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Laser Doppler Perfusion Imaging in the Mouse Hindlimb

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TITLE:
Laser Doppler Perfusion Imaging in the Mouse Hindlimb

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SUMMARY:
Here, we present a protocol that demonstrates the technique and necessary controls for Laser Doppler perfusion imaging to measure blood flow in the mouse hindlimb.

ABSTRACT:
Blood flow recovery is a critical outcome measure after experimental hindlimb ischemia or ischemia-reperfusion. Laser Doppler perfusion imaging (LDPI) is a common, noninvasive, repeatable method for assessing blood flow recovery. The technique calculates overall blood flow in the sampled tissue from the Doppler shift in frequency caused when a laser hits moving red blood cells. Measurements are expressed in arbitrary perfusion units, so the contralateral non-intervened upon leg is usually used to help control measurements. Measurement depth is in the range of 0.3-1 mm; for hindlimb ischemia, this means that dermal perfusion is assessed. Dermal perfusion is dependent on several factors—most importantly skin temperature and anesthetic agent, which must be carefully controlled to result in reliable readings. Furthermore, hair and skin pigmentation can alter the ability of the laser to either reach or penetrate to the dermis. This article demonstrates the technique of LDPI in the mouse hindlimb.

INTRODUCTION:
Skin ulceration with inadequate wound healing is a leading cause of amputations in human patients[1]. Adequate wound healing requires higher levels of arterial perfusion than are needed to maintain intact skin, which is compromised in patients with peripheral arterial disease[2-4]. Several other rheumatologic conditions and diabetes can also lead to disturbed and inadequate skin microcirculation to heal wounds[5,6]. Many diabetic patients have concomitant peripheral arterial disease, placing them at especially high risk for amputation. Laser Doppler perfusion imaging (LDPI) is used in clinical situations to evaluate the skin microcirculation, as well as in research situations to evaluate blood flow and blood flow recovery after experimental hindlimb ischemia, ischemia-reperfusion, and microsurgical flaps[7].

The LDPI system projects a low power laser beam that is deflected by a scanning mirror to move over a region of interest. This differs from Laser Doppler flowmetry, which provides a perfusion measurement for the small area of tissue in direct contact with the flowmetry probe[8]. When the laser beam interacts with moving blood in the microvasculature, it undergoes a Doppler frequency shift, which is photodetected by the scanner and converted to arbitrary perfusion units. Because LDPI is a light-based technique, it is limited in terms of depth of penetration to 0.3-1 mm, meaning that for the most part dermal perfusion is assessed[7]. Dermal flow can be altered by skin temperature and the sympathetic nervous system, which may be affected by various anesthetic agents[9]. Measurements from the optical laser are also affected by ambient lighting conditions, skin pigmentation, and can be blocked by overlying fur or hair[7].

LDPI is the most commonly used research technique to monitor perfusion recovery after ischemia because it is noninvasive, does not require contrast administration, and has quick scan times allowing data collection on multiple animals. This makes it ideal to help determine whether treatments aimed at therapeutic arteriogenesis or angiogenesis are effective in small animal models. Blood flow recovery after hindlimb ischemia as measured by LDPI correlates well with collateral artery development when assessed by other means such as Microfil casting or micro-CT[10,11]. The goal of this protocol is to demonstrate the assessment of hindlimb perfusion using LDPI.

PROTOCOL:

Animal experiments were performed according to a protocol approved by the University of Washington Institutional Animal Care and Use Committee.

1. **Scanner preparation**

1.1 Adjust the scanner height so that the distance to the scanned subject is approximately 30 cm.

1.2 Turn on the imager and launch the associated software.

1.3 Open the **Measurement** program. If the software is correctly communicating with the scanner, the infrared laser turn on warning will appear.

1.4 Calibrate the machine with manufacturer provided standards (not shown in the video and will depend on the specific model of machine being used).

1.5 Adjust the scanner settings to be appropriate for the background material and lighting setup in the room.

1.5.1 Set the gain levels **DC FLUX** and **CONC**, per the manufacturer's instructions (not shown in the video).

1.5.2 Set the Background Threshold by pointing the laser beam at the black background material, and press **Auto BK Set**.

2. **Mouse pre-scanning preparation**

2.1 Set up the isoflurane induction chamber with appropriate scavenging of the waste gas.

NOTE: Placing the induction chamber on a warming pad will help prevent mouse temperature loss during anesthesia induction.

2.2 Turn on the homeothermic blanket, which is placed in the scanning area underneath a nonreflective surface (in this case a black neoprene fabric). Set the homeothermic blanket to maintain a body temperature of 37 °C.

2.3 Position the temperature probe for the homeothermic blanket and lubricant so they are ready for insertion.

2.4 Place the anesthesia mask and scavenging system in the scanning area.

2.5 Anesthetize the mouse with an isoflurane vaporizer. Set the oxygen rate to 1 L/min of flow and adjust the isoflurane to 4% for anesthesia induction. Turn on the flow to the anesthesia induction chamber, and the mouse breathing rate will slow. Adequate anesthesia is achieved when the mouse loses its righting reflex.

2.6 Transfer the mouse to an anesthetic mask/nose cone with attached waste gas scavenger and adjust the isoflurane to 1.5%.

NOTE: This anesthesia level is generally adequate to keep the mouse lying relatively still during scanning, but is not intended to provide surgical levels of anesthesia, so the depth of anesthesia is not checked. Changing the isoflurane level causes changes in heartbeat, respiration, and dermal perfusion, so a consistent percentage should be used throughout any time course experiment and for all experimental subjects. Alternative anesthetic techniques such as IP injection of ketamine xylazine can also be used, but the same anesthetic technique should be used throughout any time course study as different anesthetics affect dermal perfusion differently.

2.7 (Optional depending on scanning area) If the planned region of interest to be scanned is covered by fur, use a small electric trimmer or depilatory cream to remove the hair from the region of interest.

NOTE: The depilatory cream should be completely removed, and the mouse skin dried prior to scanning.

2.8 Place the mouse in the appropriate scanning position on the black nonreflective surface covering the homeothermic blanket, confirming that both hindlimbs remain on the heat source throughout equilibration and scanning (**Figure 1**).

NOTE: It is important to maintain both feet on the homeothermic blanket to prevent regional variation in temperature.

2.9 Insert the lubricated rectal temperature probe associated with the homeothermic blanket.

2.10 Equilibrate the mouse temperature to desired scanning temperature (37 °C); approximately 5-10 minutes.

2.11 Select **Scanner Setup**, which can be accessed from the top menu or from the scanner setup icon. Adjust the scan area by changing the X-Y coordinates to accommodate the region of interest. Scan speed will depend on the scan resolution. Higher resolution will result in longer scan times. For repeat scanning

focusing on global perfusion, as opposed to higher resolution focusing on anatomic perfusion, a scan speed of 4 ms/pixel is adequate.

NOTE: Higher resolution and single scan should be considered if the researcher is attempting to directly study the developing collateral circulation (best imaged in the ventral thigh and calf where it is closer to the skin). Repeated scan at lower resolution/speed (e.g., 4 ms/pixel) is adequate when assessing global perfusion to the end organ of the mouse footpad. The software shown in the video automatically loads the previously used template for scanning area, speed, and resolution when restarted, or it can be retrieved from a stored file if different regions of interest are being used for various experiments.

2.11 If performing repeat scans, select the **Repeat** and **Line Scan** tab. The number of scans can be changed (in this case 3 scans) as well as the repeat interval. The minimum time for the repeat interval would be the estimated scanning time, which is shown in the grayed-out area on the right of the box determined by scan area and scan resolution. Adding a few seconds allows the user to pause and potentially reposition the mouse if needed between scans.

3. **Scanning**

3.1 Select the **Image Scan** tab and select the **Mark** button. The laser will move to outline the scanning area. Adjust the mouse position so that the target to be scanned is within the marked area.

NOTE: For footpad or footpad and calf scanning, prone positioning with the hindlimbs extended provides a more consistent region of interest than supine positioning. The femoral artery and saphenous artery and collaterals are very close to the ventral surface of the thigh and calf, so supine positioning is preferred if using these regions of interest.

3.2 Start repeated measurement by selecting the repeat scanning icon and press the **Play Button** to initiate the scan.

3.3 Select the scanning distance in the pop-up window.

3.4 Monitor the mouse during scanning for mouse movement; if the mouse moves sufficiently that the hindpaws are no longer in the scanning region in the middle of a scan, restart the scan. Small variations in mouse hindpaw position can be accommodated for in the analysis software.

3.5 Monitor the mouse temperature during the scanning process as it may fluctuate even with the use of the homeothermic blanket. If there is too much variation in the mouse temperature, this may result in significant variation between scans. Generally, a temperature range of 36.8-37.2 °C will result in acceptable data.

3.6 Save the captured scan under the **Save as** window with a file name that includes mouse identifier and timepoint for easier data analysis. Enter mouse and timepoint details if desired in the subject details window.

3.7 Turn off the isoflurane and remove the rectal temperature probe.

3.8 Disinfect the rectal temperature probe with 70% ethanol so it is ready for use in the next mouse.

3.9 Allow the mouse to recover from anesthesia to the point where it displays a righting reflex by flipping from the supine position to the prone position prior to returning it to the cage.

NOTE: Recovery can be carried out either on a warming blanket for isoflurane since recovery is very quick or in a warmed recovery cage for ketamine/xylazine.

4. **Capturing LDPI data (Figure 3)**

4.1 Open the imaging review software program.

4.2 Go to the file menu, open, and locate the saved file.

4.3 Select the **ROI** icon from the toolbar.

4.4 Select the **Add Polygon** button.

4.5 Trace the region of interest (ROI) for the control hindlimb using the mouse. Polygon tracing does not have to be exact as the gray background will not be included in the calculated averages.

4.6 Repeat steps 4.3 and 4.4 for the surgical hindlimb.

4.7 Choose the **Statistics** icon to open the **Image ROIs Statistics Results (PU)** window.

4.8 Export the results for Polygon 1 (control hindlimb) and Polygon 2 (surgical hindlimb) to a data collection worksheet via copy/paste.

5. **Analysis**

5.1 Capture the data as Surgical/Control ratio for each scan.

5.2 Use the averaged surgical/control for all three scans for the data point for that particular mouse at that timepoint. Because of biologic variability in the response to hindlimb ischemia, in general 8-10 mice are required per timepoint to achieve reproducible results with ~10% standard error.

NOTE: Before allowing the mouse to recover from anesthesia, it is worthwhile to perform a quick analysis of the repeated scans to check if the data is too variable (e.g., more than 100-150 perfusion units different between scans 1-3—this corresponds to more than 10% of typical perfusion value for the control hindlimb). High variation between repeated scans suggests the mouse was not completely equilibrated during the scan (**Figure 2**), and a repeat scan can be performed without losing a datapoint, which would occur if the images are not analyzed until a later date. Changing the color palette to optimize the dynamic range of displayed flux values may be necessary to better display scan variation (**Figure 2**)

REPRESENTATIVE RESULTS:

Successful LDPI should result in consistent repeated measures scans, with no more than 100-150 perfusion unit variation (corresponding to about 10% of the usual mean perfusion for the mouse footpad) between the three scans (**Figure 2**). As demonstrated in **Figure 2**, repeat scans help determine that the mouse has been appropriately equilibrated so that the ischemic/control ratio best reflects the underlying blood flow as opposed to variation in dermal perfusion caused by temperature variation. Using single

scans for datapoints will increase the variability leading to the need for more experimental mice. When used for hindlimb ischemia, the surgical hindlimb should have decreased global perfusion when compared to the control hindlimb. Results are expressed as a ratio of surgical hindlimb perfusion/control hindlimb perfusion. As mice initially vasodilate and develop their intrinsic collateral network over time, blood flow recovery by LDPI should be seen over a postoperative time course (**Figure 4**). The degree of recovery is dependent on the mouse strain and severity of the hindlimb ischemia model.

FIGURE AND TABLE LEGENDS:

Figure 1. Mouse positioning for laser Doppler perfusion imaging of the ventral footpads. Anesthetized mouse using isoflurane nose cone (A) is placed in the prone position with hindlimbs extended to allow scanning of the ventral footpads. Rectal temperature probe (P) for homeothermic blanket is in place to maintain consistent body temperature during the scan. The homeothermic blanket pad is underneath the nonreflective neoprene material used to provide background for the scan. Laser indicating the middle of the scanning region is visible next to the mouse's tail.

Figure 2. (**A**) Laser Doppler repeated scans with significant variation caused by mouse core temperature variation during the repeated scans, which is visible based on the perfusion units translated to color on the repeated flux scans. (**B**) Changing the dynamic range on the color palette (shown on the left of the scan window) from 0-1000 in A to 0-1500 (red arrow) in (**B**) makes the variation more obvious. (**C**) Statistics showing mean perfusion values for the region of interest (circled in red) for the control hindlimb (Polygon 1 in black on the rfx image in **A** and **B**) ranges between 655 for the 1st scan to 791 on the 3rd scan and mean perfusion for the region of interest for the ischemic hindlimb (Polygon 2 in red on the rfx image in A and B) showed less variation (361 to 400), leading to significant differences in the ischemic/control ratio between the repeated scans (0.60, 0.53, and 0.46). (**D**) Window to change the dynamic range of the color palette in the measurement software (left panel) and image review software (right panel). Red arrows show where to increase or decrease the upper range.

Figure 3. Data capture for laser Doppler perfusion imaging with repeated scans. (**A**) Top toolbar with 1. Add ROI icon. 2. Add Polygon icon. 3. Subject details icon (accesses window pop-up in B). 4. Statistics icon (accesses window pop-up in C). (**B**) Subject details window. (**C**) Statistics window showing the mean perfusion values (circled in red) for each ROI. (**D**) Repeated scan with polygon ROI traced around control hindpaw (black) and ischemic hindpaw (red).

Figure 4. Time course experiment with LDPI data. P27 knockout mice (3-5 month old female CDKN1b$^{-/-}$ mice on a C57Bl/6 background) after femoral artery ligation with (n=6) and without (n=10) oral doxycycline treatment compared to age-matched female wildtype C57Bl/6 mice with (n=11) and without (n=9) oral doxycycline treatment (unpublished data from the author). Error bars represent standard error of the mean (SEM).

DISCUSSION:

Consistent technique is critical for obtaining reliable results with LDPI. The same anesthetic, temperature settings, mouse position, and region of interest should be used throughout the entire time course. Different anesthetic agents will result in higher or lower perfusion values[9]. Isoflurane anesthesia is convenient because of its rapid onset and emergence as well as overall safety. A consistent percentage of isoflurane should be used as depth of anesthesia with this vasodilatory agent may alter skin perfusion. If the region of interest includes fur, then the same method of hair removal should be used each time, as chemically depilated mice will have higher perfusion values than mice whose fur was removed with electric clippers[7]. Mouse temperature has a large effect on perfusion imaging, with mice at 36 °C having

significantly lower perfusion values than mice at 38 °C[7,12]. The ischemic hindlimb may also react differently to regional temperature variation than the control hindlimb (**Figure 2**). In this protocol, a homeothermic blanket is used to maintain mouse temperature during scanning, which provides more consistent temperature control during the scanning process than pre-equilibrating the mouse on a 37 °C warming plate for five minutes and then scanning on a non-warmed surface as shown by Niiyama et al.[13]

If only the footpads are chosen as the region of interest, then prone positioning is preferred because of reproducibility in the scanned region of interest. The advantage of this approach is that it studies the area furthest from the heart, and the most clinically relevant area corresponding to where ischemic foot ulcers are common. The footpads are hairless, so clipping or depilation is not necessary, simplifying preparation and time for measurement. Also, non-white mice may have patches of pigmentation in the skin of the calf or thigh, which can interfere with LDPI measurement. If the chosen region of interest includes the calf and thigh, then supine positioning is preferred because the femoral and saphenous artery run along the ventral surface of the hindlimb and can be imaged using LDPI[7]. From the supine position, consistent imaging of the foot is difficult to capture, as the side and top surfaces may be variably imaged.

Collateralization and blood flow recovery after hindlimb ischemia is dependent on a number of different factors including hindlimb ischemia model, mouse strain, gender, and age. Certain strains of mice such as C57Bl/6 have robust baseline collaterals, with a less dramatic drop in perfusion after induction of acute hindlimb ischemia, whereas others such as BALB/c have poor collaterals[14,15]. Female mice have worse recovery than male mice. Older mice also have worse blood flow recovery than younger mice[16]. Therefore, mice need to be strain, age, and gender matched for reliable conclusions to be drawn regarding blood flow recovery using LDPI data. Even with rigorous matching and using inbred strains of mice, there is a certain amount of biologic variability to the mouse response to hindlimb ischemia, so adequate mouse numbers (usually 8-10 mice per timepoint) are required for valid data. Furthermore, normalization of LDPI does not necessarily mean restoration of normal levels of arterial flow as the measurement is done in anesthetized mice who do not have any skeletal muscle demand. Finally, because of the limitations in depth of penetration, detailed anatomic studies of collateral pathways that may run through the deeper musculature of the thigh and calf are not possible with LDPI.

Several other methods have been used to assess blood flow recovery including perfusion-based imaging of the skin such as laser speckle imaging[17-19] or deeper structures such as contrast-enhanced ultrasound of skeletal muscle[20], MRI[21], and (13)N-ammonia PET[22]. Also used are anatomic-based imaging of collateral vessels such as micro-CT[10], OCT[23], and contrast-enhanced ultrasound with intravital microscopy[24]. Because of fast scan time, relative ease of data capture and analysis, and avoidance of the need for intravenous contrast, LDPI is the predominant method used by most groups in the literature. Weaknesses include that the technique measures blood flow velocities and provides data in arbitrary perfusion units rather than measuring absolute tissue perfusion, the scanning depth is relatively shallow, and it provides relatively poor anatomic detail.

LDPI is most commonly used to assess recovery after various hindlimb ischemia models[7]. It has also been used in ischemia-reperfusion research both in the hindlimb[25] as well as in deeper splanchnic organs or the spinal cord[26-28]. Assessment of deep structures however requires surgical exposure of the structure to be scanned, making repeated measurements more difficult because of scarring. A further application is assessment of flap reperfusion after microsurgery[29].

In conclusion, LDPI is an effective, easily performed, and repeatable method for measuring hindlimb dermal perfusion as a reflection of overall arterial perfusion. Consistent technique is required when using LDPI to obtain reliable data.

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DISCLOSURES:

Dr Tang has no conflicts of interest to disclose.

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Figure 1

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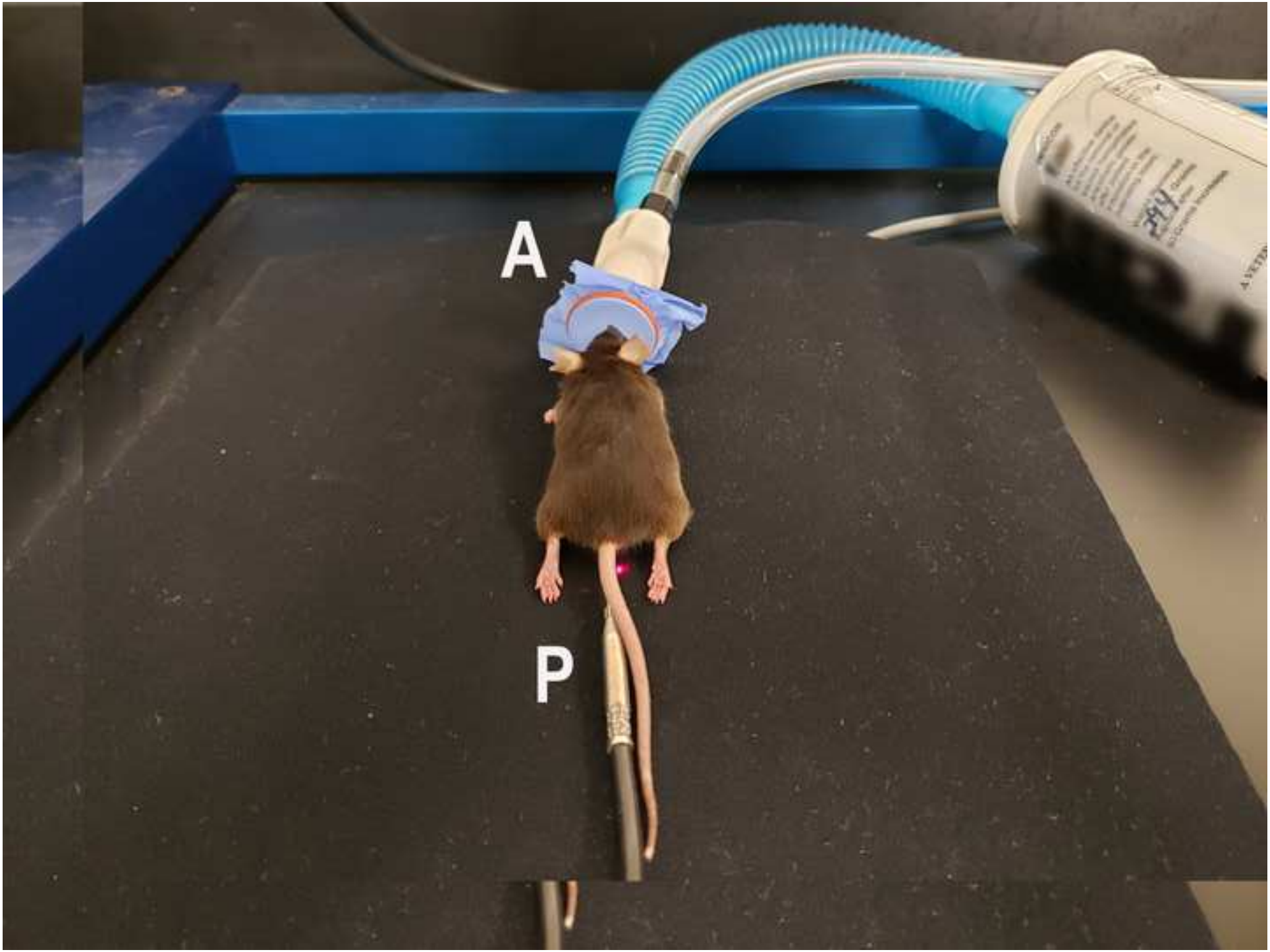

Figure 2

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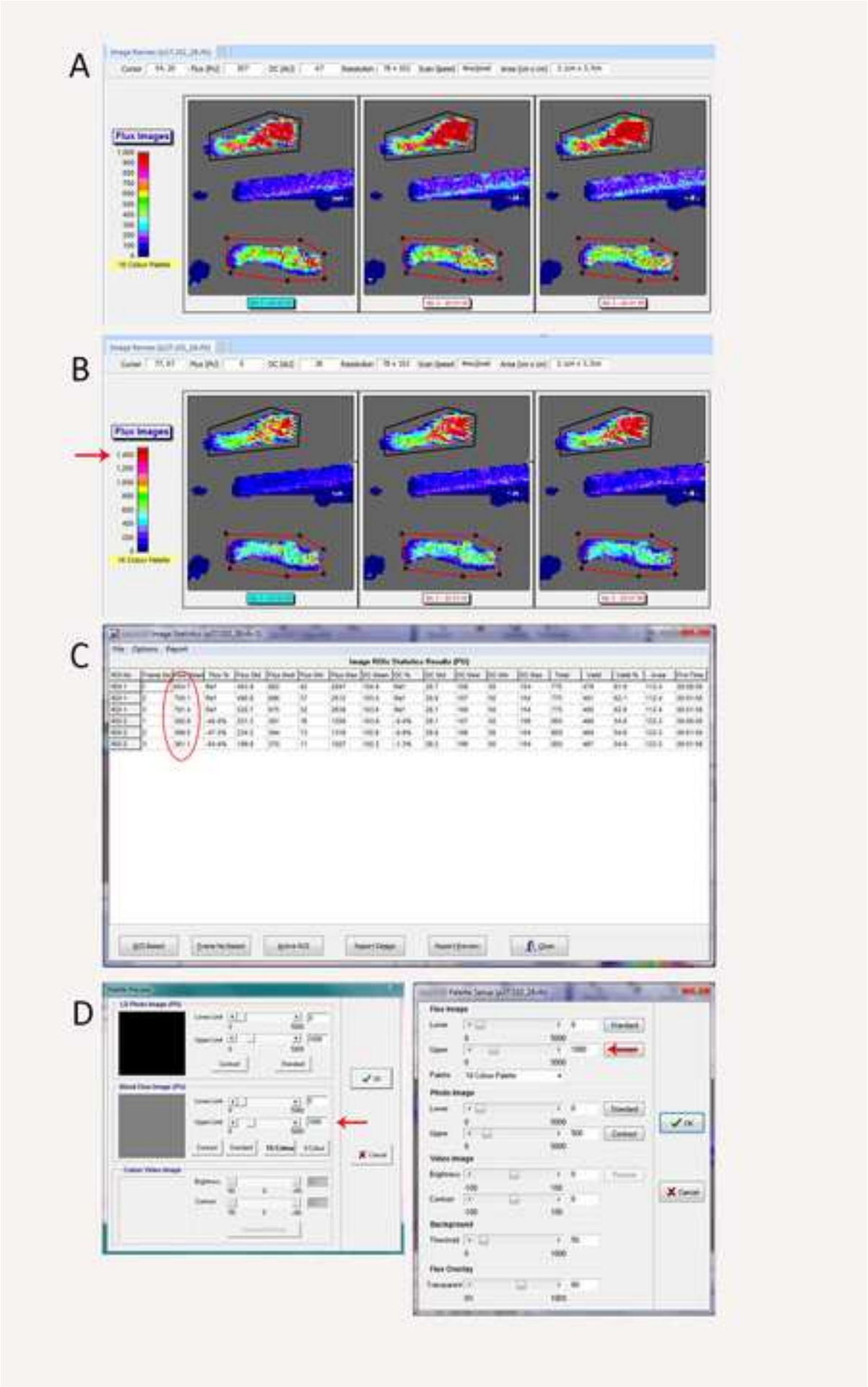

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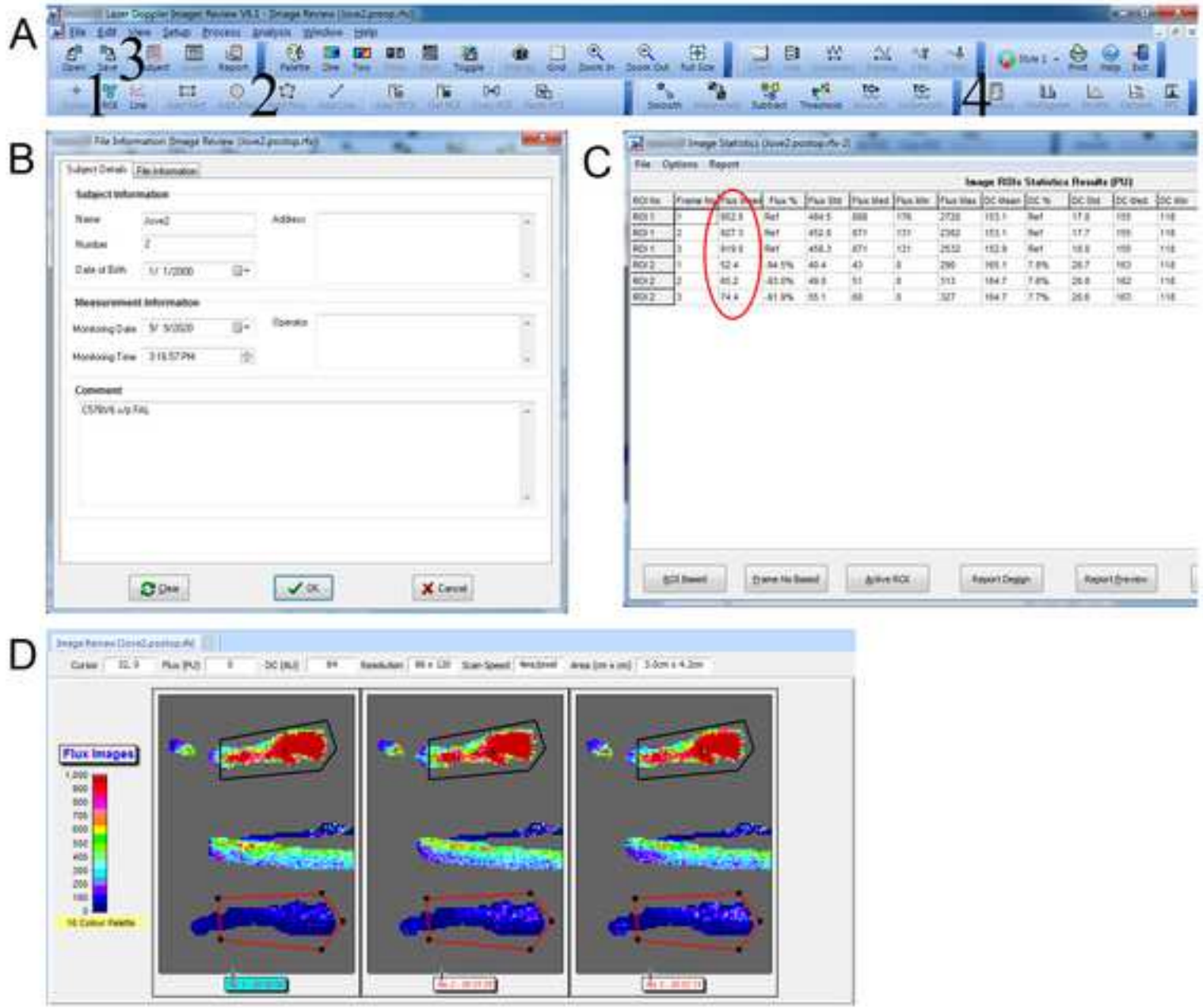
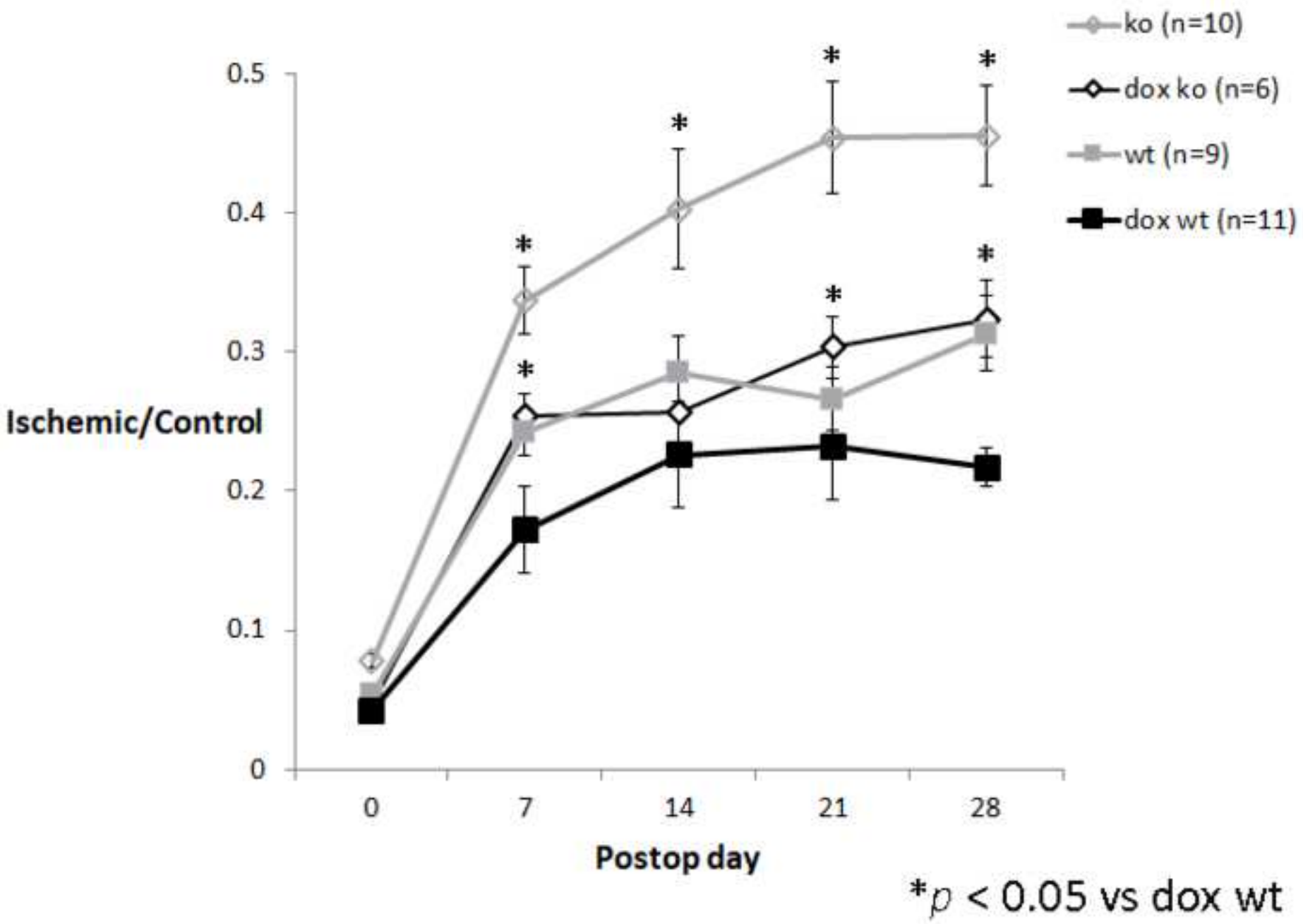


Figure 4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Black nonreflective material			Fabric store, black neoprene recommended by company
F/air cannister	A.M. Bickford Inc	80120	
Homeothermic blanket with rigid metal probe	Harvard Apparatus		Also comes with flexible probe, but this is less durable
Isoflurane Anesthesia machine	Drager		Multiple manufacturers
Isoflurane induction chamber	VetEquip	941444	2 L chamber
Moor laser Doppler perfusion imager	Moor Instruments	MoorLDI2-IR	Higher resolution imager (MoorLDI2-HIR)
Mouse Anesthesia nose cone			Multiple manufacturers
Nair	Nair		
Oxygen tank			Multiple manufacturers
Surgilube	Multiple distributors		

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2. Please provide an email address for each author.

Author information has been added to the manuscript

3. Please use complete sentence for the summary section and ensure that summary is between 10-50 words: "Presented here is a protocol …"

The summary section has been changed to read as follows:

Presented here is a protocol that demonstrates the technique and necessary controls for Laser Doppler Perfusion Imaging to measure blood flow in the mouse hindlimb.

4. 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 and Reagents. For example: moorLDI2-IR laser Doppler Imager (Moor Instruments, Wilmington, Delaware), Nair, Excel, etc.

All commercial language has been removed from the manuscript and replaced with generic terms.

5. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. Please use generic term for the dopler imager used in the manuscript.

The specific laser Doppler perfusion imager brand name has been removed from the manuscript and the video narration.

6. Please reformat the protocol to show the use of the equipment presented to address a specific research question using a clear dataset.

We have added a slide showing femoral artery ligation to the introduction. We have also added to the representative results section with an example mouse as well as Figure 4 to the video to show the use of LDPI experimentally. See also the response to editor's comment #15.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

The Protocol has been renumbered as requested.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. 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."

The Protocol section has been modified as requested.

9. Please include more details in the protocol section, associated with your experiment. Please include all actions to show how each step is performed.

The focus of the video is on the specific imaging technique of laser Doppler perfusion imaging. We have focused the protocol on this and the appropriate controls and setup to achieve reliable results from LDPI rather than on the induction of murine hindlimb ischemia, which is well covered in the prior JOVE video, Reference 8 by Niiyama.

10. Please include single line space between each step, sub-step, and notes.

A blank line has been added as requested.

11. Please use complete sentences throughout.

We have revised the manuscript such that complete sentences are now used throughout.

12. Please include details of the mice used for the experiment presented- age, sex, strain, etc.

The mouse details are now included in the legend for Figure 4.

13. Please include how do you check for the depth of anesthesia.

We have added a note to protocol regarding anesthesia level during scanning.

NOTE: This anesthesia level is generally adequate to keep the mouse lying relatively still during scanning, but is not intended to provide surgical levels of anesthesia, so the depth of anesthesia is not checked. Changing the isoflurane level causes changes in heartbeat, respiration, and dermal perfusion, so a consistent percentage should be used throughout any time course experiment and for all experimental subjects.

14. Please include how do you start the repeat measurement- what is being measured, settings, etc. What is the scanning distance in your experiment? Rationale for using these settings, etc.

The scanning distance for this particular model of LDPI scanner is fixed at 30cm, as mentioned in the video and within the manuscript protocol. The repeat measurement is triggered by using

the repeat measurement function of the software and it automatically performs all three scans under the same settings as chosen by the user for repeated measurement.

We have added to the Representative Results section to explain the rationale for using repeat measurement:

As demonstrated in Figure 2, repeat scans help determine that the mouse has been appropriately equilibrated so that the ischemic/control ratio best reflects the underlying blood flow as opposed to variation in dermal perfusion caused by temperature variation. Using single scans for datapoints will increase the variability leading to the need for more experimental mice.

15. Please describe and expand the result section with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

We have added two figures to the representative results section. The first shows the flux images over time with derived ratio with narration derived from the manuscript.

"Results are expressed as a ratio of surgical hindlimb perfusion/control hindlimb perfusion. As mice initially vasodilate and develop their intrinsic collateral network over time, blood flow recovery by LDPI should be seen over a postoperative time course. The degree of recovery is dependent on the mouse strain and severity of the hindlimb ischemia model.

We have also included Figure 4 from the manuscript in the video as a representative results section to increase the homogeneity between the written manuscript and the video and to demonstrate the efficacy of the LDPI technique. We have added the following to the video narration.

"This figure shows a completed time course experiment over 28 days where LDPI was used to measure footpad blood flow recovery over time after femoral artery ligation in p27 knockout and widltype C57Bl/6 mice. Both p27 knockout and wildtype mice had impaired blood flow recovery when treated with oral doxycycline, a generalized matrix metalloproteinase inhibitor, suggesting that MMP activity facilitates arteriogenesis."

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

None of the figures are from prior publication. The representative results are from unpublished data from the author's laboratory.

17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have specifically covered all of these points within the Discussion.

Changes to be made by the Author(s) regarding the video:
1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

We have increased the homogeneity between the video and the written manuscript as requested.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word-for-word reading of the written protocol. Please use imperative tense in the narration as well.

We have changed the video narration and written manuscript so they are better aligned, and imperative tense is used throughout the narration.

3. Please ensure that chapter headings are the same in the video and the text.

We have changed the chapter headings so that they correspond in the video and the text.

4. For the introduction and conclusion section, please either include interviews or include separate slides. Please do not show the title card.

We have created video and slides for the Introduction section and a more wideshot video of the laser Doppler scanning process for the Conclusion section to replace the title cards during the narration of these sections.

5. Please hide the commercial term Moor DPI, Moor Instruments and Harvard instrument from the scanner.

We have reshot the video to hide the commercial terms as requested.

6. 3:06- 3:18: At around this time the hands are covering the action. Please show the action being performed.

We have reshot the video to better show this portion.

7. 3:26: Please zoom the temperature part and do not show the commercial terms in the instruments.

The video has been altered to remove the commercial terms in the instruments.

8. 3:40: Please use the generic term for MoorLDI in the narration and do not show this in the video. We cannot have commercial terms.

This has been removed from the video as requested.

9. Please include a representative result section which should have figures/tables to show the efficacy of the technique presented.

See the response to editor's comment #15.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
this manuscript describe the method for measuring hindlimb perfusion after hindlimb ischemia

Major Concerns:
the protocol need to be applicable also if the readers have another LDPI or software. The actual description of the use of the software is only relevant if scientist have the same Machine and software. The choices / options chosen need to be clarified better (e.g. not only by saying press yes to all options but the options need to clarified and made useful for general application)

Given the machine calibration is completely dependent on the specific machine and instructions provided by the manufacturer, we have removed this from the manuscript (and it was never included in the video).

We have also removed the troubleshooting step regarding the order of scanner turn on and when the software program is opened as this is also specific to this particular machine.

Both of the major manufacturers for LDPI have specific software that have similar features in terms of capturing a region of interest and displaying the averaged perfusion value for this region of interest. We have demonstrated in the video these specific steps with the software for the machine that we have in the laboratory as per JOVE instructions for authors.

Minor Concerns:
none

Reviewer #2:
Manuscript Summary:
This protocol describes the operation of the laser Doppler (Moor) for measurement of blood perfusion in mice. It goes through preparation steps, scanning steps, trouble shooting, and evaluation of the raw data. Generally it is well written but seems overly focused on imaging of the foot pad only. As a general protocol, it would be helpful to scan other parts of the body as well.

Major Concerns:

1. This protocol seems specific for scanning of the foot pad. However, it would be more informative if other example regions could be provided to illustrate the generality of this tool. The areas that are shown as examples should be specified in the background section.

We have added scanning of the ventral hindlimb in the supine position to the video to address this concern. The introduction video now also shows scanning of a human hand.

2. The authors suggest that the variability in measurements may be due to non-equilibrium. Can the authors comment on why does that happen, and how to prevent it from happening? Is it enough simply to allow the animals to remain anesthetized longer before scanning to reach equilibrium?

The primary sources of measurement variability occur because of local temperature variation and anesthetic depth. We have added the importance of temperature control to the video narration to stress this point. We have also noted that leaving the mice under isoflurane anesthesia too long causes measurements that are inconsistent with the rest of the time course. In general, if an acceptable scan is not achieved after two attempts, waking up the animal and allowing it to recover before reattempting the scan provides more consistent data. The duration of anesthesia with IP ketamine/xylazine is not fixed and redosing with ketamine/xylazine is not as safe as isoflurane and leads to a less consistent level of anesthesia, so this method may create problems if significant time is required for temperature equilibration.

3. Can the authors describe more detail about the differential effects of different anesthetic agents? Is isoflurane better than ketamine, for example?

This subject is well-covered by Reference 8, so we have not provided further details. We discussed why we prefer isoflurane in the first paragraph of the Discussion of the manuscript. Please also see the answer for comment #2.

4. In the Discussion, the authors should compare their approach to another JOVE protocol related to laser Doppler of tissues (Niiyama et al. J Vis Exp 2009 Jan 21;(23):1035. doi: 10.3791/1035.)

We added to the discussion the major difference in technique between the Niiyama JOVE protocol vs our protocol in terms of using a homeothermic blanket for temperature control as opposed to equilibrating the mouse on a warming plate as follows:

In this protocol, a homeothermic blanket is used to maintain mouse temperature during scanning, so that temperature control is maintained during the scanning process as opposed to pre-equilibrating the mouse on a 37 degree warming plate for five minutes and then scanning on a non-warmed surface as shown by Niiyama et al.[13]

Minor Concerns:
5. Pre-scan steps involving removing hair from the animals using Nair should specify allowing the skin to dry in advance of scanning.

We have added a Note to the protocol as follows:

NOTE: The depilatory cream should be completely removed, and the mouse skin dried prior to scanning.

6. Warming pad temperature should be specified.

The homeothermic warming pad temperature is specified in the manuscript to 37 C. The temperature for the warming pad under the induction chamber is less critical as the plastic is relatively thick and will not be overly hot even at the maximum temperature of the homeothermic warming blanket. We usually use 42 C for a circulating fluid warming blanket or 37 C for a spare homeothermic warming pad. However, as the rectal probe is exposed to room air, this is the equivalent of maximum heat generation.

7. How is the core temperature maintained during scanning? Should animals remain on warming pad during scanning?

As stated in the protocol, a homeothermic blanket is used to maintain core temperature during scanning so the animal remains on a warming pad during the scanning process. A rectal probe is used to measure core temperature and provide feedback to the homeothermic blanket. Some researchers using this technique

8. What kind of scanning position (ie supine)?

Scanning position is covered in the Discussion. We have added a short discussion of scanning position to the video narration.

9. Should the region of interest be in contact with the warming blanket during the heating phase?

This is mentioned in the protocol, but was not mentioned in the video narration. We have modified the video narration to make it more homogenous with the protocol and to include this important point.

10. Please indicate approximate scan times.
This has been added to the video narration.

Reviewer #3:
1. Does this manuscript describe a protocol that will be of interest to other scientists? Please note that novelty is NOT a requirement for publication, but the efficacy of the protocol must be demonstrated. Yes

This is a very useful and precise protocol which certainly provides excellent guidance to the use of LDPI in the experimental setting. It highlights the pitfalls of LDPI and details requirements to minimise those.

Here some minor points which may improve the protocol:
I.d please consider specifying which questions

This has been removed from the protocol.

I.g please clarify if you mean 'repeat' of the calibration process

This has been removed from the protocol

II.2. please add important purpose of blanket

We have added this to the video narration and the protocol as follows

"NOTE: It is important to maintain both feet on the homeothermic blanket to prevent regional variation in temperature."

"3.1 Monitor the mouse temperature during the scanning process, as it may fluctuate even with the use of the homeothermic blanket. If there is too much variation in the mouse temperature, this may result in significant variation between scans. Generally a temperature range of 36.8-37.2 will result in acceptable data."

II.8. please add an estimate of time scale for sufficient equilibration of the animal

We have added a statement to the manuscript and the video about the time scale for equilibration. However, this will vary depending on whether hair removal was required or not, as the mouse generally loses temperature during the hair removal process.

Equilibrate the mouse temperature to desired scanning temperature (37 C); approximately 5-10 minutes.

III.5. you might consider 'experimental' details instead of 'postoperative' details - this would make more generalizable

We have changed this to "timepoint" rather than postoperative details. As LDPI data is usually captured for a timecourse, including the timepoint within the name of each data file simplifies later transfer to a data collection sheet.

III.8. please rephrase / explain 'righting' reflex (it's well demonstrated in the video)

We have altered the narration and the written protocol to better explain this:

"3.1 Allow the mouse to recover from anesthesia to the point where it displays a righting reflex by flipping from the supine position to the prone position prior to returning it to the cage.

> NOTE: Recovery can be carried out either on a warming blanket for isoflurane since recovery is very quick or in a warmed recovery cage for ketamine/xylazine."

Representative Results: please briefly explain how you arrived at / provide a reference for the threshold of 100 perfusion unit variations (this could also go into the discussion)

The video provides a very clear visual of the provided information.

We chose this value because the standard mouse control footpad has an average perfusion value of ~1000-1500 perfusion units. 100-150 perfusion units corresponds to more than 10% variation from the standard average perfusion value. This has been added to the Note for this portion of the protocol as follows.

"NOTE: Performing a quick analysis of repeated scans (see Section IV) to check if the data is too variable (eg more than 100-150 perfusion units different between scans 1-3—this corresponds to more than 10% of typical perfusion value for the control hindlimb)."

Altogether a very valuable protocol which will help any researcher, or even clinician, employing LPDI with useful insight.

2. Is there sufficient introduction for the protocol? Is there an unbiased discussion of the protocol? Yes

The introduction gives a general overview on LDPI, a specific use of Laser Doppler Flowmetry, applied to clinical practice. The discussion is very valuable providing a balanced and informative appraisal of LDPI. LDPI uses the principles of Laser Doppler flowmetry, a technique utilised experimentally / clinically - this might be added to the introduction. The protocol is also valuable for general use of Laser Doppler flowmetry and this should be reflected in the keywords.

Laser Doppler flowmetry has been added to the keywords.

We have added the following sentence to the Introduction to mention the differences between Laser Doppler flowmetry and Laser Doppler perfusion imaging/scanning:

"This differs from Laser Doppler flowmetry, which relies on a probe in contact with a specific point of tissue and provides a measurement for that specific point of interest.[8]"

3. Does this manuscript need additional copy-editing? Please do not copy-edit the manuscript as we provide in-house copy-editing services. No

The language is clear. I would suggest to take some of the information provided in the Figure legends into the main body of the protocol, eg the rationale for the homeothermic blanket.

The importance of the homeothermic blanket has been moved to the main body of the protocol as requested.