Fluorescence Microangiography for Quantitative Assessment of Peritubular Capillary Changes after AKI in Mice

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ABSTRACT
AKI predicts the future development of CKD, and one proposed mechanism for this epidemiologic link is loss of peritubular capillaries triggering chronic hypoxia. A precise definition of changes in peritubular perfusion would help test this hypothesis by more accurately correlating these changes with future loss of kidney function. Here, we have adapted and validated a fluorescence microangiography approach for use with mice to visualize, analyze, and quantitate peritubular capillary dynamics after AKI. A novel software-based approach enabled rapid and automated quantitation of capillary number, individual area, and perimeter. After validating perfusion in mice with genetically labeled endothelia, we compared peritubular capillary number and size after moderate AKI, characterized by complete renal recovery, and after severe AKI, characterized by development of interstitial fibrosis and CKD. Eight weeks after severe AKI, we measured a 40%±7.4% reduction in peritubular capillary number (P<0.05) and a 36%±4% decrease in individual capillary cross-sectional area (P<0.001) for a 62%±2.2% reduction in total peritubular perfusion (P<0.01). Whereas total peritubular perfusion and number of capillaries did not change, we detected a significant change of single capillary size following moderate AKI. The loss of peritubular capillary density and caliber at week 8 closely correlated with severity of kidney injury at day 1, suggesting irreparable microvascular damage. These findings emphasize a direct link between severity of acute injury and future loss of peritubular perfusion, demonstrate that reduced capillary caliber is an unappreciated long-term consequence of AKI, and offer a new quantitative imaging tool for understanding how AKI leads to future CKD in mouse models.


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BRIEF COMMUNICATION www.jasn.org

AKI is a strong risk factor for the future development of CKD and ESRD. In a recent meta-analysis, Coca and colleagues compared the risk for CKD, ESRD, and death in patients with or without AKI in 13 cohort studies that included >1.4 million patients.1 They demonstrated that patients with AKI are at high risk to develop CKD, with a pooled adjusted hazard ratio (HR) of 8.8 (95% confidence interval [95% CI], 3.1 to 25.5); ESRD (pooled HR, 3.1; 95% CI, 1.9 to 5); and mortality (pooled HR, 2.0; 95% CI, 1.3 to 3.1).1 Possible mechanisms underlying the link between AKI to future CKD include nephron loss, glomerular hypertrophy, inflammation, interstitial fibrosis, epithelial cell-cycle abnormalities, and endothelial injury with capillary rarefaction.2 Acute ischemic injury causes epithelial injury and death, but the tubule can repair completely after mild-to-moderate injury (restitutio ad integrum).3,4 After multiple ischemic events or severe ischemia-reperfusion injury (IRI), the repair process is often incomplete, resulting in fibrogenesis and development of CKD.2,5,6

In contrast to renal tubules, the microvasculature lacks substantial regenerative capacity, resulting in a persistent vascular rarefaction after severe or recurrent injury.7,8 Indeed, the loss of the renal microvasculature is hypothesized to be a key component in fibrogenesis and CKD progression after AKI. As the molecular pathways regulating capillary rarefaction begin to be unraveled, preventing this process has been proposed as a therapeutic target.9,10 Capillary rarefaction induces focal hypoxia, activating an injury cascade with inflammation and ultimately interstitial fibrosis.2,11 It has been described in a variety of rodent AKI models,7,12–15 and in human patients with CKD the loss of capillary density correlates with the severity of fibrosis.16–18
Despite the established association between AKI and peritubular capillary loss, the precise nature of microvascular changes occurring after AKI are poorly defined because high-resolution techniques have not been used to examine this question. To date, microvascular density has been measured by immunostaining and genetic labeling of the endothelium, techniques that assess the surface area of endothelial cells or the number of visually identified capillaries but not the actual capillary lumen. Endothelial cell antigens used for immunostaining, such as CD31 or genetic labeling (such as Tie 2), are reported to be expressed on nonendothelial cell types, which may introduce error. Other approaches have included Microfil infusion and radiation microangiography. Whereas radiation microangiography does not give information about the capillaries, the other techniques allow an estimation of microvascular density; however, they do not provide high-resolution information about perfusion characteristics, such as single capillary cross-sectional area and perimeter. A further limitation to date is the absence of a nonbiased and high-throughput means of quantitating capillary changes. Finally, when the endothelium is used as a surrogate for capillary perfusion, overestimation is possible because endothelial cells of clogged or collapsed vessels with microembolism will still be stained, even though the capillary is actually nonfunctional.

Recently, Advani and colleagues reported use of fluorescence microangiography (FMA) by renal artery injection in the rat 5/6 nephrectomy model. They describe changes in glomerular perfusion and a global reduction in peritubular perfusion in this CKD model. Here we modify this technique for use in mice by intracardiac injection and show that it allows visualization of microvasculature in all solid organs tested.

To more precisely define renal peritubular capillary dynamics during progression of AKI to CKD, wild-type mice were subjected to severe IRI (28 minutes of clamping), moderate IRI (23 minutes of clamping), or sham surgery followed by FMA 8 weeks after the surgery. Whereas the BUN levels in the moderate group showed a complete recovery at 14 days after surgery, BUN levels remained significantly elevated at day 14 and week 8 after severe IRI, indicating a progression to CKD.
Figure 2. Severe ischemia reperfusion injury leads to CKD with interstitial fibrosis. (A) Wild-type mice were subjected to severe bilateral IRI (clamping for 28 minutes), moderate bilateral IRI (clamping for 23 minutes), or sham surgery and were euthanized following FMA at 8 weeks. (B) BUN measurement revealed significantly increased BUN values in both IRI groups at day 1 after surgery. After moderate IRI, mice showed a complete recovery, with BUN levels returned to normal values at day 14 after surgery, whereas severe IRI lead to persistently increased...
group did not differ significantly compared with the sham group (Figure 2, C and D). All mice were subjected to the FMA procedure immediately before euthanasia at 8 weeks after surgery. Confocal microscopy pictures of the kidneys demonstrate that FMA allows precise delineation of the peritubular capillary network, including by three-dimensional reconstruction of Z-stack images (Figure 3A, Supplemental Videos 1 and 2).

Our software-based analysis (script available in Supplemental Material) demonstrates a significant 62%±2% reduction of total cortical perfused area (μm²/high-power field of the inner cortex) in the severe IRI group compared with the sham group and a significant 52%±1% reduction compared with the moderate IRI group (Figure 3B). This reduction of total perfused cortical area was due to both a reduction of capillary number (Figure 3C) and individual capillary cross-sectional area and perimeter (Supplemental Figure 2).

The use of genetic lineage tracing is limited by the specificity of the Cre drivers used. During this study, we noted that 15%±1% of F4/80+ macrophages were labeled in VE-Cadherin−; tdtomato+ kidneys (Supplemental Figure 3). Because macrophage infiltration intensifies in the postischemic kidney, this macrophage labeling would induce an error during quantitative measurements after injury. Therefore, we decided to compare the performance of FMA with standard CD31 immunostaining instead. Comparison between FMA and CD31 staining following IRI demonstrated a larger reduction of the perfused FMA+ capillary area compared with the CD31+ endothelial cell surface area (Supplemental Figure 4), suggesting that some capillaries might lack perfusion following IRI. Indeed, we observed areas where CD31+ capillaries did not contain FMA+ luminal signal following IRI (Supplemental Figure 4).

For the automated software-based analysis of the FMA sections, we used a lower cutoff value of 4.9 μm² because it has been previously reported that the critical diameter at which erythrocytes cannot pass through capillaries is 2.5 μm (π×r²=4.9 μm²); to exclude venules, glomerular capillary convolutes, and other larger vessels (arterioles, arteries, veins), we used an upper cutoff value of 100 μm² (Supplemental Figure 5). Importantly, the raw data obtained without applying these cutoffs still showed the same significant differences after IRI (Supplemental Figure 5).

The loss of total perfused peritubular cross-sectional area (μm²/high-power field), peritubular capillary number, and individual capillary cross-sectional area (μm²/capillary) and perimeter (μm/capillary) at week 8 correlated very closely with BUN at day 1, and to a lesser degree at day 14 and week 8 after IRI (Figure 4B, Supplemental Figure 6), suggesting that severity of initial injury is a critical factor for future peritubular capillary rarefaction.

In summary, this method reveals novel insight into capillary dynamics in response to AKI, including a previously unappreciated reduction not only in the number but also the caliber of surviving peritubular capillaries. Basile et al. previously used Microfil injection to demonstrate that rats develop a significant reduction of peritubular capillary density in the kidney (cortex, −25% to 30%; inner stripe of outer medulla, −35% to 40%) at 4–40 weeks after IRI. Horbelt et al. subsequently identified endothelium by immunostaining of postischemic mice and also reported a 45% decrease of microvascular density at 30 days after IRI.14 Although the experimental protocols differ, our results confirm a substantial reduction in perfusion after acute injury but also report that surviving capillaries are smaller. Thus, an advantage of FMA in assessing peritubular microvasculature is its ability to define perfused capillaries and their precise architecture.

Several future applications can be envisioned. This mouse FMA approach should be useful for the study of the microvasculature, not just in the kidney but in a variety of solid organs, where capillary rarefaction after injury is also considered to be an important component of chronic disease progression.27–29 It should also enable a more precise definition of changes within the interstitial space that accompany injury and repair, such as visualizing pericyte migration away from endothelium during CKD, which has been proposed to underlie fibrogenesis.19,30,31 Finally, mouse FMA may serve as a useful functional readout for therapeutics targeting vascular survival, such as angiogenic growth factors or drugs.19

BUN levels at 8 weeks after surgery. (C and D) α-Smooth muscle actin staining and quantification revealed induction of interstitial fibrosis only after severe IRI. (Graphs show mean±SEM; *P<0.05; **P<0.01; ***P<0.001, one-way ANOVA with post hoc Bonferroni correction). DAPI, 4′,6-diamidino-2-phenylindole.
Figure 3. High-throughput software–based analysis of fluorescence microangiography reveals reduced capillary number and caliber after severe IRI. (A) FMA after sham surgery and moderate and severe IRI, together with CD31 immunostaining, demonstrates capillary rarefaction in response to severity of injury (arrows indicate capillaries with red CD31+endothelial cells surrounding the green FMA solution; all scale bars are 50 μm). (B and C) Severe IRI results in a significant reduction of the total cortical cross-sectional capillary area per high-power
CONCISE METHODS

All mouse experiments were performed according to the animal experimental guidelines issued by the Animal Care and Use Committee at Harvard University. Wild-type mice were 8- to 10-week-old C57Bl/6J males from Charles River Laboratories (Wilmington, MA). The Ve-Cadherin-Red Rosa reporter mice were created by crossing homozygous VE-Cadherin-Cre (Tg(Cdh5-cre)7Mlia) with homozygous R26-tdTomato (Gt[ROSA]26Sor tm9[Cre-loxP-tdTomato] ) reporter mice (The Jackson Laboratories; stock # 006137 and 007909). IRI was performed as previously described. Briefly, mice were anesthetized with pentobarbital sodium (60 mg/kg body wt intraperitoneally); buprenorphine (0.1 mg/kg body wt intraperitoneally) was used to achieve analgesia; kidneys were exposed through flank incisions; and mice were subjected to ischemia by clamping the renal pedicle with nontraumatic microaneurysm clamps (Roboz, Rockville, MD) for 28 minutes (severe IRI) or 23 minutes (moderate IRI) or no clamping (sham surgery). Reperfusion was visually verified. During the surgery, 1 ml of 0.9% NaCl was subcutaneously administered. Body temperatures were controlled at 36.5°C–37.5°C throughout the procedure. Mice were bled at 24 hours, 14 days, and 8 weeks after the surgery via the tail vein. BUN was measured using the Infinity Urea assay (Thermo Fisher Scientific) according to the manufacturer instructions.

Figure 4. Severity of initial injury determines shift in capillary size and extent of capillary rarefaction. (A) Distribution of capillary size demonstrates a loss of larger capillaries (＞15 μm²) after moderate and severe IRI, with a slight increase in counted small capillaries (＜15 μm²) (data represent three mice per group). (B) FMA assessed total capillary cross-sectional area (total perfused area, μm²/high-power field), individual capillary cross-sectional area (μm²), and capillary number (number/high-power field) shows highly significant correlation with day 1 BUN and lower correlation with week 8 BUN.

FMA

The agarose-fluorescent microbead mixture was made immediately before the procedure by microwaving low-melting-point agarose (Lonza; #50080) 1% by mass in distilled water (4.5 ml dH20+0.05 g agarose/mouse), according to the protocol published by Advani et al. Following complete dilution of the agarose, 0.02 mm FluoSpheres sulfate (Invitrogen; #F8845, yellow-green) were added to the mixture such that they were 10% by volume (i.e., 500 μl FluoSpheres plus 4.5 ml 1% agarose/mouse). Mice were anesthetized with pentobarbital (60 mg/kg of body wt intraperitoneally); buprenorphine (0.1 mg/kg body wt intraperitoneally) was given to achieve analgesia; and mice were placed on a surgical heating pad (37°C). The abdomen and thorax were cut via a midline incision extending from the symphysis pubis to the jugulum. We did not achieve satisfactory results when following the Advani protocol in mice until all solutions were prewarmed to 41°C rather than room to field [×400, inner cortex] (B) and a significant reduction in capillary number (C). (D and E) The cortical individual capillary cross-sectional area (D) (mean±SEM, sham: 30.31±0.42 μm²; moderate IRI: 26.29±0.28 μm²; severe IRI: 24.77±0.35 μm²) and perimeter (E) (sham: 30.19±0.31 μm; moderate IRI: 29.13±0.22 μm; severe IRI: 24.77±0.35 μm) was significa ntly reduced after both moderate and severe IRI. (Of note, data represent n=3 mice in the sham group and severe IRI group and n=6 mice in the moderate IRI group; mean±SEM in B and C; box and whiskers with 10th–90th percentiles in D and E; + indicates mean in D and E. **P<0.01; ***P<0.001, one-way ANOVA with post hoc Bonferroni correction). DAPI, 4’,6-diamidino-2-phenyindole.
body temperature. One milliliter of heparinized saline (100 IU/ml heparin [Sagent Pharmaceuticals] in 0.9% NaCl) followed by 1 mm of 3 M KCl (41°C) was injected in the beating left ventricle using a 27-gauge butterfly catheter (Exel, Corp., #26709). The inferior vena cava was then cut and the mouse was perfused with 41°C prewarmed PBS (10 ml), immediately followed by 5 ml of the agarose-microbead mixture (41°C). (Note: A rapid switch between the different syringes is critical [i.e., PBS → agarose].) Immediately after the perfusion, kidneys, heart, and liver were excised and carefully placed in an ice bucket (surrounded by ice) for 10 minutes. Thereafter, the tissues were fixed in 4% paraformaldehyde on ice for 2 hours, then incubated in 30% sucrose at 4°C overnight and optimum cutting temperature (OCT) embedded (Sakura Finetek). OCT-embedded organs were cryosectioned into 7- to 40-μm sections and mounted on Superfrost slides (Thermo Fisher Scientific) using ProLong Gold Antifade reagent (Invitrogen). Sections were washed in 1× PBS (3×5 minutes), stained with 4′,6-diamidino-2-phenylindole and mounted in ProLong Gold (Life Technologies). For immunofluorescence staining, sections were blocked in 10% normal goat serum (Vector Labs) and incubated with an primary antibody specific for CD31 (1:100; eBioscience; #14–0311), F4/80 (1:200; Abcam, Inc.; #ab6640) or α-smooth muscle actin (1:200; Sigma-Aldrich; #A52457) followed by a Cy5-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories). All images were obtained by confocal microscopy (Nikon C1 Eclipse; Nikon, Melville, NY).

Ten images of each kidney section (×400 magnification) were taken at random (inner cortex), or seven images of each kidney medulla were obtained using the same laser power and gain intensity for all pictures with the Nikon C1 Eclipse confocal microscope. All images were split in RGB channels using ImageJ (National Institutes of Health, Bethesda, MD), and the green channels were saved in grayscale as a PNG file. Pictures were then automatically analyzed using a MATLAB-based script (Supplemental Material, Supplemental Figure 1). The script removes the background noise and creates a binary image of the capillaries. Through an array loop, it sorts out measurements that do not meet the basic requirements for a capillary. For the analysis, the script was written to exclude measured areas smaller than the size of an erythrocyte (i.e., 4.9 μm²) as background noise (i.e., probably no functional capillary) and areas >100 μm² as too large for a capillary (i.e., arteries, arterioles, venules, and glomerular convoluted). The same analysis was run without the array loop to include all data without any cutoff value (Supplemental Figure 5). Quantification of α-smooth muscle actin–positive surface area was performed by taking random cortical pictures (×200; n=5/kidney) of each mouse using the number of stained pixels per total pixels in Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA).

Statistical Analyses

Data are presented as mean±SEM. For multiple group comparisons, ANOVA with post hoc Bonferroni correction was applied. All statistical analyses, including linear regression analyses, were performed using GraphPad Prism software, version 5.0c (GraphPad Software Inc., San Diego, CA). A P value<0.05 was considered to indicate a statistically significant difference.

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REFERENCES


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Supplementary Material:

Fluorescence Microangiography for Quantitative Assessment of Peritubular Capillary Changes after Acute Kidney Injury

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Supplementary figure S1: Our MATLAB script allows automated high-throughput analysis of the microvasculature.

A-C: The channels of the original confocal picture of inner cortex (RGB format, A) were splitted in ImageJ (NIH) and the green channel was saved as a grayscale picture (PNG file, B) this picture will be automatically processed by the MATLAB script which generates a binary image of the capillaries (C). D-E: The MATLAB script automatically generates an Excel sheet with number, area (µm²) and perimeter (µm) of the capillaries and also demonstrates the area and perimeter data as a histogramm (D, E). Note, through an array loop the script can sort out measurements that do not meet the user defined requirements for a capillary. For example, if the measured area is smaller or larger than a certain value (cut-off values in our case <4.9µm² and >100µm²) it will be counted as a false and is sorted as a zero.
Supplementary Figure S2: Fluorescence microangiography of the renal medulla.

A: Fluorescence microangiography (FMA) of the renal medulla after sham, moderate and severe ischemia-reperfusion injury (IRI) demonstrates a dramatic capillary rarefaction after severe IRI. B: B-C: Severe IRI results in a significant reduction of the total cortical cross-sectional capillary area per high power field [hpf /400x, medulla] (B) and a significant reduction of capillary number (C). D-E: The medullary individual capillary cross sectional area (D: (mean±SEM, sham: 31.67±0.49µm²; moderate IRI: 26.47±0.36µm²; severe IRI: 18.97±0.54µm²) and perimeter (E: sham: 31.08±0.34µm; moderate IRI: 26.41±0.252µm; severe IRI: 22.41±0.41µm) was significantly reduced after both moderate and severe IRI. (of note data represents n=3 mice in sham and severe IRI and n=6 mice in moderate IRI; mean with SEM in B and C; box and whiskers with 10 to 90 percentile in D and E, + indicates mean in D and E; *=p<0.05, **=p<0.01;***=p<0.001 one way ANOVA with posthoc Bonferroni) (all scale bars are 50µm)
**Supplementary Figure S3:** Cdh5 is expressed in kidney macrophages.

**A-B:** Immunostaining against the macrophage marker F4/80 in non-injured kidneys of Ve-CadherinCre⁺, R26Tomato⁺ mice (A) and quantification of positive cells revealed that about 15% of macrophages in the kidney also express VE-Cadherin (B).

**C:** Representative picture of a non-injured kidney from a CadherinCre⁺, R26Tomato⁻ mouse stained for the endothelial surface marker CD31.
Supplementary Figure S4: Reduction of perfused vascular area compared to endothelial cell surface area following ischemia reperfusion injury.

**A:** To compare the reduction of the perfused vascular surface area with the reduction of the endothelial cell-surface area following IRI we performed immunostaining for the endothelial-cell surface marker CD31 in kidney sections after application of FMA. The MATLAB based FMA analysis of the perfused vascular area was performed in the same image as the quantification of the CD31 positive surface area (representative picture in A).

**B:** Quantification of the total CD31^+ surface area per high power field (hpf n=7 / kidney) demonstrates a reduction of 23±11% after moderate IRI and a reduction of 53±19% following severe IRI whereas analysis of the FMA^+ vascular luminal area revealed a slightly higher reduction of 35±14% following moderate IRI and 78±2% following severe IRI. (Of note: no
cut-off values where applied because this would have induced an error in the comparison between CD31+ surface area and FMA+ surface area because application of a cut of value would exclude more FMA+ areas then CD31 as for example glomera would be automatically excluded regarding their size >100µm² in the FMA but would be included in the analysis based on their CD31+ surface area.

C: Representative picture of a kidney 8 weeks after moderate IRI demonstrating that some capillaries surrounded by CD31+ endothelial cells do not show a luminal FMA signal (arrows) suggesting that these capillaries lack perfusion.

supplementary figure S5

Supplementary figure S5: High throughput software based analysis of fluorescence microangiography without cut-off values remains significant.

Each picture was automatically analyzed using the MATLAB script without the array loop to include all data points without application of cut-off values demonstrating a significant reduction of the average capillary cross sectional area (A) after moderate or severe ischemia reperfusion injury (IRI) (mean±SEM, moderate IRI: 28.19±0.65µm²; severe IRI: 18.01±0.67 µm²) when compared to the sham group (36.39±1.12µm²). Similar results where obtained when the average individual capillary perimeter (B) was determined without cut off values (mean±SEM, sham: 30.03±0.61µm; moderate IRI: 26.14±0.39µm; severe IRI: 20.92±0.47 µm). (box and whiskers with 10 to 90 percentile, + indicates mean; ***=p<0.001 one way ANOVA with posthoc Bonferroni)
Supplementary figure S6: Correlations between FMA assessed capillary parameters and blood urea nitrogen. FMA assessed total capillary cross sectional area (total perfused area, µm²/ high power field-hpf), average individual capillary cross sectional area (µm²), capillary number (number / hpf), average total capillary perimeter (µm/ hpf) and average individual capillary perimeter (µm/capillary) show highly significant correlation with day 1 blood urea nitrogen (BUN) and to a lower degree with day 14 and week 8 BUN.
**Supplementary Figure S7: Labeling strategies of kidney capillaries**

**A:** Staining of CD31 in the kidney of a VE-Cadherin+^,tdTomato+ mouse shows that the endothelial cell marker CD31 is expressed on the surface of tdTomato+ cells.

**B:** Comparison of capillary demarcation using genetic tagging of endothelial cells via VE-Cadherin (Cdh5) expression and the fluorescence microangiography (FMA) indicating that some endothelial cells might be missed by the genetic strategy alone (arrows). Of note the genetic Ve-Cadherin labeling strategy seems to miss also some endothelial cells of the arteriole (arrowheads).

**C:** Comparison of capillary demarcation using immunostaining of the endothelial cell-surface antigen CD31 and FMA indicating that some capillaries might be missed by CD31 staining (arrows).
**Supplementary Video 1:** Confocal Z-Stack (26.4µm) of renal cortex with fluorescence microangiography (FMA) 8 weeks after sham surgery.

**Supplementary Video 2:** Confocal Z-Stack (26.4µm) of renal cortex with fluorescence microangiography (FMA) 8 weeks after severe ischemia reperfusion injury.
**Fluorescence Microangiography (FMA) detailed protocol:**

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**FMA Protocol** (in our experience the best results are achieved with 2 persons performing this procedure)

1. Prepare the following solutions (for 1 mouse):
   A) 1ml 0.9 % NaCL + 100 IU heparin
   B) 1ml 3M KCl
   C) 10ml 1x PBS
   D) FMA solution: Prepare the solution in a 20ml glass scintillation vial 4.5ml dH2O + 0.05g low melting temperature agarose (Lonza #50080), microwave until the agarose is dissolved add 500µl 0.02µm FluoSpheres sulfate (Invitrogen #F8845, yellow-green)
      ➔ transfer the solutions into a syringe using a 20g needle (2x3ml syringe for solution A+B and 1x 10ml syringe for solution C and 1x 5ml syringe for solution D, place the syringes in a water bath at 41°C until immediately before injection into the mouse (set up the water bath next to the surgical heating pad where you are planning to perform the procedure with the mouse).

2. Anesthetize mouse with pentobarbital (60mg / kg bodyweight) via intraperitoneal injection and additional analgesia with 0.1mg/kg bodyweight of buprenorphine i.p.

3. Place the mouse on a surgical heating pad to remain the bodytemperature at 37°C (to prevent an early gelation of the injected agarose).

4. Cut abdomen and thorax via a midline incision extending from the symphysis pubis to the jugulum.

5. Inject solution A (100 IU/ml heparin, 0.9% NaCl, 41°C) using a 27 gauge butterfly catheter (Exel Corp. #26709) directly into the beating left ventricle of the mouse, keep the needle in the left ventricle and switch the syringe to solution B (3M KCl, 41°C), inject solution B slowly (the perfusion works best with 2 persons, person A can make
sure that the butterfly catheter remains in the left ventricle and person B injects the
different solutions)

6. Cut the vena cava inferior directly proximal of the bifurcation (be careful not to injure
the aorta and to remain the butterfly catheter needle in the left ventricle during this
procedure)

7. Inject solution C (10ml 1x PBS, 41°C) slowly via the butterfly catheter

8. Inject solution D (FMA solution), monitor the perfusion i.e. the green solution should
exit the circulation through the vena cava incision.

9. Remove the kidney (and / or other organs of interest) carefully using tweezers and
scissors and place them directly on ice (make a little hole in the ice bucket for each
organ to make sure that it’s completely surrounded by ice). After 10 min, cut the
kidney carefully into half using a razor blade and transfer it in 4% paraformaldehyde
at 4°C for 2 hours, then 30% sucrose overnight.

10. Embed the tissue in OCT (Sakura Finetek) and store at -80°C, you can now make
cryosections mount them on Superfrost Plus slides (we did 7-40µm) and store them at
-80°C.

11. You can perform a DAPI counterstaining or a immunostaining of your antigens
(optimaly using a Cy5 secondary antibody to prevent bleed trough). For this you
should carefully wash the slides using 1x PBS (3x5min) and then incubate with your
antibodies (follow a standard immunofluorescence protocol) or DAPI (1mg/ml 1:1000
for 5 min). Wash again for 2x5 min (1xPBS) after the DAPI or 3x10min (1xPBS) after
the secondary antibody.

12. Add one drop of the Prolong Gold Antifade reagent directly onto the tissue and add a
coverslip (remove the air bubbles using weak pressure of a pipette tip). Let the
coverslips dry overnight in the dark at room temperature.
13. Seal the coverslips with nail polish and proceed to confocal microscopy (for later quantification using the MATLAB script (below) please make sure that you are using the same laser intensity and gain for all pictures.

**MATLAB script for the automated high-throughput analysis of the microvasculature:**

1.) This script generates all raw data of capillary area (in $\mu$m²) and perimeter (in $\mu$m) without any cut off value (cut off values can be choosen within the excel sheet or by analyzing the pictures with script #2:

```matlab
folder_name = uigetdir; %Prompts user to select folder
filename = uigetfile; %Prompts user to select file to be analyzed
uiimport = (filename); %Imports selected file name
I = imread(filename); %Reads imported file
background = imopen(I,strel('disk', 15)); %Standardizes background and threshold
figure, surf(double(background(1:8:end,1:8:end))),zlim([0 255]);
set(gca,'ydir','reverse');
I2 = I - background; %Removes excess noise
imshow(I2);
level = graythresh(I2);
bw = im2bw(I2, level);
bw = bwareaopen(bw,50); %States capillary area
cc = bwconncomp(bw,4);
cc.NumObjects;
labeled = labelmatrix(cc);
whos labeled;
RGB_label = label2rgb(labeled, @spring, 'c', 'shuffle'); %colors individual capillaries
figure, imshow(RGB_label);
capillarydata = regionprops(cc,'all'); %reads all perimeter data of the capillaries
```
capillary_peri = [capillarydata.Perimeter];
capillary_area = [capillarydata.Area];
[min_perim, idx] = min(capillary_peri);
capillary = false(size(bw));
capillary(cc.PixelIdxList{idx}) = true;

% Converts perimeter data to micrometers
PDataInMicrons = capillary_peri*0.30120';
% Insert conversion factor here in microns per pixel

% Converts Area data to Micrometers
ADataInMicrons = capillary_area*0.0907';
% Insert conversion factor here in microns-squared per pixel-squared

nbins = 50;
figure, hist(ADataInMicrons, nbins) % Generates capillary Area histogram
title('Histogram of Capillary Area Data')
figure, hist(PDataInMicrons, nbins) % Generates capillary Perimeter histogram
title('Histogram of Capillary Perimeter Data')
SA = ADataInMicrons';
SP = PDataInMicrons';
csvwrite('AreaQuant1.csv', SA) % Writes data to area excel sheet
csvwrite('PerimQuant1.csv', SP) % Writes data to perimeter excel sheet

B) This script generates data of capillary area (in $\mu m^2$) and perimeter (in $\mu m$) with a cut off value of individual capillary area (>4.9 $\mu m^2$ and < 100 $\mu m^2$) to automatically exclude arterioles, arteries, veins, venules and glomerular capillary convolutes:

csvwrite('AreaQuant1.csv', SA) % Writes data to area excel sheet
csvwrite('PerimQuant1.csv', SP) % Writes data to perimeter excel sheet

folder_name = uigetdir; % Prompts user to select folder
filename = uigetfile; % Prompts user to select file to be analyzed
uiimport = (filename); %Imports selected file name
I = imread(filename); %Reads imported file
background = imopen(I,strel('disk', 15)); %Standardizes background and threshold
figure, surf(double(background(1:8:end,1:8:end))),zlim([0 255]);
set(gca,'ydir','reverse');
I2 = I - background; %Removes excess noise
imshow(I2);
level = graythresh(I2);
bw = im2bw(I2, level);
bw = bwareaopen(bw,50); %States capillary area
cc = bwconncomp(bw,4);
cc.NumObjects;
labeled = labelmatrix(cc);
whos labeled;
RGB_label = label2rgb(labeled, @spring, 'c', 'shuffle'); %colors individual capillaries with pretty colors
figure, imshow(RGB_label);
capillarydata = regionprops(cc,'all'); %reads all perimeter data of the capillaries
capillary_peri = [capillarydata.Perimeter];
capillary_area = [capillarydata.Area];
[min_perim, idx] = min(capillary_peri);
capillary = false(size(bw));
capillary(cc.PixelIdxList{idx}) = true;
%Converts perimeter data to micrometers
PDataInMicrons =capillary_peri*0.30120';
%Insert conversion factor here in microns per pixel
%Converting Area data to Micrometers

ADataInMicrons = capillary_area * 0.0907;

%Insert conversion factor here in microns-squared per pixel-squared

%Counter variable

n = 1;

%Number of entries in the data set you are looking for

arraysz = length(ADataInMicrons);

%While loop: look at each point, if it is greater than 10, put in into new vector

while n < (arraysz + 1)

if ADataInMicrons(n) > 4.9

if ADataInMicrons(n) < 100

Data1Sorted(n) = ADataInMicrons(n);

end

end

n = n + 1;

end

%Reset your counter

n = 1;

nbins = 50;

figure, hist(Data1Sorted, nbins) %Generates capillary Area histogram

title('Histogram of Capillary Area Data')

figure, hist(PDataInMicrons, nbins) %Generates capillary Perimeter histogram

title('Histogram of Capillary Perimeter Data')

SA = Data1Sorted';

SP = Data2Sorted';

csvwrite('AreaQuant1.csv', SA) %Writes data to area excel sheet
Analysis of the confocal microscopy picture using the MATLAB script (above):

1. The first thing to do is to open each picture in Image J and split the channels, save the green channel (FMA) in grayscale mods a PNG file.

2. Make a central folder (call it MATLAB) to import into MATLAB with the following files: the script called “QuantUnsort”, area.csv file, perimeter.csv file, and all the pictures you are going to analyze (all these files are available as online supplements). [the “QuantUnsort“script will give you data for all measured vessels i.e. including large arteries and veins to get only capillary data use the “QuantFinalR“ script (online supplements) instead of the “QuantUnsort” script, this will exclude all areas smaller than 4.9µm² and larger than 100µm².

3. Open MATLAB

4. Import the folder into MATLAB. You might be prompted to set this folder as the MATLAB “path”. Say yes to this.

5. Type “QuantUnsort” into the command window.

6. A window will pop up and ask you which folder you want to open. Click on the MATLAB folder and press enter. The MATLAB folder may also be pre-selected. If this is the case, just press “open”.

7. Another window will pop up and ask you to choose a file. This will be asking you which image you want to analyze. If you can’t see your file in the window, make sure the file type is set to “All Files”. A common problem is that by default MATLAB will only look for matlab files and will not “see” any .png files. Chose your image and press enter. The script will run and analyze it.

8. A series of 4 images will pop up. The first is the original image, the second is the same image but with different colors assigned to different capillaries. These are there to make sure that the right image was analyzed and to track progress. The next two images are histograms of the area data and perimeter data for the analyzed image. By this point, the data has been exported to the respective AreaQuant1.csv or PerimeterQuant1.csv files.

9. Open up Excel and open the AreaQuant1.csv and/or PerimeterQuant1.csv file. All of the measurements should be in one column. The area is in µm² and the perimeter is
in µm. Copy and paste this into another excel file where you can compile your data. After you do that, make sure you close the area.csv or perimeter.csv files or else when you analyze the next image, MATLAB won’t be able to export the new data to these files.

10. Return to matlab and type “clc” then press enter into the command window. This resets the program and allows you to analyze another image. Make sure you do this after each picture!

11. Re-do steps 1-9 for the rest of the images.