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Multiphoton Intravital Imaging for Monitoring Leukocyte Recruitment During Arteriogenesis in a Murine Hindlimb Modell

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Corresponding Author:	Hellen Ishikawa-Ankerhold Ludwig-Maximilians-Universität München: Ludwig-Maximilians-Universität München Munich, Bavaria GERMANY
Corresponding Author's Institution:	Ludwig-Maximilians-Universität München: Ludwig-Maximilians-Universität München
Corresponding Author E-Mail:	hellen.ishikawa-ankerhold@med.uni-muenchen.de
Order of Authors:	Manuel Lasch Mykhailo Vladymyrov Dominic van den Heuvel Philipp Götz Elisabeth Deindl Hellen Ishikawa-Ankerhold
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TITLE:

Multiphoton Intravital Imaging for Monitoring Leukocyte Recruitment During Arteriogenesis in a Murine Hindlimb Model

AUTHORS AND AFFILIATIONS:

Manuel Lasch^{1,2,3}, Mykhailo Vladymyrov⁴, Dominic van den Heuvel^{1,5}, Philipp Götz^{1,3}, Elisabeth Deindl^{1,3}, Hellen Ishikawa-Ankerhold^{1,5}

¹Walter-Brendel-Centre of Experimental Medicine, University Hospital, Ludwig-Maximilians–Universität München, Marchioninstr. 15, 81377 Munich, Germany

²Department of Otorhinolaryngology, Head & Neck Surgery, University Hospital, Ludwig-Maximilians–Universität München, Marchioninstr. 15, 81377 Munich, Germany

³Biomedical Center, Institute of Cardiovascular Physiology and Pathophysiology, Faculty of Medicine, Ludwig-Maximilians–Universität München, Großhaderner Strasse 9, 82152 Planegg-Martinsried, Germany

⁴TKI, University of Bern, Freiestrasse 1, 3012 Bern, Switzerland

⁵Department of Internal Medicine I and Cardiology, University Hospital, Ludwig-Maximilians–Universität München, Marchioninstr. 15, 81377 Munich, Germany

Email addresses of co-authors:

Manuel Lasch	(Manuel_Lasch@gmx.de)
Mykhailo Vladymyrov	(Mykhailo.Vladymyrov@tki.unib.ch)
Dominic van den Heuvel	(Dominic.Van@med.uni-muenchen.de)
Philipp Götz	(P.Goetz@med.uni-muenchen.de)
Elisabeth Deindl	(Elisabeth.Deindl@med.uni-muenchen.de)

Corresponding author:

Hellen Ishikawa-Ankerhold (hellen.ishikawa-ankerhold@med.uni-muenchen.de)

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Multiphoton intravital imaging, arteriogenesis, femoral arterial ligation, leukocyte live trafficking, leukocyte extravasation, drift correction tools, ViVoFollow drift correction software, tissue motion

SUMMARY:

The recruitment of leukocytes and platelets constitutes an essential component necessary for the effective growth of collateral arteries during arteriogenesis. Multiphoton microscopy is an efficient tool for tracking cell dynamics with high spatio-temporal resolution *in vivo* and less photo-toxicity to study leukocyte recruitment and extravasation during arteriogenesis.

ABSTRACT:

Arteriogenesis strongly depends on leukocyte and platelet recruitment to the perivascular space of growing collateral vessels. The standard approach for analyzing collateral arteries and leukocytes in arteriogenesis is *ex vivo* (immuno-) histological methodology. However, this technique does not allow the measurement of dynamic processes such as blood flow, shear stress, cell-cell interactions, and particle velocity. This paper presents a protocol to monitor *in vivo* processes in growing collateral arteries during arteriogenesis utilizing

intravital imaging. The method described here is a reliable tool for dynamics measurement and offers a high-contrast analysis with minimal photo-cytotoxicity, provided by multiphoton excitation microscopy. Prior to analyzing growing collateral arteries, arteriogenesis was induced in the adductor muscle of mice by unilateral ligation of the femoral artery.

After the ligation, the preexisting collateral arteries started to grow due to increased shear stress. Twenty-four hours after surgery, the skin and subcutaneous fat above the collateral arteries were removed, constructing a pocket for further analyses. To visualize blood flow and immune cells during *in vivo* imaging, CD41-fluorescein isothiocyanate (FITC) (platelets) and CD45-phycoerythrin (PE) (leukocytes) antibodies were injected intravenously (i.v.) via a catheter in the tail vein of a mouse. This article introduces intravital multiphoton imaging as an alternative or *in vivo* complementation to the commonly used static *ex vivo* (immuno-) histological analyses to study processes relevant for arteriogenesis. In summary, this paper describes a novel and dynamic *in vivo* method to investigate immune cell trafficking, blood flow, and shear stress in a hindlimb model of arteriogenesis, which enhances evaluation possibilities notably.

INTRODUCTION:

Despite intensive research interest during recent years, cardiovascular diseases, e.g., ischemic heart disease and stroke, are still the leading global cause of death¹. Current treatments for these diseases are highly invasive therapies such as percutaneous transluminal angioplasty, percutaneous transluminal coronary angioplasty, or bypass surgery². Therefore, the development of alternative, non-invasive therapeutic options is urgently needed. The body can create natural bypasses around a stenosed or occluded vessel to redirect the interrupted blood flow to the distal part of the stenosis. This process is called arteriogenesis². Many recent studies have shown that increased fluid shear impacts leukocyte recruitment, which plays an important role during arteriogenesis³. The main current options to analyze the recruitment of leukocytes during arteriogenesis are *ex vivo* (immuno-) histological analyses or fluorescence-activated cell sorting (FACS) methodology⁴. To enable the assessment of leukocyte dynamics during arteriogenesis, this paper presents an intravital imaging protocol with multiphoton microscopy.

Leukocytes are the major blood cells recruited during the process of arteriogenesis³. This protocol uses multiphoton imaging to show the crawling of adherent leukocytes labeled with injected anti-CD45-PE antibodies in collateral arteries, 24 h after the induction of arteriogenesis by femoral artery ligation (FAL) employing a murine hindlimb ischemia model^{5,6}. Alternatively, immune cells can be labeled *ex vivo* and carefully injected into mice, as shown in studies on angiogenesis using intravital microscopy⁷. The blood flow inside vessels and arteries can be visualized by CD41-FITC (to label platelets), dextran-FITC (plasma), or by the second harmonic generation (SHG), which visualizes collagen type 1 present in the basement membrane of some part of the vascular tree. SHG is a unique free labeling effect of the multiphoton excitation. Multiphoton imaging allows long-term cell tracking without harming the tissue and activating the cells by laser power excitation. Multiphoton microscopy is the imaging method of choice for visualizing fluorescently labeled cells and structures in living animals due to its ability to excite the fluorophores deeper into the tissue/organs with minimal phototoxicity⁸.

The use of tuned infrared lasers with pulses delivered within femtosecond intervals excites the fluorochrome only at the focal plane, with no excitation above and below the focal plane⁸. Thus, multiphoton microscopy allows high spatio-temporal resolution, less photo-damage, and increased tissue

penetration imaging for studying dynamic biological events inside the organs. It is an ideal microscopy tool for live-animal imaging. However, multiphoton and any other art of intravital microscopy is limited by tissue motion due to heartbeat, respiration, peristaltic movements, muscle tonality, and other physiological functions, which disturbs imaging acquisition and analysis. As these movements impair temporal and spatial resolution and sometimes even prohibit subsequent analysis, they must be addressed appropriately to enable accurate data analysis and interpretation. Several strategies have been developed to lower or prevent artifacts from tissue motion. This protocol applies an *in situ* drift correction software called VivoFollow⁹ to correct tissue drift during image acquisition. This approach provides the required image stabilization, enabling long-term imaging and cell-tracking analysis.

PROTOCOL:

This study was approved by the Bavarian Animal Care and Use Committee (approved by ethical code: ROB-55.2Vet-2532.Vet_02-17-99); these experiments were carried out in strict accordance with the German animal legislation guidelines.

1. Animals and femoral artery ligation (FAL)

NOTE: To induce sterile inflammation and arteriogenesis, 8–10 weeks old male C57BL/6J mice were used. None of the mice died or suffered from wound infection or wound healing disturbance during or post-FAL or sham surgery, respectively.

1.1. Anesthesia

NOTE: Before injection of the MMF-mix anesthesia, it is recommended to perform pre-analgesia with 5% isoflurane until the mouse is sleeping.

1.1.1. Weigh the mouse and prepare MMF mix with a dose of midazolam 5.0 mg/kg, medetomidine 0.5 mg/kg, and fentanyl 0.05 mg/kg.

1.1.2. Fill a 1 mL syringe with MMF-mix and inject a final volume of 100 µL s.c. using a 30 G needle (after pre-analgesia with 5% isoflurane).

1.1.3. Place the mouse on a warm pad and wait until the mouse is completely anesthetized. Set the timer to 35 min. After this time, inject s.c. half of the dose (50 µL) of the MMF-mix.

1.1.4. Check the depth of anesthesia by testing the pedal and interdigital reflexes (by firm toe pinch) of the mouse.

NOTE: An absence of response indicates deep analgesia.

1.2. Femoral artery ligation (FAL)

NOTE: Use sterile surgical instruments and sutures. Disinfect the skin with disinfectant before opening and

133 after closing.
134
135 1.2.1. Place the anesthetized mouse on a heating plate (35–37 °C) to maintain physiological body
136 temperature. Apply eye cream (dexpanthenol) to each eye to prevent drying of the eyes under anesthesia.
137
138 1.2.2. Transfer the anesthetized mouse to a Laser Doppler imaging (LDI) chamber before and after FAL to
139 check blood flow and perfusion (**Figure 1A–C**).
140
141 1.2.3. Remove hair, disinfect the skin, and make a cut to access the femoral artery. Under a surgical
142 microscope, ligate the right femoral artery of the mouse hind limb using a braided silk suture (0/7) and
143 perform a sham operation on the left femoral artery to serve as an internal control as previously described⁶
144 (**Figure 1D–K**).
145
146 1.2.4. Close the skin using a braided silk suture (0/6). Administer buprenorphine (0.1 mg/kg) s.c. every 12
147 h, starting 10 min before antagonizing the anesthesia for pain relief. Observe the animal for signals of pain,
148 i.e., reduced grooming activity, reluctance to move, or hunched posture for 24 h post-surgery.
149
150 1.2.5. Inject (s.c.) a combination of naloxone (1.2 mg/kg), flumazenil (0.5 mg/kg), and atipamezole (2.5
151 mg/kg) to antagonize the anesthesia after finishing the FAL and closing the skin.
152
153 1.2.6. After surgery, keep the mouse on the warm pad and continuously monitor the animal until it can
154 maintain an upright posture and start walking normally around the cage.
155
156 1.2.7. When the mouse is fully awake, place it back in the cage together with the other mice and return
157 the cage to the animal housing room.
158

159 **2. Tissue preparation for multiphoton intravital imaging**

160 2.1. Tail vein injection

161
162 NOTE: The animal needs to be under anesthesia before starting the preparation for intravital imaging.
163 Before introducing the needle of the vein catheter, it is recommended to apply a skin disinfectant on the
164 tail. This will also help to visualize the vessels.
165
166

167 2.1.1. Prepare a tail vein injection catheter using a 2 x 30 G needle, 10 cm of a fine bore polythene tubing
168 (0.28 mm internal diameter and 0.61 mm outer diameter), a 1 mL syringe, and a histoacryl glue to fix the
169 catheter on the mouse tail for further i.v. injections during imaging (**Figure 2A,B** and **Figure 2F**).
170

171 2.1.2. Prepare the antibody solution for i.v. injection: CD45-PE and CD41-FITC (20 µg/mouse) at a final
172 volume of 100 µL.
173

174 2.1.3. Check the tail for the laterally located tail veins and dam the vein to identify the tail vein. Disinfect
175 the tail, insert the vein catheter, and fix it using tissue histoacryl glue (**Figure 2B**).
176

177 2.1.4. Inject the antibodies prior to imaging (at least 15 min before) and after finishing the tissue
178 preparation.

179 180 2.2. Tissue preparation

181
182 NOTE: Always use a warm pad and administer a drop of eye cream (dexpanthenol) in each eye before tissue
183 preparation. Use sterile surgical instruments and sutures. Disinfect the skin with disinfectant before open
184 and after closing.

185
186 2.2.1. Place the anesthetized mouse on a stable and portable pad. Fix the mouse in a supine position with
187 a 4 point fixation on the pad using adhesive tape (**Figure 2B**).

188
189 2.2.2. Mold two pieces of modeling clay and place the pieces under the upper hind limb of the mouse to
190 ensure a plane position of the adductor muscle (**Figure 2B**, indicated by red arrows).

191
192 2.2.3. To ensure a stable body temperature of 37 °C of the mouse, place the pad with the mouse on a
193 heating plate under a surgical microscope with a 0.64-4.0-fold zoom (**Figure 1L**).

194
195 2.2.4. Remove the hair from both legs, disinfect the skin, and cut the skin in a circle around the scar of the
196 earlier femoral artery ligation of the right hind limb (**Figure 1M**).

197
198 2.2.5. Remove the skin and fat layer from the top of the leg (**Figure 1N,O**), and pull aside the remaining
199 skin using sutures to create a pocket around the adductor muscle (**Figure 1P,Q**).

200
201 2.2.6. Remove subcutaneous fat to get a clear vision of the adductor muscle and the profound artery/vein
202 as well as the collateral vessels (**Figure 1Q,R**).

203
204 2.2.7. Carefully start removing the superficial muscle layer on top of the collateral vessels by carefully
205 dissecting it out with fine forceps (**Figure 1R**).

206
207 NOTE: The collateral artery and the collateral vein are clearly visible and ready for imaging (**Figure 1S**).

208
209 2.2.8. Fill the prepared pocket with saline to avoid drying of the tissue and continue with the same
210 procedure for the sham-operated left hind limb. Before imaging, add ultrasonic gel in both pockets, which
211 prevents drying and serves as an immersion medium for optical coupling with the objective (**Figure 2C** and
212 **Figure 2F**).

213 214 3. Intravital multiphoton microscopy

215 216 3.1. Preparation

217
218 NOTE: Keep a syringe with ultrasonic gel inside the incubator chamber of the microscope to maintain a
219 warm temperature for refilling the pockets during long-term imaging.

220

3.1.1. Remove the saline from the two prepared pockets and replace it with ultrasonic gel to cover the collateral vessels (**Figure 2C**).

3.1.2. Inject the antibody solution through the tail vein catheter. After 15 min of antibody injection, transfer the pad with the fixed mouse into the warmed multiphoton imaging chamber (**Figure 2F**).

3.1.3. After completion of the image acquisition, euthanize the mouse by cervical dislocation under deep narcosis.

3.2. Multiphoton microscopy image acquisition

NOTE: Turn on the microscope 30 min before imaging to allow for laser stabilization. For the best optical stability of the microscope, it is recommended to keep the microscope chamber permanently heated.

3.2.1. Turn on the key of the Ti:Sapphire laser box, the electronic interfaces boxes, the heating unit of the incubation chamber, the fluorescent lamp, and the computer (**Figure 2D,E**).

3.2.2. Launch the acquisition software (Inspector software). Set the wavelength to 800 nm and open the microscope shutter (**Figure 3A ii**).

3.2.3. In the **Measurement Wizard dialog**, choose the **Instrument Mode (Single beam)** and in **Measurement Mode (3D-scan Time lapse)** (**Figure 3A i**).

3.2.4. Transfer the anesthetized mouse into the pre-warmed incubation chamber of the microscope. Position the area with the ultrasonic gel (step 2.2.8) directly in contact with the objective front lens (**Figure 2F**).

3.2.5. Open the epifluorescence microscope shutter, choose an adequate filter/dichroic setting for FITC visualization (4) to find the area of interest by following the blood flow under epifluorescence illumination. After bringing the area of interest into the center field of view, close the epifluorescence microscope shutter.

3.2.6. In the **xy-Scanner dialog**, set the following parameters: Image size (554x554), Pixel (512x512), Frequency (400), Line average (1) (**Figure 3A iii**). Adjust the laser power (5%) (**Figure 3A iv**), and set the photomultiplier (PMT) gain for the channels green (66), red (66), and blue (63) (**Figure 3A v**).

3.2.7. Select an adequate objective for intravital imaging and drift correction settings (see details in 3.2.11).

NOTE: Here, the selected objective was the Nikon LWD 16x (**Figure 3A vi**).

3.2.8. Define the image stack range by **starting the preview acquisition mode** by pressing the red button on the top right corner of the **Measurement Wizard dialog** window (**Figure 3A i**).

3.2.9. Define the area and structure of interest by focusing to obtain a good image. Then, while observing the screen, change focus by moving the objective up until the image disappears, and set it as zero (**first= 0**). Change focus in the opposite direction by moving the objective down until the image disappears from the screen again, and set it as the last (**end= 40 μm**) position in the axial direction (**Figure 3A vii**).

3.2.10. Set the step size to 2 μm . Click the red button on the top right corner of the **Measurement Wizard dialog** to stop the preview scanning.

NOTE: The number of images will be calculated and set automatically (21 images), as indicated in **Figure 3A vii**.

3.2.11. If the microscope is equipped with live-drift correction⁹, start the drift correction software (e.g., VivoFollow) and follow the steps described below.

3.2.11.1. In the **Measurement Wizard dialog**, choose the Python axis (**Python Ax**) (**Figure 3A viii**) as the first axis device; **DO NOT** activate the auto save mode. Check the **auto save (AS)** box only on the time axis (**Time Time**). Press the **Python** icon on the **Available Devices** window (**Figure 3A ix**) to open the **Python dialog** window.

3.2.11.2. In the **Python dialog Axis** settings, enter in **From** (0 zero) and **To** (-40). Enter **Number of steps** (21) as indicated in **Figure 3A x**.

3.2.11.3. Go to the **xyz Stage Z dialog** window and enlarge the **Scan Range** by 200 μm on both ends: in **Start**, enter (200 μm) and in **End**, enter (-240 μm), the range will automatically be set (-440 μm). Keep the step size (2 μm) as shown in **Figure 3A xi**.

3.2.11.4. Open the **VivoFollow Frontend** dialog and click the **Motion Profile box** (**Figure 3A xii**).

3.2.11.5. Start image acquisition by pressing the green arrowhead in the top left corner of the **Measurement Wizard dialog** of the Inspector Software (**Figure 3A i**).

3.2.11.6. If live-drift correction is used, look for a dialog for the drift correction configuration to appear as shown in **Figure 3A xiii**. Set the acquisition channel that will be used as an immobile landmark reference (for this protocol, choose channel 2, where the vascular tracer signal is to be recorded). Enter the maximal correction offset in μm that was added to the first and last z positions (here, 200 μm) and click **OK**.

3.2.11.7. Monitor the current drift offset in x, y, and z in real time during the image acquisition in the **VivoFollow fronted dialog** window **Figure 3A xiv**.

3.2.11.8. Stop imaging acquisition after ~35 min when another cycle of anesthesia re-injection is required. Inject half a dose of the MMF-mix (50 μl s.c) and re-start imaging acquisition by pressing the green arrowhead again (step 3.2.11.5).

3.2.11.9. Repeat this procedure until the experiment is finished.

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REPRESENTATIVE RESULTS:

Multiphoton microscopy offers a high spatio-temporal resolution for leukocyte tracking, wherein cell migration steps and speed can be tracked and monitored (**Figure 4A,B**). However, the physiological motion of the sample poses a challenge, especially for long-term intravital microscopy image acquisitions. Therefore, a good tissue preparation and holders to fix the tissue and tools, such as real-time imaging correction for tissue drift, are required for successful and stable imaging acquisition. Here, a live-drift tool, VivoFollow, was employed to correct for the tissue drift generated during long-term imaging acquisition.

The application of this tool allows long-term image acquisition and enables the collection of high-quality data, suitable for tracking of cells for speed measurements. As shown in **Figure 3B**, without drift correction, the region of interest progressively drifts away from the recording view, impacting the ability to track cells for speed analysis. However, as shown in **Figure 3C**, with the drift correction software, stable movies can be recorded, and more cells can be tracked over a long period. The drift correction software can also provide a visualization of the x, y, and z offsets over time that the system corrected for, as shown in **Figure 3D**.

FIGURE AND TABLE LEGENDS:

Figure 1: Laser Doppler Imaging setup and preparation for femoral artery ligation and tissue preparation for intravital imaging. (A) LDI imaging setup. (B) The mouse is placed inside the LDI warming chamber for image acquisition. (C) LDI images of the right and left legs before (upper panel) and after FAL (lower panel) to check the hindlimb perfusion after ligation. The right femoral artery was ligated/occluded, while the left femoral artery was sham-operated (Sham) and served as an internal control. In the color bar, blue indicates low blood perfusion, and red indicates higher perfusion. (D–K) Images represent the surgery steps of the femoral arterial ligation. Scale bar = 10 mm. (L–S) Tissue preparation for intravital imaging after FAL. The area to be imaged is indicated by the black circle, where the collateral vessels are located. Scale bar = 5 mm. Abbreviations: LDI = Laser Doppler imaging; FAL = femoral artery ligation; Occ = occlusion.

Figure 2: Setup for multiphoton intravital imaging. (A) Vein catheter preparation; 1-Fine polythene tubing of 10 cm, 2: 2x 30 G needle; 3: mounted catheter connected with 1 mL syringe; 4: tissue histoacryl glue; 5: needle holder. (B) The mouse positioned in supine position with both hindlimbs placed on pieces of black modeling clay (red arrows) prepared for imaging. (C) Occluded (Occ) right hindlimb and left, sham-operated hindlimb ready for imaging. (D) Multiphoton microscope setup showing laser boxes, fluorescence lamp box, tubes for heating the incubator chamber. (E) Incubator chamber of the multiphoton microscope. (F) Mouse placed inside the microscope chamber with the 16x objective touching the tissue covered with ultrasonic gel. Detail showing the vein catheter fixed on the tail vein for antibody injection.

Figure 3: VivoFollow drift correction software setup. (A) (i-xiv) Software setup steps as described in protocol step 3.2.2–3.2.11.9. (B) Representative time series images showing the imaging drifting without drift correction software turned on. Collateral artery is delimited by the white discontinued lines (**Video 1** and **Video 2**). (C) Representative time series images showing the stable time series when the live drift correction is applied during imaging acquisition. Leukocytes were labeled with CD45-PE antibodies (red), platelets were labeled by CD41-FITC antibodies (green) and collagen type 1 with the SGH (blue). The

353 collateral artery is delimited by the white discontinued lines. (D) Line chart showing the live correction
354 along x, y, and z directions. Abbreviations: PE = phycoerythrin; FITC = fluorescein isothiocyanate; SGH =
355 second harmonic generation.

356
357 **Figure 4: Representative results.** (A) Leukocyte speeds measured in collateral arteries of sham-operated
358 (Sham) and femoral artery-ligated hindlimbs. (B) Representative images show the cells tracked (magenta)
359 with the tracks color-coded. The color code bar represents the cell speed with the slower cells shown by
360 blue tracks and faster cells with red tracks. Leukocytes were labeled with injected CD45-PE antibodies (red),
361 platelets were labeled by injected CD41-FITC antibodies (green), and the collagen type 1 with the SGH
362 (blue). Results from three individual experiments. Scale bar = 20 μm . See **Video 3** and **Video 4**.
363 Abbreviations: Occ= occluded/femoral artery-ligated; PE = phycoerythrin; FITC = fluorescein
364 isothiocyanate; SGH = second harmonic generation.

365
366 **Video 1: Multiphoton intravital imaging of a collateral artery without drift correction.** Four-dimensional
367 images were recorded with a pixel size of 554 x 554 μm , frequency 600 Hz, an interval of 1100 ms/frame,
368 step size of 2 μm , and range of 40 μm . The video shows the image drifting without the application of the
369 VivoFollow drift correction software. The collateral artery is shown in the lower part of the video, and the
370 collateral vein appears after 3 min in the upper part of the movie. Scale bar = 50 μm .

371
372 **Video 2: Multiphoton intravital imaging of a collateral artery with applied drift correction.** Four-
373 dimensional images were taken with a pixel size of 554 x 554 μm , frequency 600 Hz, an interval of 1100
374 ms/frame, step size of 2 μm , and range of 40 μm . The video was recorded with the live drift correction
375 software, VivoFollow, and shows the imaging stability promoted by applying the drift correction software.
376 Scale bar = 50 μm .

377
378 **Video 3: Cell-tracking imaging in a collateral artery after sham operation.** Leukocytes labeled with injected
379 anti-CD45-PE antibodies were imaged in a resting collateral artery of the sham-operated leg and tracked
380 using Imaris software with the plugin for tracking cells. Tracked cells were selected and represented by the
381 magenta dots. The tracks were color-coded according to the cell speed. Slower cells are represented by
382 blue tracks and faster cells by red tracks. Scale bar = 50 μm . Abbreviation = PE = phycoerythrin.

383
384 **Video 4: Cell-tracking imaging in a collateral artery after FAL.** Leukocytes labeled with anti-CD45-PE
385 antibodies were imaged in a growing collateral artery 24 h after the induction of arteriogenesis by FAL and
386 tracked by using Imaris software with the plugin for tracking cells. Tracked cells were selected and
387 represented by the magenta dots. The tracks were color-coded according to the cell speed. Slower cells
388 are represented by blue tracks and faster cells by red tracks. Scale bar = 50 μm . Abbreviations: FAL =
389 femoral artery ligation; PE = phycoerythrin.

390
391 **Table 1: Troubleshooting.**

392
393 **DISCUSSION:**
394 The described method of multiphoton *in vivo* analysis of leukocyte recruitment represents an addition to
395 commonly used tools for leukocyte recruitment studies such as (immuno-) histological or FACS analyses.
396 With this imaging method, it is possible to visualize in greater detail the dynamic processes in leukocyte

adherence and extravasation during arteriogenesis¹⁰. Despite the added value of this method, the offered protocol includes some critical steps and limitations. See **Table 1** for more troubleshooting tips. During the removal of the superficial muscle layer on collateral arteries, these arteries could be damaged and will not be useful for intravital imaging. The catheter must be well-fixed for antibody injection; otherwise, slipping of the tail vein catheter can cause the extravasation of the antibodies into the perivascular space of the tail, which makes replacement and additional correction for injection of the antibodies a challenging task. The correct identification of the collateral artery and the collateral vein during intravital microscopy represents another critical step of this protocol.

Given that the mouse is not mechanically ventilated during intravital microscopy, the dynamic imaging time is limited to avoid physical injury to the mouse as a result of excessively long anesthesia. One of the shortcomings of intravital microscopy is the mouse physiologic motion, generated by respiration, heart beating, and peristaltic movements, that can impact image acquisition and therefore, the quality of data analyses. Improving tissue holders, mounting, and fixation are steps that further reduce the effects of motion. In addition to the physiological motion, long-term imaging may contribute to tissue drifting during imaging. As described in this protocol, a real-time tissue drift correction software, VivoFollow, was employed to obtain imaging data suitable for subsequent cell tracking and cell speed analysis. This VivoFollow software corrects for the sample displacement in x, y, and z directly during image acquisition. It relies on immobile anatomical structure, for example, when vessels are visualized with vascular tracers that serve as reference landmarks for the correction procedure.

Although there are other post-acquisition drift correction tools available, such as those within Bitplane Imaris or plugins for ImageJ, they are very limited in their capacity for correction as they do not allow for restoration of the region of interest. In principle, they cannot correct for severe offsets when the region of interest moves out of the field of view. However, this protocol can be applied even when the live-drift correction is unavailable, and post-acquisition correction becomes necessary. However, this requires frequent training of personnel in tissue mounting and fixation. In conclusion, multiphoton intravital microscopy is a useful dynamic method that can be applied in parallel to static established methods such as (immuno-) histological analyses to get a more detailed picture of the dynamic components of leukocyte recruitment.

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DISCLOSURES:

The authors have no conflicts of interest to declare.

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Figure 1

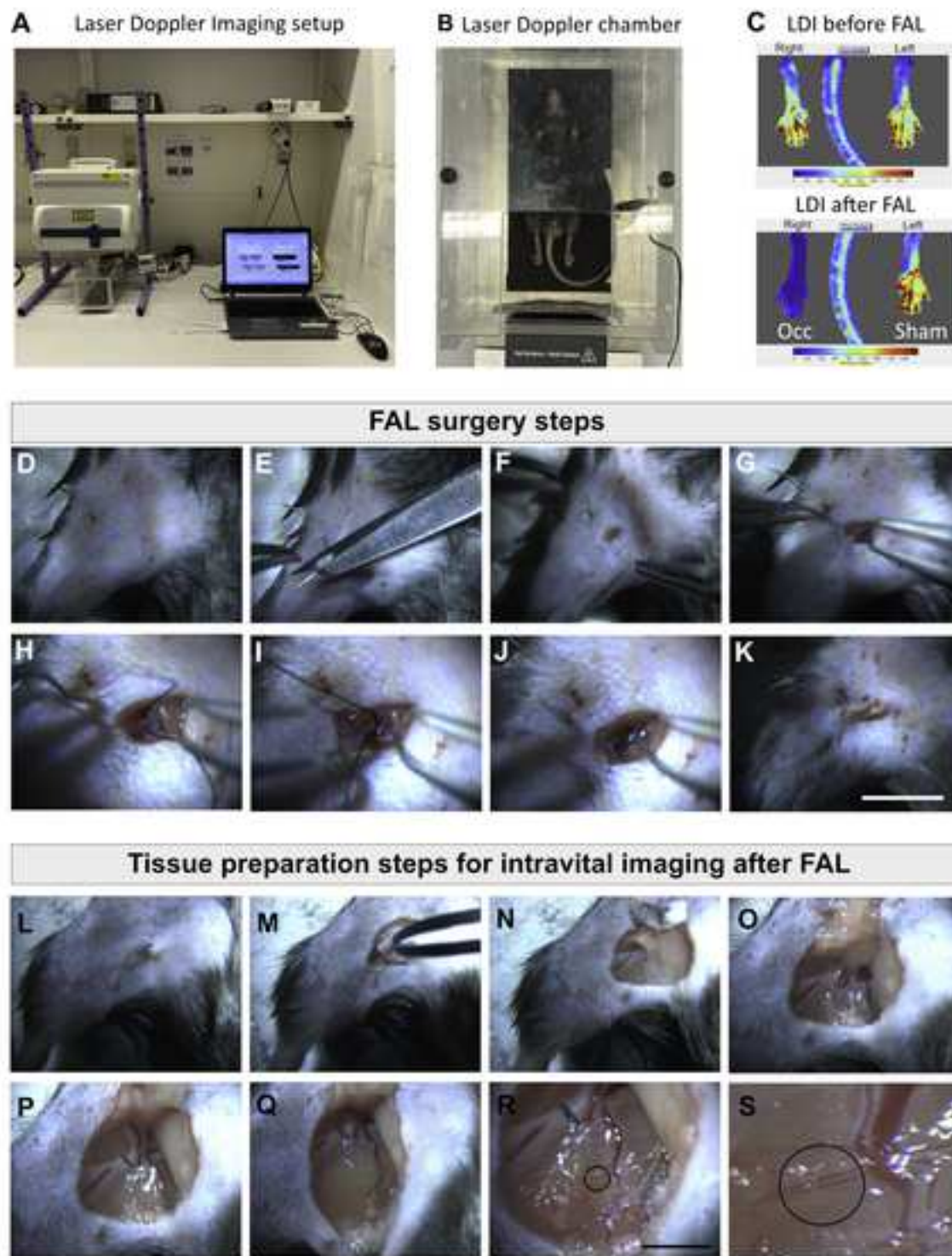


Figure 2

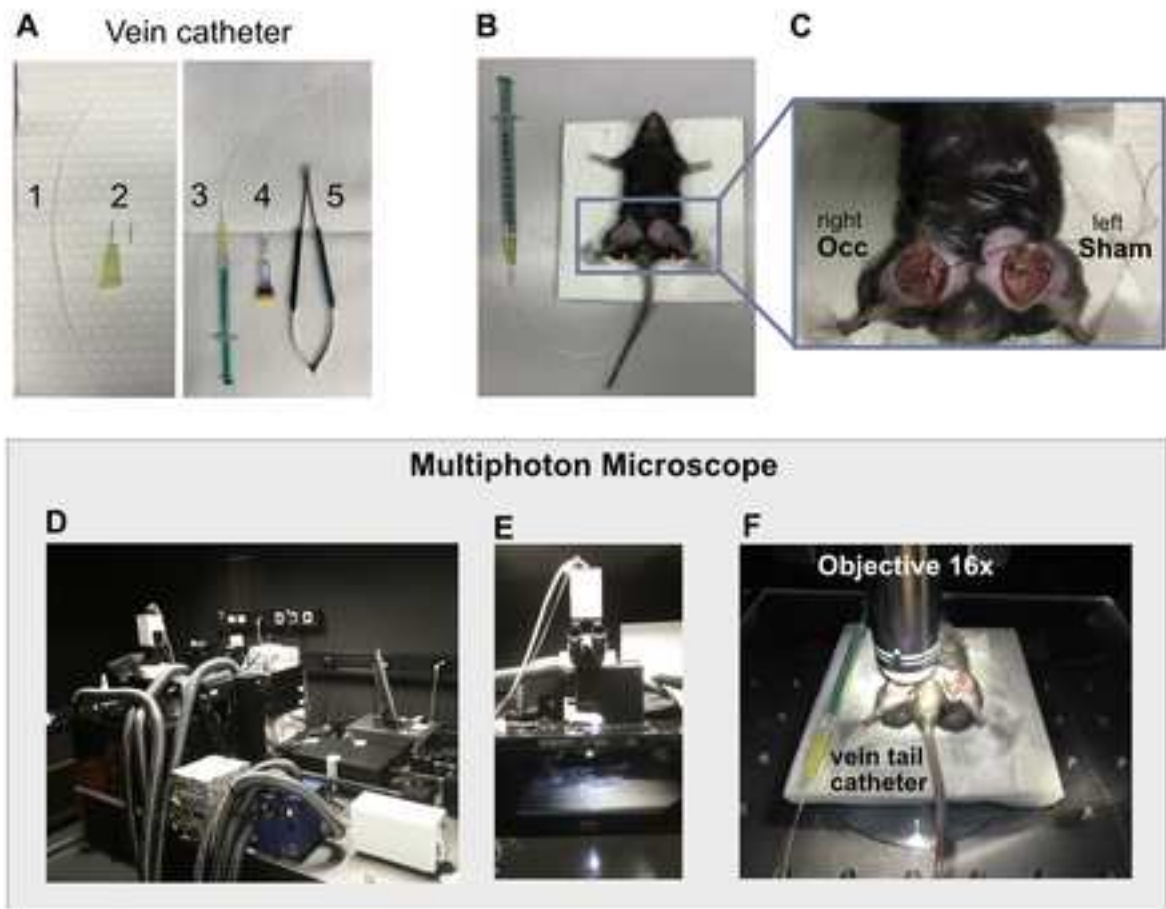


Figure 3

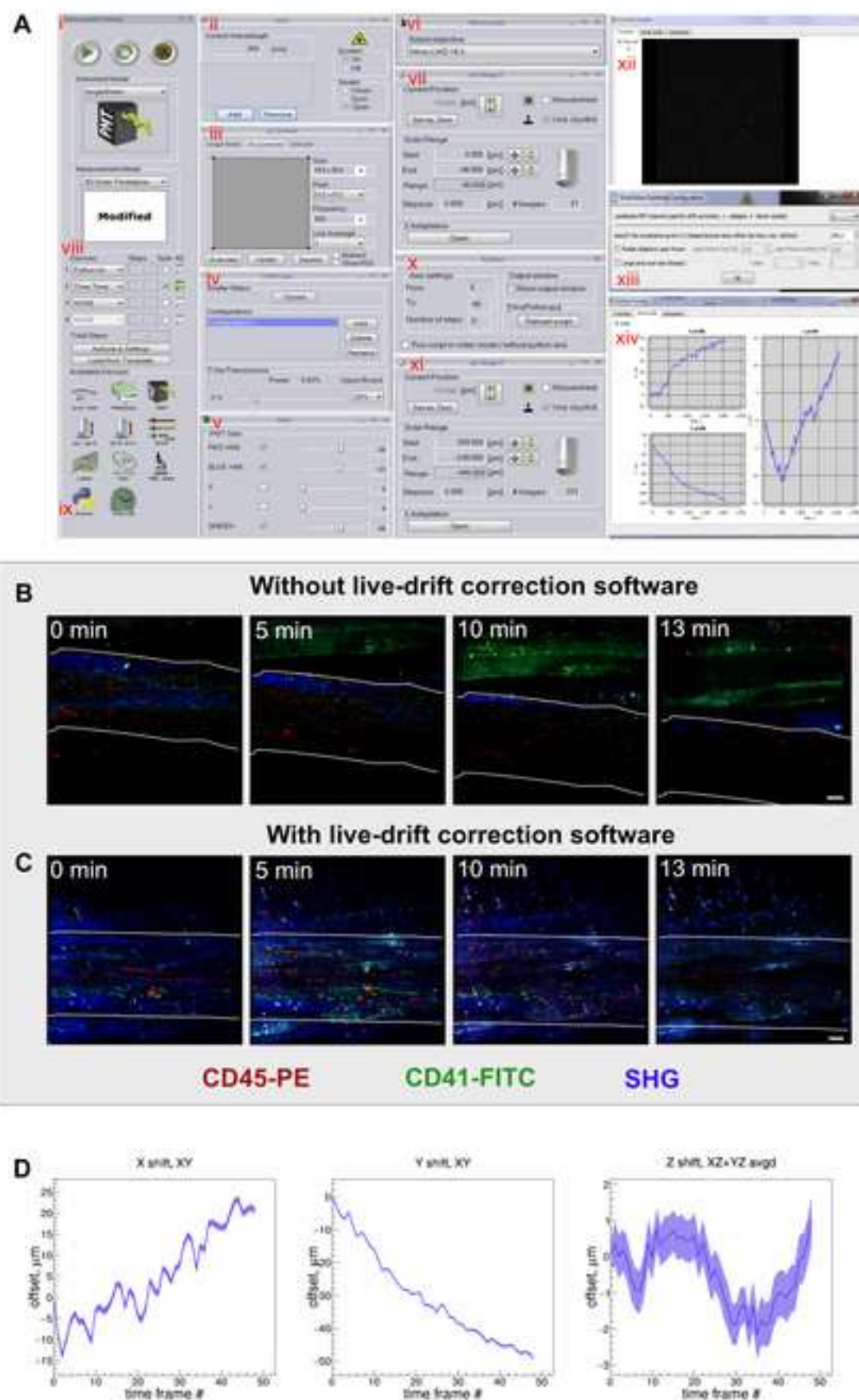
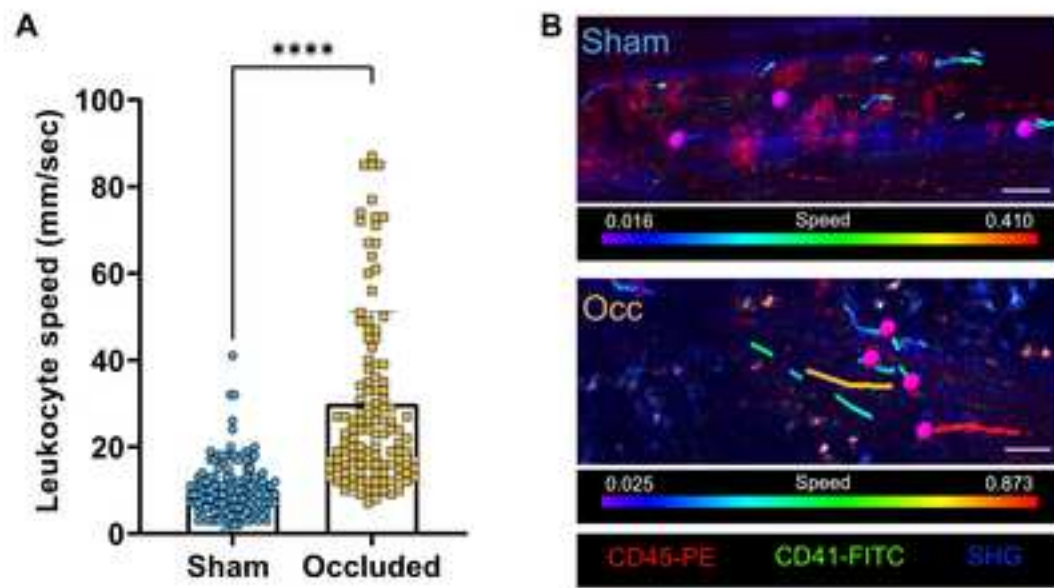
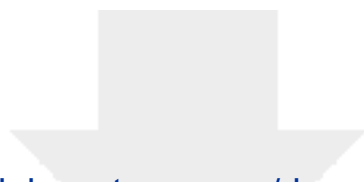


Figure 4

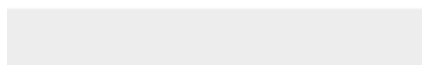
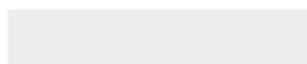


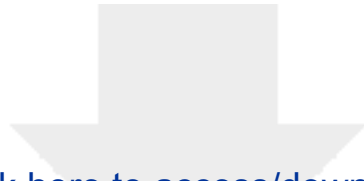


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Video or Animated Figure

Movie 1-without drift correction.mp4



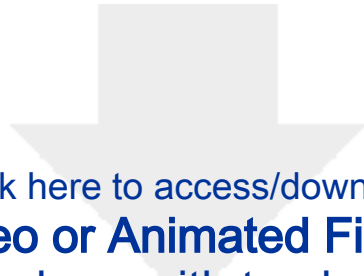


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Video or Animated Figure

Movie 2-with drfit correction (1).mp4





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Video or Animated Figure

Movie 3-sham with tracks (1).mp4



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Video or Animated Figure

Movie 4- FAL with tracks (1).mp4





Table 1. Troubleshooting
Technical Problems

<p>➤ In case the tail vessels are not visible for introducing the catheter</p>
<p>➤ In case the tail catheter cannot be fixed for antibody injection</p>
<p>➤ When the motion of tissue impedes imaging stability</p>
<p>➤ When the image quality starts decreasing</p>
<p>➤ When the arteries are damaged during preparation for imaging</p>

Proposed solutions

✓ Use a warm compress surrounding the mouse tail for 3-5 min to promote local vasodilation. It will help to make the vein visible.

✓ Use a 1 mL syringe with 30 G needle to introduce into the vein and directly inject the antibodies and dyes before imaging.

✓ In some cases (depending of the antibodies) the injection can be applied i.p. 1 h before imaging.

✓ Try to re-position and re-fix the mouse upper hind leg to avoid the physiological abdominal movements.

✓ Check whether the microscope table has enough air to cancel vibration.

✓ Make sure that the mouse is in deep anesthesia and does not wake up.

✓ Monitor the breathing of the mouse. When the mouse is breathing too fast, it can have an impact on the imaging motion.

✓ Ensure the ultrasonic gel does not dry out.

✓ Re-fill the ultrasonic gel.

✓ Make sure that the objective is clean (clean up the dried ultrasonic gel on the objective whenever a new re-filling is done).

✓ In this case, it will not be possible to acquire images. It is not possible to run the experiment.

|



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Table of Materials

JoVe Materials_revised (1).xls



Answers to editor/reviewers

Resubmission of the manuscript Ref. No.: JoVE62969:

Title: Multiphoton intravital imaging as a tool for monitoring leukocyte recruitment during arteriogenesis *in vivo* in a murine hindlimb model

AUTHORS:

Manuel Lasch^{1,2,3}, Mykhailo Vladymyrov⁴, Dominic van den Heuvel^{1,5}, Philipp Götz^{1,3}, Elisabeth Deindl^{1,3}, Hellen Ishikawa-Ankerhold^{1,5}

Editorial comments:

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. Please define all abbreviations at first use.

A: We proofread the manuscript again and define all abbreviations at first use.

2. Please provide an email address for each author.

A: We now provide an email address for each author.

3. Please revise the following lines to avoid overlap with previously published work: 85-86, 205-206, 209-212.

A: We revised those sentences.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

A: We revised the text especially the protocol part and removed the personal pronouns.

5. 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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

A: We checked the text in the protocol carefully to avoid usage of could/should/would and we use imperative tenses.

6. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows

A: We now provide this statement.

the animal care guidelines of your institution. Indicate whether your study was approved by an ethics committee.

b) What happened to the mice after the study? Please specify the euthanasia method without highlighting any steps.

A: We added these information's, lines 103-105 and 244-245.

c) Please mention how proper anesthetization is confirmed.

A: We added the information, please see point 1.4.

d) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

A: We specified the use of ointment, please see lines 135-136 and 188-189.

e) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

A: After surgery the mice received buprenorphine (0,1mg/kg) each 12h s.c to avoid postsurgical pain. By administration of buprenorphine we did not observe relevant signals of pain, as reduced grooming activity or reluctance to move, which is described now on lines 145-147.

f) Discuss maintenance of sterile conditions during survival surgery.

A: All surgical instruments are sterilized before use. Before cutting the skin and after closing the skin, it is disinfected with Cutasept disinfectant, to avoid infection during and after surgery. We have added this information

on lines 129-130 and 191-192.

g) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

A: After surgery the mice are monitored by checking the abdominal breathing until having wakeup movements by start to walk and moving around the cage. We provide this information on lines 148-149.

h) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

A: We now provide this information on lines 152-153

7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. Please convert lines 101 to 113 to action steps, keeping only notes relevant to those steps and moving the rest of the discussion or background to discussion or introduction, respectively.

A: We now changed this paragraph as requested.

8. 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 ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

A: We have added all the details to be followed by the video recording of the mouse model tissue preparation for Multiphoton intravital imaging.

9. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video. Avoid highlighting notes.

A: We now format the manuscript as requested.

10. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

A: We added the missing scale bars to Figures 1 and 4.

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

A: We used the JoVE style for our references and all references appear in the requested style.

12. Please sort the Materials Table alphabetically by the name of the material.

A: The table is now sorted alphabetically.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript is well written in detail. I have almost nothing else to mention to add.

Major Concerns:

I just wonder if you can calculate the speed of blood flow in each group at the same time some way, in order to exclude the effect of blood flow to leukocytes.

A: Thanks for your question. Yes, the blood flow speed can be calculated by tracking the path of a single labelled platelet or by injection of FluoSpheres (beads with 1.0-2.0 μm diameter), that can flow freely together with the blood through vessels/arteries. However, the tracking of such beads or singles platelets exceeds what we are able to reliably accomplish on our multiphoton system. As such, in our experimental setup the focus was on the crawling cells on the vascular wall and measuring their trajectories and velocities. It is known that the shear stress (which depends on the flow speed, vessel size, etc) does not affect the T-cell crawling speed (<https://www.sciencedirect.com/science/article/pii/S0006349512051181>), and in fact in in-vitro experiments under flow chamber modeling of the blood-brain barrier the T cells crawl isotropically in all directions: with as well as against the flow, thus, their speeds should not be affected by the shear stress.

Minor Concerns:

L325 ")" should be deleted.

A: The bracket in this line was deleted.

Reviewer #2:

Manuscript Summary:

Cardiovascular disease is the leading cause of death worldwide. Artery is the key vessel affecting cardiovascular disease, so the study on the mechanism of artery growth and repair is of great significance. The murine hindlimb ischemia model is a widely used mouse model to study arteriogenesis in detail. In ischemic vascular diseases, leukocyte recruitment and polarization are crucial for revascularization and tissue repair. Studies of hindlimb ischemia in mice with deficient leukocyte recruitment have shown impaired angiogenesis, arteriogenesis, and restoration of blood perfusion. In this study, the authors combined multiphoton microscopy and hindlimb ischemia mice model together to visualize leukocytes trafficking. With this method, it is possible to study the dynamic processes in leukocyte migration and extravasation during arteriogenesis.

Major Concerns:

No

Minor Concerns:

This method is complicated and needs delicate technique. It would be better if the authors give a trouble shooting table.

A: We have made a troubleshooting table. See it below.

Table 2. Troubleshooting

Technical Problems	Proposed solutions
➤ In case the tail vessels are not visible for introducing the catheter	✓ Use a warm compress surrounding the mouse tail for 3-5 min to promote local vasodilation. It will help to make the vein visible.
➤ In case the tail catheter cannot be fixed for antibody injection	✓ Use a 1 ml syringe with 30G needle to introduce into the vein and directly inject the antibodies and dyes before imaging. ✓ In some cases (depending of the antibodies) the injection can be applied i.p. 1h before imaging.
➤ When the motion of tissue impede imaging stability	✓ Try to re-position and re-fix the mouse upper hind leg to avoid the physiological abdominal movements. ✓ Check whether the microscope table has enough air to cancel vibration. ✓ Make sure that the mouse is in deep anesthesia and does not wake up. ✓ Monitor the breathing of the mouse. When the mouse is breathing too fast it can have an impact on the imaging motion.
➤ When the image quality starts to decrease	✓ Check whether the ultrasonic gel did not dry out. ✓ Re-fill the ultrasonic gel. ✓ Make sure that the objective is clean (clean up the dried ultrasonic gel on the objective whenever a new re-filling is done).
➤ When the arteries are damaged during preparation for imaging	✓ In this case it will be not possible to acquire image. It is not possible to run the experiment.



Walter-Brendel-Zentrum für Experimentelle Medizin

Dr. Hellen Ishikawa-Ankerhold

Marchioninstraße 15, 81377 München

Dr. Carmine Rocca/Dr. Vidhya Iyer
Guest Editor/Review Editor
Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA

TEL: +4989 2180 76585

FAX: +4989 2180 76503

Email: hellen.Ishikawa-Ankerhold@med.uni-muenchen.de

Munich, July 20st, 2021

Resubmission of the manuscript Ref. No.: JoVE62969:

Multiphoton intravital imaging as a tool for monitoring leukocyte recruitment during arteriogenesis *in vivo* in a murine hindlimb model

Dear Dr. Rocca and Dr. Iyer,

Thank you very much for giving us the opportunity to revise our above entitled manuscript.

We have considered all your points and the review comments by addressing all issues and concerns in detail.

Please find enclosed our revised manuscript as well as detailed point by point reply to the editor's and reviewer's comments.

All changes made in the manuscript are in font color blue.

We would be very pleased if you and the reviewers will find our manuscript suitable for publication in JoVE.

Kind regards,



Hellen Ishikawa-Ankerhold