/metastasis of circulating tumor cells into/from the tumor, and interactions of immune system with perfused organs and tissues.

**INTRODUCTION:**

Isolated organ perfusion was originally developed to study organ physiology for transplantation[1-3], and enabled understanding of functions of the organs without interference from other body systems. For example, isolated kidney and heart perfusion was immensely useful in understanding basic principles of hemodynamics and effects of vasoactive agents, whereas liver perfusion was important to understanding the metabolic function, including drug metabolism in healthy and diseased tissue[4-7]. In addition, perfusion studies were critical in understanding viability and function of organs intended for transplantation. In Cancer Researchearch, isolated tumor perfusion has been described by several groups using mouse, rat, and freshly resected human tissues[8,9]. In some isolated tumor perfusion, the tumor was implanted in the ovary fat pad to force the growth of tumor supplying blood vessels from the mesentery artery[10]. The Jain group performed pioneering studies using isolated perfusion of colon adenocarcinomas to understand tumor hemodynamics and metastasis[8,11-13]. Other innovative engineered ex vivo setups include a 96-well plate-based perfusion device to culture the primary human multiple myeloma cells[14] and a modular flow chamber for engineering bone marrow architecture and function research[15].

In addition to physiology and pathology studies, organ perfusion has been used to study the basic principles of drug delivery. Thus, one group described isolated rat limb perfusion and studied accumulation of liposomes in implanted sarcomas[16], whereas another group performed dissected human kidney perfusion to study the endothelial targeting of nanoparticles[17]. Ternullo et al. used an isolated perfused human skin flap as a close-to-in vivo skin drug penetration model[18].

Despite these advancements in perfusion of large organs and tissues, there have been no reports on in situ perfusion models in mice that: a) bypass clearance organs such as liver, spleen and kidneys; b) include pelvic organs, skin, muscle, reproductive organs (in male), bladder, prostate and bone marrow. Due to the small size of these organs and the supplying vasculature, ex vivo cannulation and establishment of a perfusion circuit has not been feasible. The mouse is the most important animal model in cancer and immunology research, and drug delivery. The ability to perfuse small mouse organs would allow interesting questions regarding drug delivery to these organs, including to tumors implanted in the pelvis (bladder, prostate, ovary, bone marrow), to be answered, as well as studies of basic physiology and immunology of diseases of these organs. To address this deficiency, we developed an in situ perfusion circuit in mice that can potentially avoid tissue injury and is much better suited for functional research than isolated organ perfusion.

**PROTOCOL:**

All methods described here have been approved by the University of Colorado's Institutional

Animal Care and Use Committee (IACUC).

## 1. Pre-heat the perfusion system

1.1. Prepare the perfusion system before surgery by starting a 37 °C circulating water bath for all water-jacketed components (perfusate reservoir, moist chamber, and lid) as shown in a customized configuration in **Figure 1A**. Make sure the tubing is clean and replace if necessary. To limit perfusate volume, use a bubble trap within a moist chamber as the perfusate reservoir (**Figure 1B-6**).

## 2. Vascular catheterization

2.1. Induce anesthesia in an 8-10 week old BALB/c mouse using an isoflurane veterinary anesthesia machine with 3-5% isoflurane and oxygen flow rate at 0.3 L/min. As an alternative, use ketamine/xylazine or any other type of intraperitoneal anesthesia. Evaluate the depth of anesthesia by 2 methods: toe-pinch and corneal reflex.

2.2. Prewet a 4-0 silk suture with needle in double distilled water.

2.3. Place the anesthetized mouse in a supine position on a Styrofoam board with the head facing the surgeon and immobilize forelimbs and hind limbs with tape. Wipe the abdomen with isopropyl alcohol and cut the abdomen along the midline in a "T" shape with scissors. Stop bleeding around the edge of the incision by electrocoagulation (cauterizing).

2.4. Push the stomach, jejunum and colon to the right side of the abdomen to reveal the abdominal aorta, vena cava, and common iliac and iliolumbar arteries and veins.

2.5. Under a dissection microscope, find and ligate the iliolumbar artery/vein in the male, and ovarian artery/vein and the iliolumbar artery/vein in the female using 4-0 silk sutures (**Figure 2** yellow lines).

2.6. Under a dissection microscope, loop two 4-0 silk sutures underneath the abdominal aorta and inferior vena cava (about 1 cm above iliac artery and vein, 1 mm apart, **Figure 2**), and make a loose knot in the suture closest to the iliac vessels (**Figure 2**, white dotted line). Alternatively, a 6-0 silk suture can be used for this knot.

2.7. Under a dissection microscope, horizontally align and stretch both the inferior vena cava and the abdominal aorta with porte-aiguille. Use a 24 G winged shielded I.V. catheter to puncture the abdominal aorta, push the button to retract the needle core and insert the catheter about 5 mm into the vessel.

2.7.1. Repeat the same procedure with the inferior vena cava and tie up knots of both sutures around the catheterized vessels.

NOTE: The needle can easily puncture through the blood vessel; therefore, keep the vessels stretched and needle parallel with the vessel. Retract the needle core as soon as the needle penetrates about 1 mm into the vessel. The abdominal aorta is underneath the inferior vena cava and much thinner and more elastic due to being encased in connective tissue. Therefore, the aorta can "hold on" to the catheter, and should thus be catheterized before the vena cava to reduce the likelihood of the catheter slipping out.

2.8. Apply instant glue to immobilize the catheters to the erector spinae, replace the abdominal organs, and end the surgery while maintaining anesthesia.

NOTE: Organs that cannot be completely replaced into the abdominal cavity will need to be periodically moistened with perfusion medium during the perfusion process.

## 3. Set up the perfusion system

3.1. Transfer the mouse into the water-jacketed moist chamber prewarmed to 37 °C on a silicon pad and immobilize the catheter wings to the pad with 19 G needles.

3.2. Fill the arterial catheter's end (inlet) with prewarmed perfusion buffer (Ringer's lactate solution supplemented with 5% BSA), and then connect the catheter end with the inlet perfusion tubing using a screw-on connector (**Figure 1B**, red arrow).

NOTE: Hold the connector with hemostatic forceps and immobilize the tubing with tape to avoid moving the catheters.

3.3. Adjust the peristaltic flow rate to 0.6 mL/min and keep the perfusion outflow (**Figure 1B**, blue arrow) open-ended for 5-10 min to wash out the blood through the venous catheter. There will be some clots in the outlet catheter; flush out the clots with perfusate buffer before closing the perfusion circuit.

3.4. Connect the venous catheter's end with the outlet tubing using a screw-on connector to close the circuit (**Figure 1B**). At this point, perform $CO_2$ gas euthanasia and verify by chest puncture or any other method.

3.5. Cover the moist chamber with the warmed lid. Check the level of perfusate periodically and add more if needed. Perfusion can be performed for up to 2 hours.

NOTE: 5 mL of perfusate will be needed to set up the closed perfusion system. If there is no leaking or edema, the volume of perfusate will decrease by less than 1 mL and additional buffer will not be needed. To avoid edema induced by peripheral circulating thrombus, perfusion buffer containing 0.002% heparin can be used in the first 10 minutes of perfusion, but should be changed to buffer without heparin to avoid the leaking at the edge of the incisions.

3.6. If needed, add a reagent of choice into the perfusion reservoir or to the injection port at any time (**Figure 1B-5**). For example, 10 µL of 10 mg/mL Hoechst33342 can be added into the perfusate to stain the cell nuclei 2 h before the end of perfusion, or 50 µL of 1 mg/mL DyLight 649-lectin to stain the vascular endothelial cells 30 min before the end of perfusion.

NOTE: If attempting to stain bone marrow with Hoechst solution, mice will need to be pre-injected 30 minutes before surgery.

3.7. After perfusion with fluorescent reagents, wash out with perfusion buffer for another 10 minutes to minimize the background fluorescence.

**4. Analysis of perfused organs**

4.1. Collect organs including testis, prostate, bladder, femur, muscle, and skin (e.g., feet). Excise a piece of organ about 1 $mm^3$ and flatten between two glass slides.

4.1.1. Study under inverted fluorescent confocal microscope using DAPI/Cy5 excitation and emission filters (excitation lasers : DAPI, 405 nm; Cy5,640 nm). Use at least 200x magnification objective with a 0.45 numerical aperture.

4.1.2. Alternatively, fix the organs with 4% formaldehyde solution for 24 h and perform hematoxylin-eosin staining[19].

4.2. To create a bone window for intact bone marrow observation, immobilize both ends of the femur or tibia and scrape away the cortical bone with the lateral edge of a 19 G needle to expose the periosteum; take care to keep a thin layer of residual bone. Place bone on a cover slip with the window facing the glass and image with inverted fluorescent confocal microscope using DAPI and Cy5 channels as described above. The cells and vascular network in the bone marrow cavity can be readily observed.

**REPRESENTATIVE RESULTS:**

We set up a closed circuit perfusion system through cannulation of the abdominal aorta and the inferior vena cava of 8-10 week old mice while keeping the volume of perfusion buffer less than 10 mL. **Figure 3A** shows confocal images after perfusing tissues with Ringer's solution containing Hoechst 33342 and DyLight 649-lectin. Muscle, bone marrow, testis, bladder, prostate, and foot skin show efficient nuclear and vascular staining. **Figure 3B** shows hematoxylin-eosin staining of organs after 2 hours of normothermic perfusion.

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Simplified perfusion setup.** (**A**) The system includes (1) a pressure/flow rate controller, (2) a circulating heated water bath, (3) a heated moist chamber with heated cover and custom Styrofoam spacer, and (4) a peristaltic pump. (**B**) The perfusion circuit shows inlet (red lines) and outlet (blue lines), (5) the injection port and (6) the customized perfusion reservoir.

**Figure 2. Location of ligated and cannulated blood vessels in male and female mice.** In order to outline the blood vessels, 5 µL/kg of 1% Evans Blue dye was added to perfusion medium 30 min before the end of the perfusion. In female mice, both iliolumbar and ovarian arteries and veins are ligated. In male mice, iliolumbar artery and vein are ligated. Yellow and white lines show approximate position of ligation knots; yellow arrows show actual sutures and knots. Both venous and arterial catheters are inserted. Intestines were removed for demonstration purposes.

**Figure 3. Confocal and H&E images show successful perfusion of organs for 2 h with no apparent tissue injury.** Confocal scale bar: 20 µm for bone marrow and 100 µm for other organs. H&E scale bar: 100 µm.

**DISCUSSION:**

The described circuit can be used to probe various research questions, for example the role of different serum components and tissue barriers in drug delivery, or immune and stem cell trafficking. Different drug delivery systems (e.g., liposomes and nanoparticles) can be added to the perfusate in order to understand the role of physiological and biochemical factors in delivery. The duration of perfusion can vary, depending on the tissue studied, scientific goals, and the composition of perfusate. We present here the results of perfusion up to 2 h using a basic perfusion media consisting of Ringer's solution with lactate and albumin. It must be noted that the goal of the present work was to establish the perfusion circuit rather than developing and optimizing perfusion medium and conditions to support optimal organ/tissue function and oxygenation. The addition of nutrients, vitamins, hormones, and blood cells have been extensively investigated in the previous literature, and numerous perfusion media and oxygenation protocols have been described in dozens of research publications in human and animal tissues[7,9-13,16,17,20,21]. Refinements of the perfusate composition as well as oxygenation can enable long-term maintenance of tissue metabolism at body temperature. Thus, some groups used red blood cells and blood oxygenation, which greatly improves viability of sensitive tissues[17]. Perfusion controls are also highly customizable; if necessary, a gas manifold to control oxygenation and pressure manometer can be added. In addition, a larger perfusate chamber can be used, and the physiological parameters such as pressure, temperature, and flow rate can be controlled by a computer.

There are several limitations in the perfusion circuit. The uterus and ovaries in female mice could not be perfused due to the ligation of ovarian artery and vein. Also, we observed that perfusion of testis was incomplete, possibly due to alternative blood supply not included in the perfusion circuit.

With sufficient practice, the cannulation procedure can be performed within 20-30 min with a success rate of over 80%. The success rate highly depends on the ability to cannulate the aorta and vena cava without puncturing the vessel as discussed in Step 2.7. It is important in step 2 to minimize injury to tissues and small blood vessels in the surgical area because of potential leaks and loss of perfusate. In step 2.5, one must always puncture the artery first, and take precaution so that the catheter will not be pulled out. In step 3, when connecting the catheters'

end to the tubing, make sure to hold the catheter tightly using porte-aiguille to avoid moving the needle. Blood pressure is directly proportional to flow rate; therefore, the flow rate of perfusate must be lower than 0.6 mL/min to maintain physiological pressure. Lastly, it is preferred to maintain the heartbeat until the perfusion circuit is closed, as this will improve the perfusion of microvasculature.

**ACKNOWLEDGMENTS:**

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure

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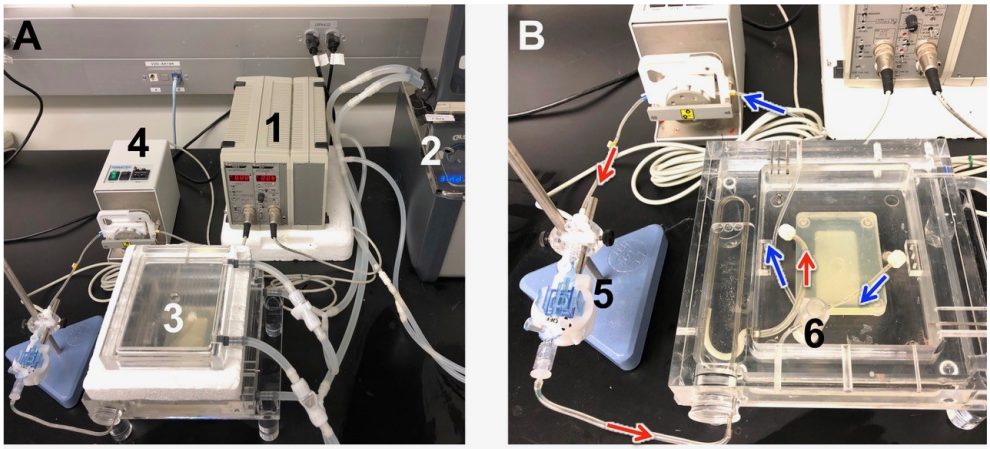

Figure

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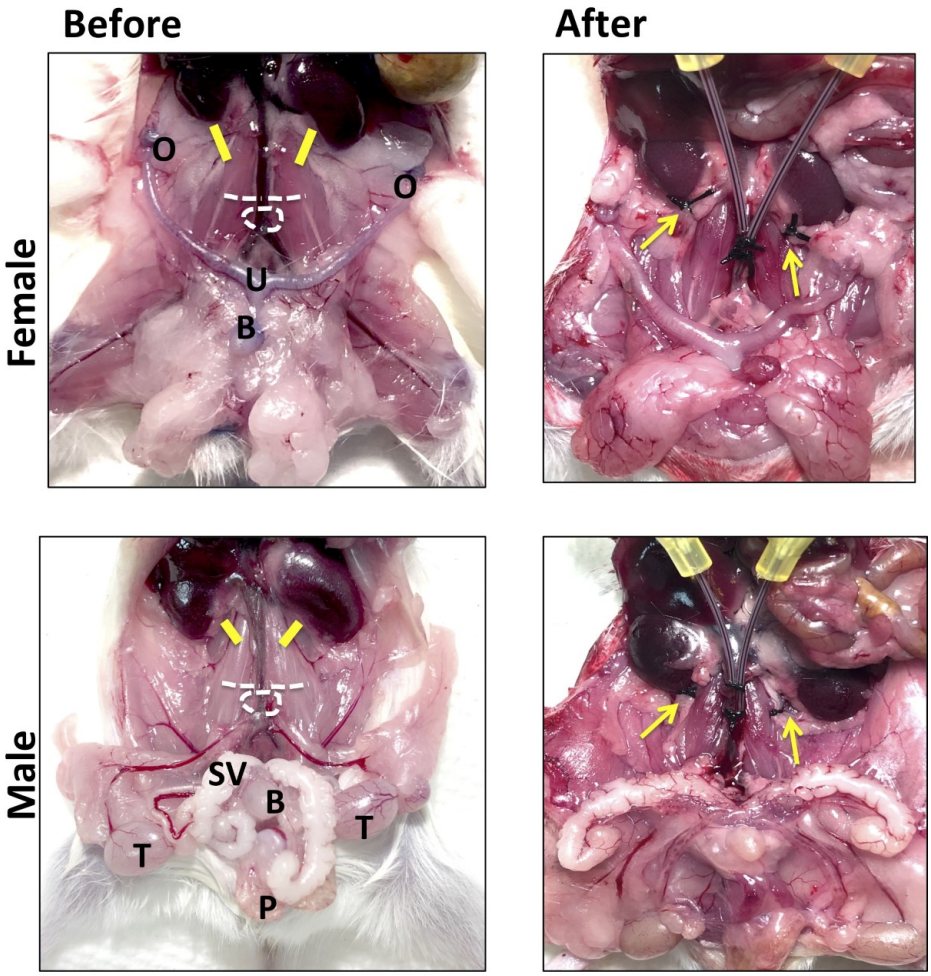

Figure3

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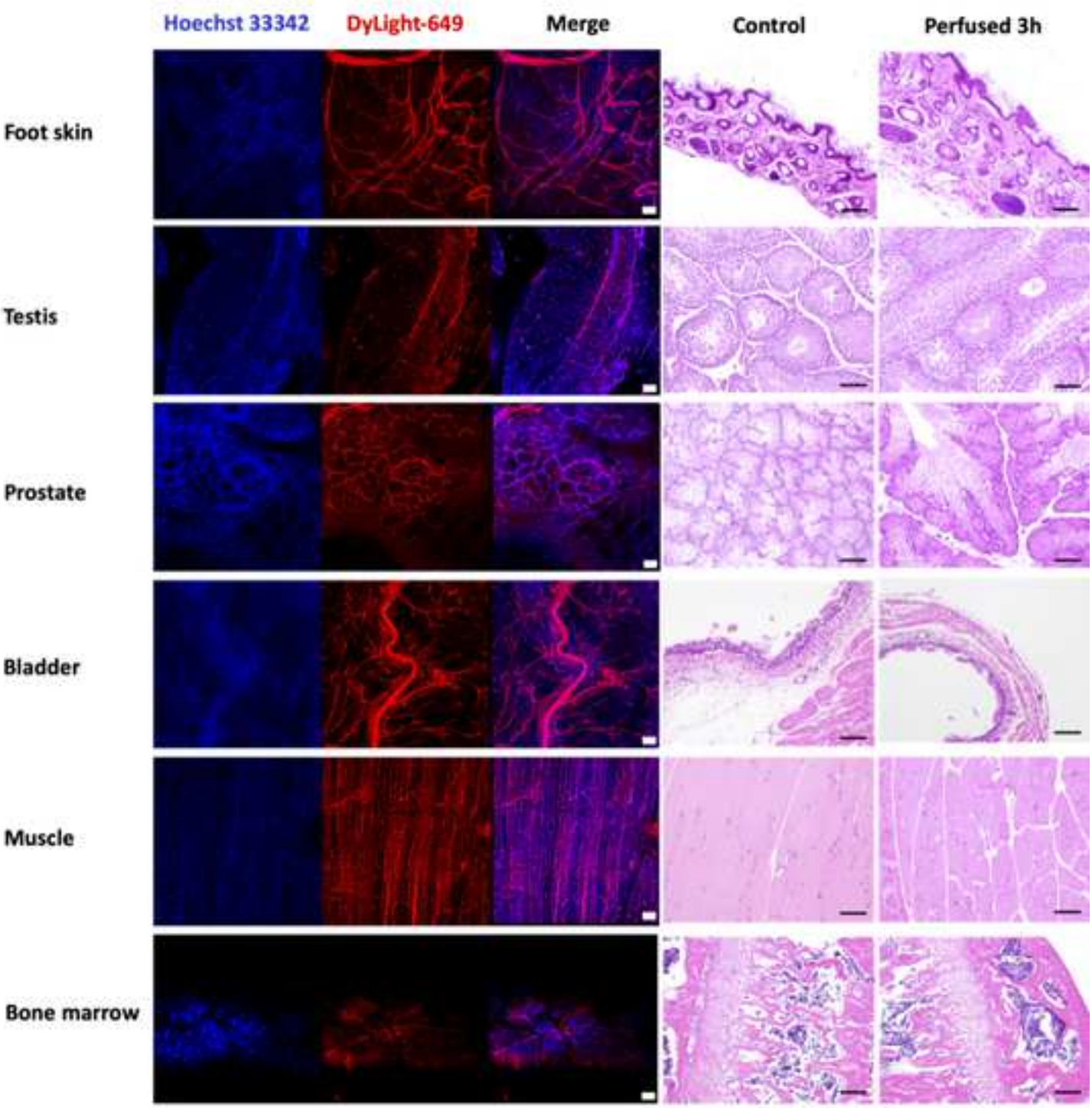

Table of Materials

Click here to access/download;Table of Materials;Table 1. Equipment and Materials-2.xlsx

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| **Equipment** | | | |
| 3.5x-90x stereo zoom microscope on boom stand with LED light | Amscope | SKU: SM-3BZ-80S | |
| Carbon dioxide, USP | Airgas healthcare | 19087-5283 | |
| Confocal microscope | NIKON | ECLIPSE Ti2 | |
| Disposable Sterile Cautery Pen with High Temp | FIAB | F7244 | |
| Moist chamber bubble trap (part 6 in Figure 1) | Harvard Apparatus | 733692 | Customized as the perfisate container; also enabled constant pressure perfusion |
| Moist chamber cover with quartz window (part 3 in Figure 1) | Harvard Apparatus | 733524 | keep the chamber's temperature |
| Moist chamber with metal tube heat exchanger | Harvard Apparatus | 732901 | Water-jacketed moist chamber with lid to maintain perfusate and mouse temperature |
| Olsen-Hegar needle holders with suture cutters | Fine Science Tools (FST) | 125014 | |
| Oxygen compressed, USP | Airgas healthcare | C2649150AE06 | |
| Roller pump (part 4 in Figure 1) | Harvard Apparatus | 730113 | deliver perfusate to cannula in the moist chamber |
| SCP plugsys servo control F/Perfusion (part 1 in Figure 1) | Harvard Apparatus | 732806 | control the purfusion speed |
| Silicone pad | Harvard Apparatus | | |
| Silicone tubing set (arrows in Figure 1) | Harvard Apparatus (TYGON) | 733456 | |
| Student standard pattern forceps | Fine Science Tools (FST) | 91100-12 | |
| Surgical Scissors | Fine Science Tools (FST) | 14001-14 | |
| Table for moist chamber | Harvard Apparatus | 734198 | |
| Thermocirculator (part 2 in Figure 1) | Harvard Apparatus | 724927 | circulating water bath for all water-jacketed components |
| Three-way stopcock (part 5 in Figure 1) | Cole-Palmer | 30600-02 | |
| Veterinary anesthesia machine | Highland | HME109 | |
| | | | |
| **Materials** | | | |
| 19-G BD PrecisionGlide needle | BD | 305186 | For immobilizing the Insyte Autoguard Winged needle and scratching the cortical bone |
| 4-0 silk sutures | Keebomed-Hopemedical | 427411 | |
| 6-0 silk sutures | Keebomed-Hopemedical | 427401 | |
| Filter (0.2 µm) | ThermoFisher | 42225-CA | Filter for 5% BSA-RINGER'S |
| Permanent marker | Staedtler | 342-9 | |
| Syringe (10 mL) | Fisher Scientific | 14-823-2E | |
| Syringe (60 mL) | BD | 309653 | Filter for 5% BSA-RINGER'S |
| | | | |

| Reagents | | | |
|---|---|---|---|
| 1% Evans blue ( w/v ) in phosphate-buffered saline (PBS, pH 7.5) | Sigma | 314-13-6 | |
| 10% buffered formalin | velleyvet | 36692 | |
| BALB/c mice ( 8-10 weeks old ) | Charles River | | |
| Baxter Viaflex lactate Ringer's solution | EMRN Medical Supplies Inc. | JB2324 | |
| Bovine serum albumin | Thermo Fisher | 11021-037 | |
| Cyanoacrylate glue | Krazy Glue | | |
| DyLight-649-lectin | Vector Laboratories,Inc. | ZB1214 | |
| Ethanol (70% (vol/vol)) | Pharmco | 111000190 | |
| Hoechst33342 | Life Technologies | H3570 | |
| Isoflurane | Piramal Enterprises Limited | 66794-017-25 | |
| Phosphate buffered saline | Gibco | 10010023 | |

Dear Editor,

Thank you for the opportunity to revise the manuscript. We addressed both the editorial and reviewers' comments, which are detailed below. We also attached a version with tracked changes in the revision. We hope that the revised manuscripts will be acceptable for publication in JoVE.
Sincerely

Fangfang Chen, PhD.

**Editorial Comments:**

• **Introduction:** Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.
We expanded the introduction accordingly.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) 2.1: Mention animal strain, age, sex.
2) 2.2: What kind of sutures?
3) 2.3: How is anesthesia induced? Mention drug dose (and oxygen flow rate in case of inhaled anesthetic).
4) 2.3: Describe how anesthesia is confirmed?
5) 4.1: describe the steps in detail.
6) 4.2: details are insufficient. Mention microscopy settings e.g., magnification, lens NA, excitation and emission filter settings etc.
We added details to each step.

• **Protocol Numbering:** Please add a one-line space after each protocol step.
Done.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
Done.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are

provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
Done.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
We expanded the Discussion to include these points.

• **Figures:** Add scale bars to fig 3.
Done.

• **References:**
1) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]
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1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
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Reviewer #1:

Manuscript Summary:
Ren et al describe a protocol for cannulation of the abdominal aorta and inferior vena cava in mice, followed by non-oxygenated normothermic perfusion. They state that this model may be a useful tool for studying mechanisms of drug delivery, migration/metastasis of circulating tumor cells into/from the tumor, and interactions of immune system with perfused organs and tissues.

Major Concerns:

However, in my opinion there are important limitations to this protocol wherefore I don't see how it can be used for the proposed applications. The most important limitation is that the perfusion is performed at normothermic temperatures without an oxygenator or oxygen carrier. This results in severe ischemia while the graft is at its full metabolic rate. The confounder of severe ischemia poses significant challenges when the above mention mechanisms were to be studied. Furthermore, the perfusate composition is of extreme importance during perfusion at (sub)normothermic perfusion temperatures as it aims to support physiologic metabolism. However, Ren et al used LR+BSA. The lack of metabolic support, in addition to the warm ischemia, creates a morbid tissue condition of which the usefulness in research is very questionable. Of note, metabolic support (e.g. oxygenation and nutrients) can omitted during hypothermic perfusion (as in some of the cited work) because the organ is not metabolically active, however, not during normothermic perfusion.

We thank the reviewer for these important comments. Indeed, we recognize that metabolic support is extremely important. It must be noted that the goal of the present work was to establish the perfusion circuit via surgical procedure rather than developing and optimizing perfusion media and conditions to support optimal organ/tissue function and oxygenation. Therefore, we omitted the term “normothermic” from the title in order to focus on the surgical procedure as the main focus of the manuscript. While our experiments have omitted this metabolic support, this method can indeed be performed with oxygenation or desired additions of vitamins etc. to the perfusion medium, and we have accordingly revised our duration of perfusion to be no more than 2 hours, and have added a discussion on the need to include oxygenation in the perfusate if desired. The perfusion media composition and oxygenation have been extensively highlighted in the previous literature and included in the new Discussion section.

Likewise, proper validation of this protocol is lacking and, in my opinion, the provided fluorescent histology images in Figure 3 are not sufficient as this merely shows the

microvasculature was perfused but yields no information about other important aspects such as tissue injury and metabolism. Also, it seems that this protocol has not been used in any peer reviewed publications.

Thank you for the suggestion, we added images of H&E staining of the perfused organs to Figure 3. H&E staining revealed no significant anatomical differences between organs harvested immediately after euthanasia and those harvested after up to 2 hours of perfusion according to our method. Together with the fluorescent histology images, this provides evidence of a successfully perfused microvasculature without damage to blood vessels or organ tissue. Again, since the focus of the publication is on the surgical technique, we did not provide specific recommendation on the perfusion media and refer the readers to the extensive bulk of literature in the Discussion.

Also, Ren et al do not acknowledge that there are several other publications that describe more advance methods to perfuse vascular composite allografts (including bone, skin, nerves, bone marrow, lymph nodes). I miss a clear explanation why the describe protocol would be preferred over these and why commonly used features like pressure control and oxygenation were omitted.

We mentioned several advantages over alternative techniques in the Introduction; however, to address your concerns, we have provided additional references to previous studies. In brief, the major advantage of our method is that it can perfuse several organs simultaneously and leaves the animal model relatively intact, allowing for more realistic observations than single-organ perfusion models. Furthermore, the above referenced-papers report perfusion of larger organs and tissues in isolated form, while perfusion of small mouse organs such as the bladder, prostate, bone marrow, and skin has not been feasible due to the very small size of the blood vessels, and is the main advance of our perfusion technique. The flow rate of perfusion buffer through the aorta is held constant at $\leq$ 0.6mL/min (described in protocol 3.3) by peristaltic pump; liquid flow rate is known to be proportional to its pressure according to laws of fluid mechanics (e.g., Hagen-Poiseuille equation). As stated above, we have accordingly added an explanation for omission of oxygenation to the “Discussion” section.

Furthermore, in general, the manuscript provides too little detail to easily understand the producers and would not be replicable as it is.
We significantly expanded the protocol; also per JoVE format, a professional video will accompany the protocol.

Finally, I have concerns about the animal welfare and ethics of this protocol. As the protocol suggests, the anesthesia (isoflurane) is stopped when the mouse it taken to the perfusion system after surgery. Mice typically start to regain consciousness within a couple minutes after isoflurane administration is stopped. However, after surgery all vital organs in the upper body are still intact, while the animals are euthanized after 10 minutes of perfusion.

Thank you. We have provided more details to our procedure, including instructions to confirm anesthesia through multiple methods and to maintain anesthesia until performance and confirmation of euthanasia, which we regrettably omitted from our initial submission (see protocol 3.4).

Reviewer #2:

Manuscript Summary:
This manuscript describes a protocol for in situ perfusion of the distal pelvic organs and hind limbs in the mouse. This technique is of interest and would be a useful addition to the literature. However, there are major issues that should be addressed in order to ensure that the protocol has maximal utility.

Major Concerns:

The main concern with the manuscript is lack of sufficient detail in order to ensure that the protocol can be successfully followed by other groups. Specifically:

1) Much greater detail, including photographs, schematic drawing and ideally videos of the different steps are required. This should include set up-of the perfusion circuit (including schematic drawing and more close-up photographs), close-up photographs of the animal connected to the perfusion circuit, precise details of the perfusion circuit used (including perfusate composition, volumes, etc), how the body temperature was monitored (rectal temp probe?), whether/how oxygenation can be achieved, etc.
2) A list of likely problems and solutions (ie, a trouble-shooting list)
3) If possible, videos should be included.
4) The authors state a success rate of 80%. What was their success rate (ie, in experienced hands). What were the causes of failure? What are the key learning curve events? These could be included in the context of a trouble-shooting table.

According to your advice, we added greater detail to our procedure and Discussion (including the success rate). In lieu of a troubleshooting list, we added comments to steps where we believe issues might arise. Videos will be professionally made and edited by the Journal of Visualized Experiments (JoVE). We believe that high quality videos should be used in lieu of schematics and photographs.

The discussion should ideally include a section on adaptation of the method for in situ perfusion of other abdominal organs. Can the technique be used for perfusion of all abdominal organs (liver, kidneys, intestine, etc). What would the required modifications be?

Thank you for the suggestion. In theory, a single organ can be perfused successfully if the correct inlet and outlet vessels for perfusion are identified. However, we do not think yet another method for perfusion of large mouse organs is needed. As explained in the Introduction and Discussion, the main advantage of our circuit is the ability to perfuse

small mouse organs for which the vasculature is not readily accessible via ex vivo perfusion.

Minor Concerns:
The title should be rephrased to refer to lower abdominal organs and hind-limbs.
The inclusion of the table of other perfusion studies is not particularly helpful (and somewhat misleading) as many refer to humans organs, rats, etc.

We changed the title to “Establishing *in situ* closed circuit perfusion of lower abdominal organs and hind limbs in mice”

Reviewer #3:

Manuscript Summary:
Description of an in situ lower body perfusion protocol that bypasses the kidneys and liver. The perfusion medium is Ringer's lactate with 5% BSA. Perfusion is performed via peristaltic pump. It is the 1st perfusion system for mouse bladder, sex organs, bone, muscle, inguinal lymph node and foot skin.

Major Concerns:
It is somehow unclear this statement in the Introduction: The perfusion medium is Ringer's lactate with 5% BSA. Perfusion is performed via peristaltic pump with blood flow... Is the perfusate only Ringer's lactate with 5% BSA or also red cells? Perfusion with blood is unlikely because of small blood volume of the mouse. What is the haematocrit of the perfusate? Also this is normothermic perfusion. Did they look at perfusate lactate or other means of organ function?

Thank you for the questions. We did indeed use Ringer's lactate with 5% BSA and no blood cells, and have therefore replaced “blood flow” with “perfusate flow” to avoid misunderstanding. While we have not measured perfusate lactate concentration, H&E staining revealed no significant differences between organs harvested immediately after euthanasia and those harvested after up to 2 hours of perfusion according to our method. While the scope of the paper is development of a surgical technique to establish the circuit rather than optimization of perfusion media, we discussed the issues of the current protocol re: oxygenation and perfusion medium in the light of the existing literature.

Minor Concerns:
Not sure the relevance of table 1 with other perfusions in literature.

Thank you. We removed Table 1 and instead reference some of the publications in the Discussion.