Supplementary Material:

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

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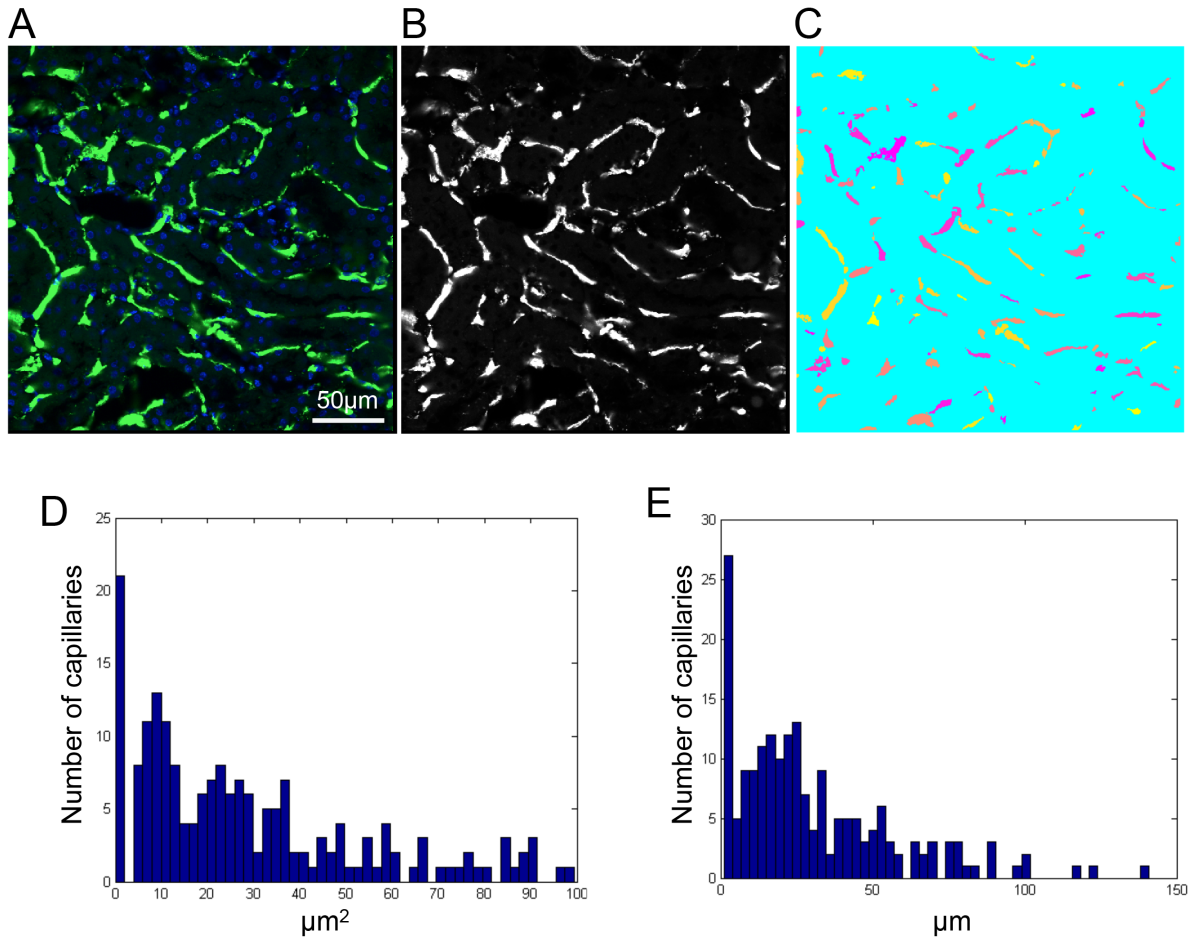
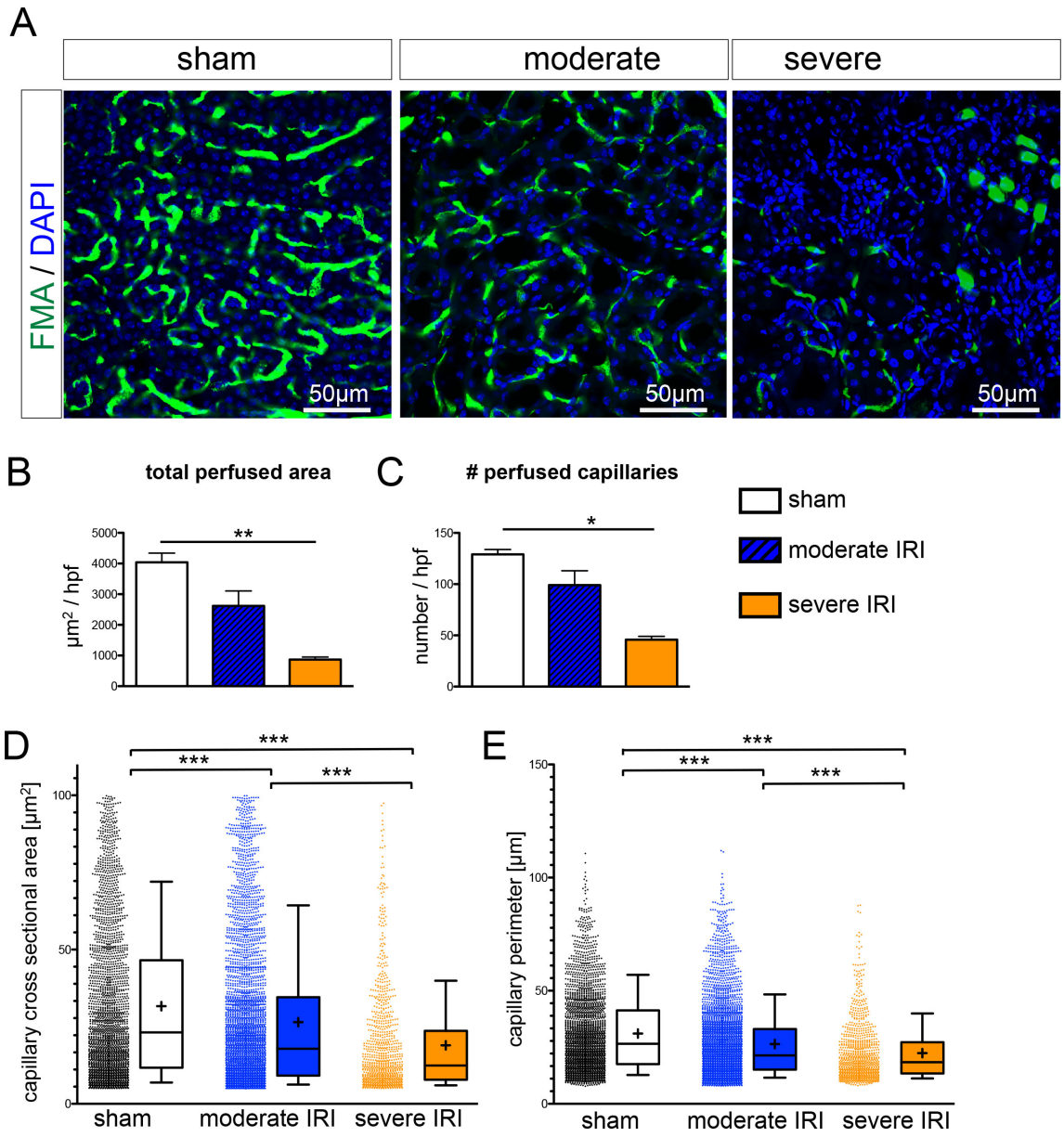
supplementary figure S1



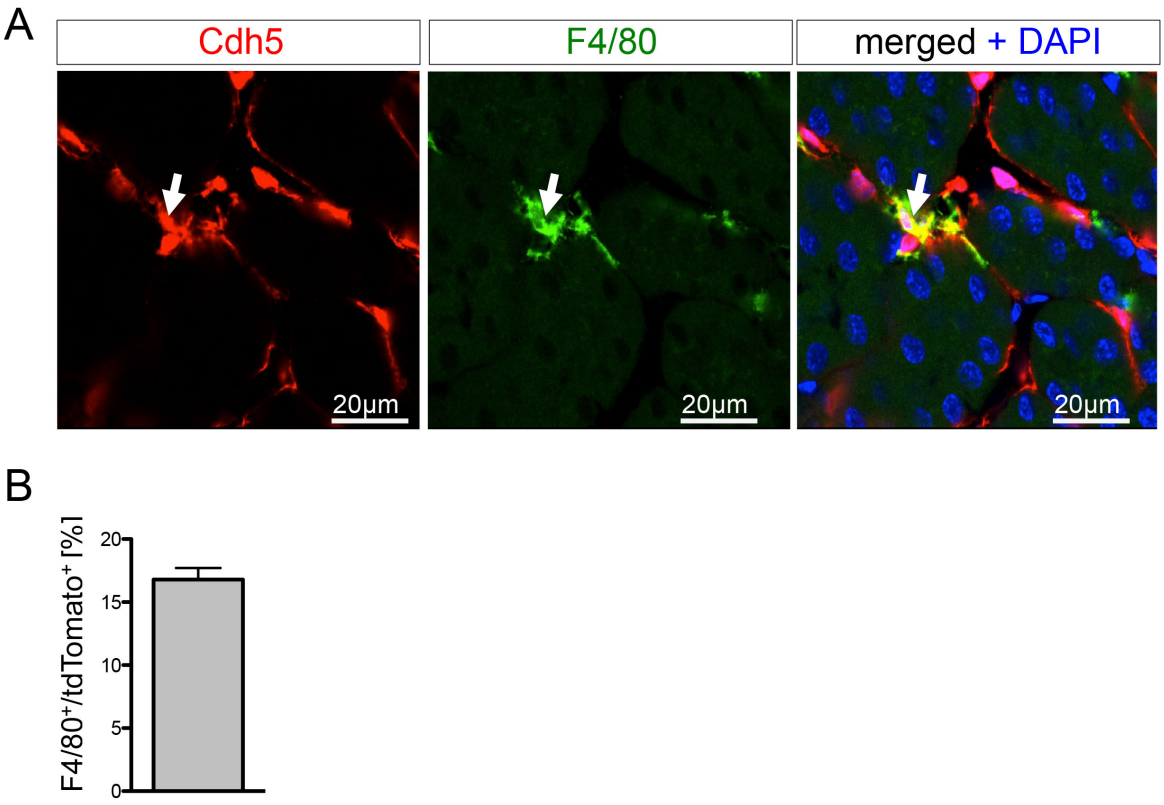
Supplementary figure S1: Our MATLAB script allows automated high-throughput analysis of the microvasculature.
A-C: The channels of the original confocal picture of inner cortex (RGB format, A) were splitted in ImageJ (NIH) and the green channel was saved as a grayscale picture (PNG file, B) this picture 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 (µm^2) and perimeter (µm) of the capillaries and also demonstrates the area and perimeter data as a histogramm (D, E). Note, Trough 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 <4.9µm^2 and >100µm^2) it will be counted as a false and is sorted as a zero.

supplementary figure S2



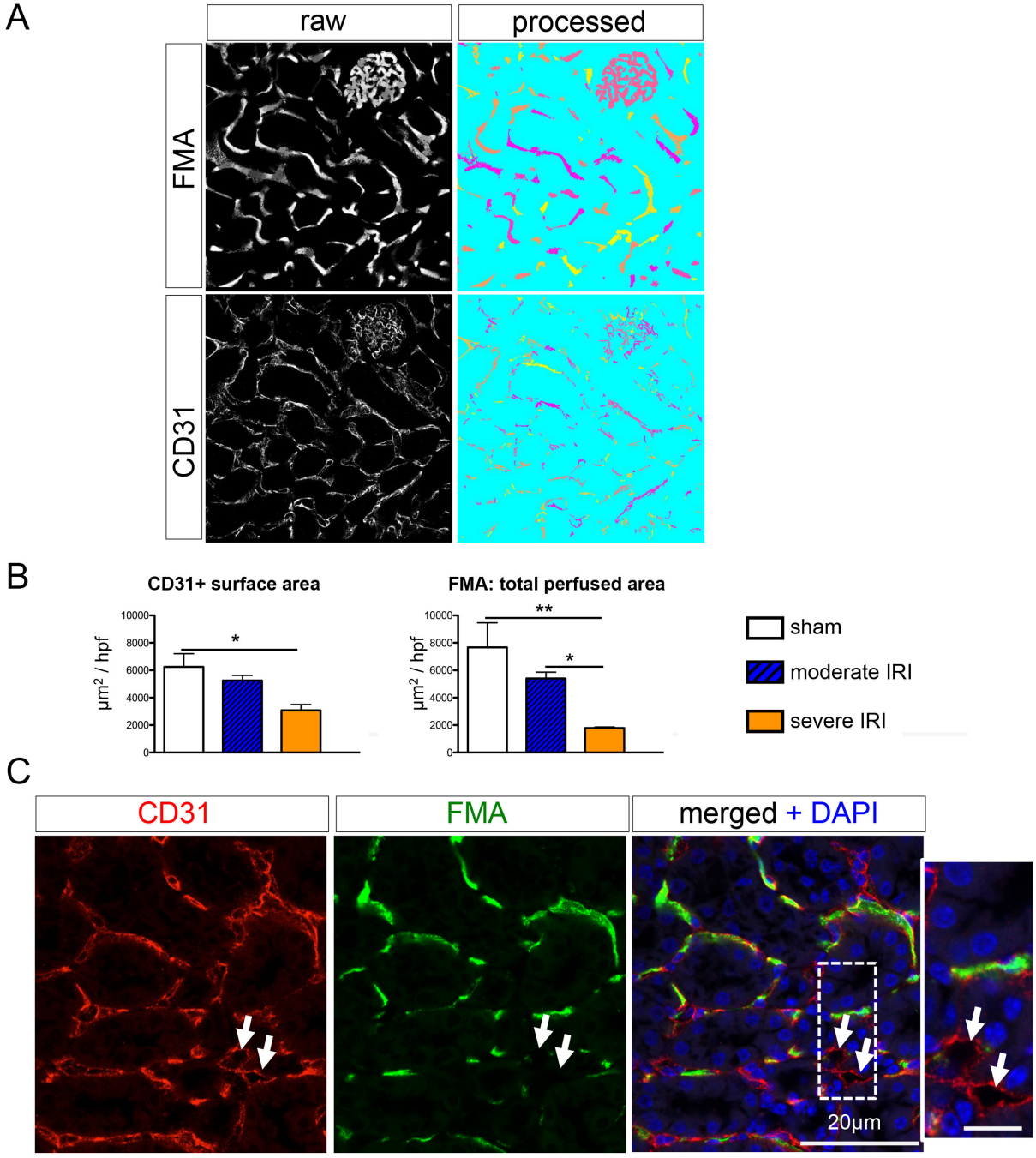
Supplementary Figure S2: Fluorescence microangiography of the renal medulla.
A: Fluorescence microangiography (FMA) of the renal medulla after sham, moderate and severe ischemia-reperfusion injury (IRI) demonstrates a dramatic capillary rarefaction after severe IRI. **B:** B-C: Severe IRI results in 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±0.49µm^2; moderate IRI: 26.47±0.36µm^2; severe IRI: 18.97±0.54µm^2) 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



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



Supplementary Figure S4: Reduction of perfused vascular area compared to endothelial cell surface area following ischemia reperfusion injury.

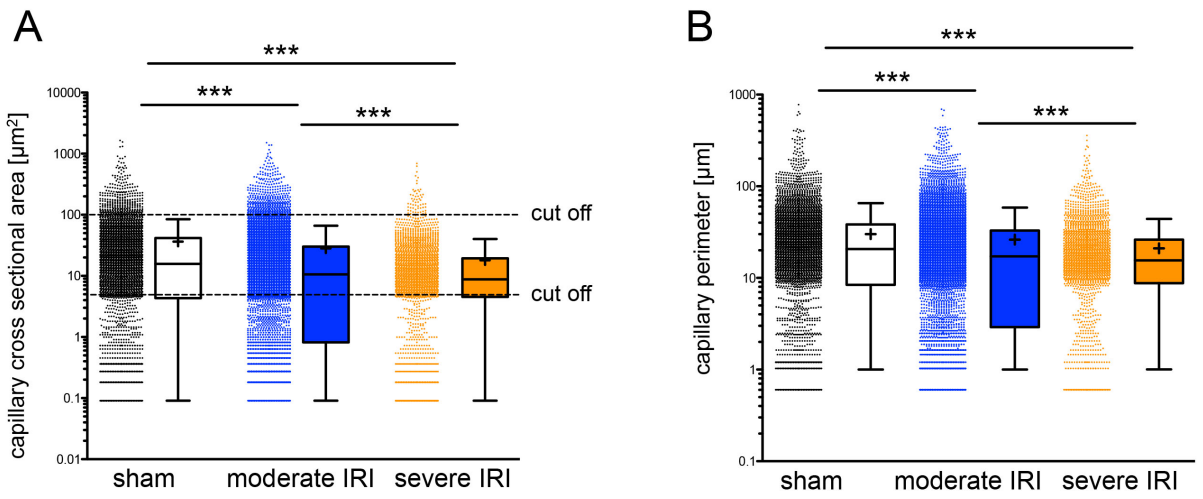
A: To compare the reduction of the perfused vascular surface area with the reduction of the endothelial cell-surface area following IRI we performed immunostaining for the endothelial-cell surface marker CD31 in kidney sections after application of FMA. The MATLAB based FMA analysis of the perfused vascular 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

cutt-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 glomerula would be automatically excluded regarding their size >100µm^2 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 surounded by CD31+ endothelial cells do not show a luminal FMA signal (arrows) suggesting a that these capillaries lack perfusion.

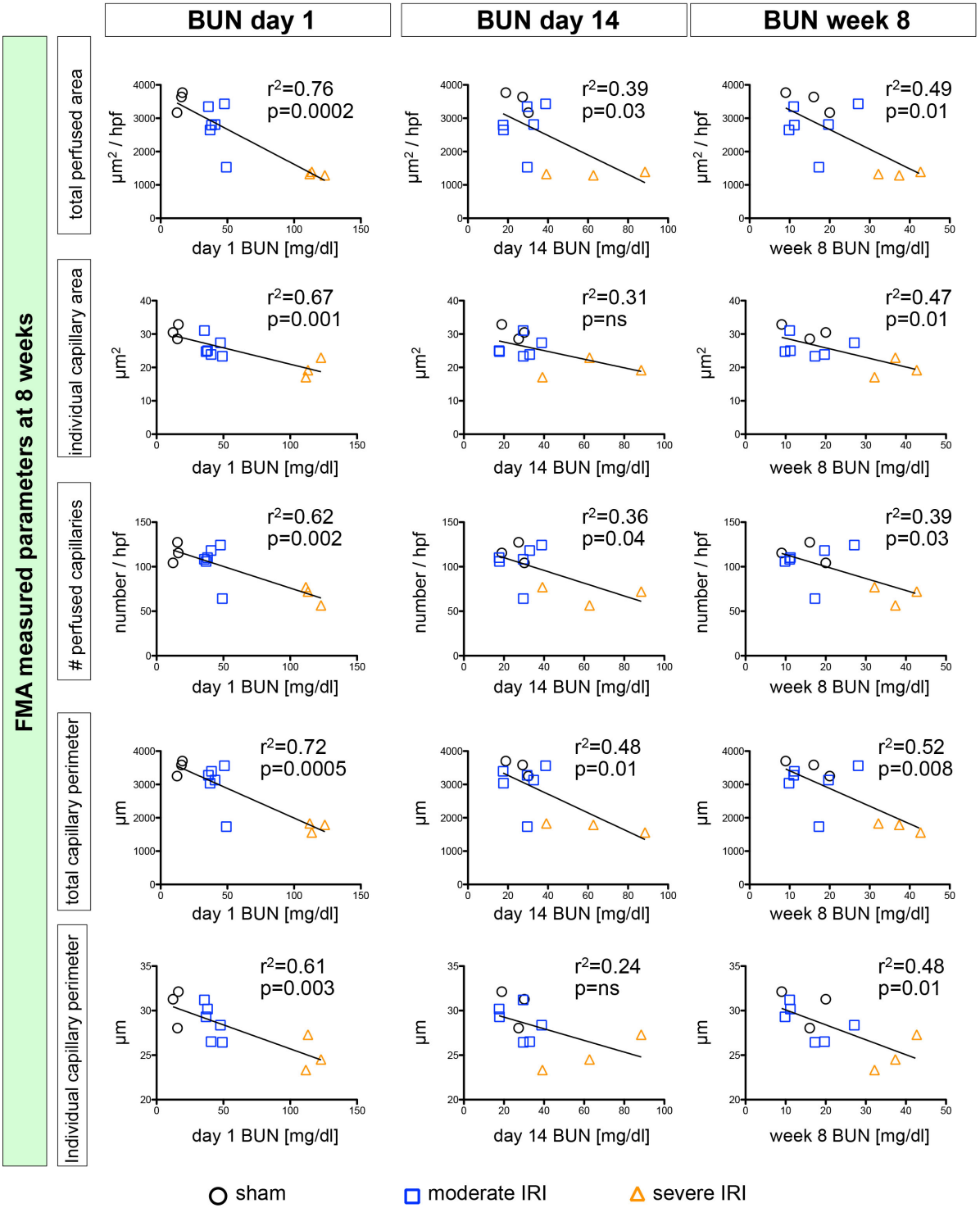
supplementary figure S5



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

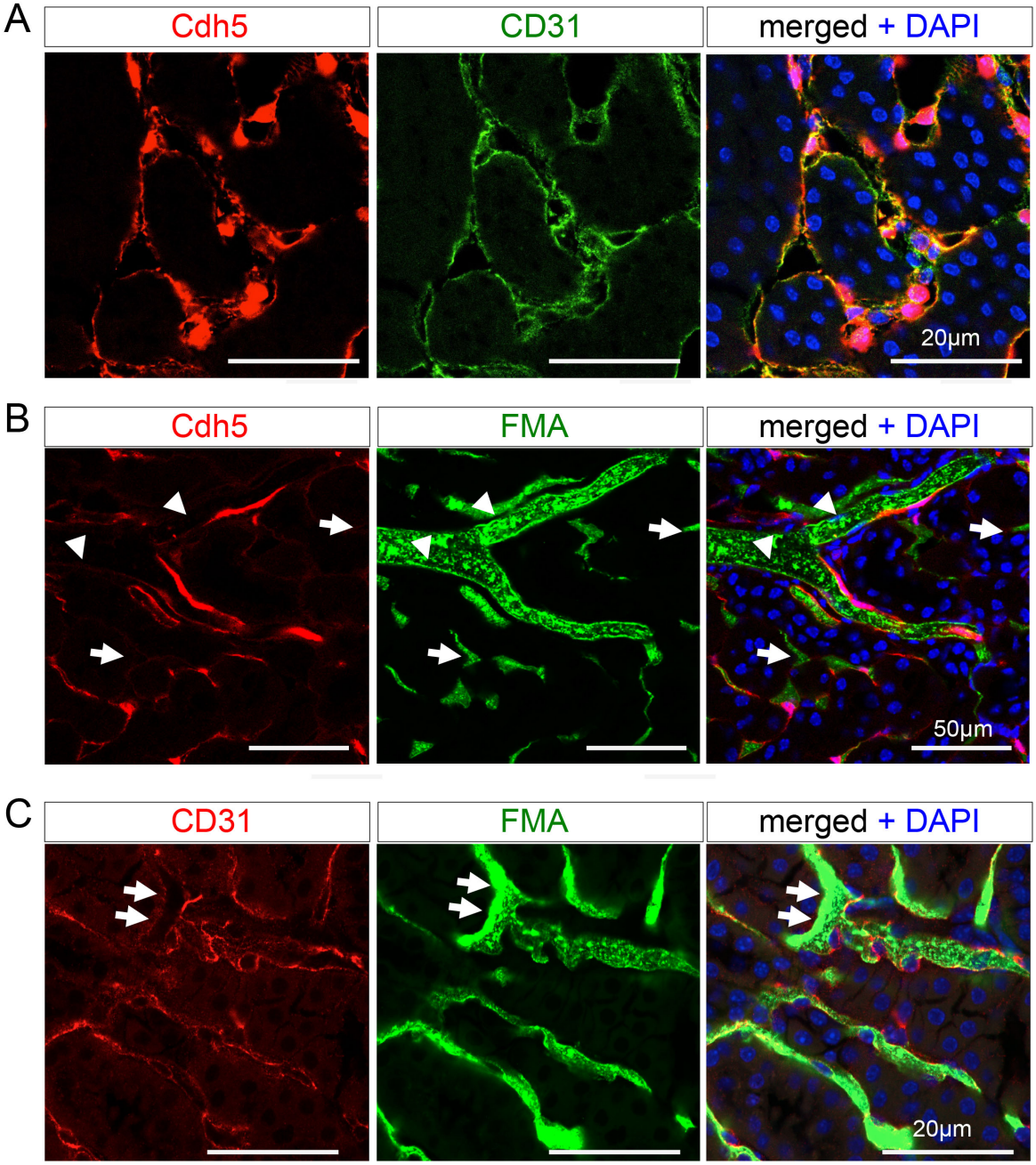
Each picture was automatically analyzed using the MATLAB script without the array loop to include all data points without application of cut-off values demonstrating a significant reduction of the average capillary cross sectional area (A) after moderate or severe ischemia reperfusion injury (IRI) (mean±SEM, moderate IRI: 28.19±0.65µm^2; severe IRI: 18.01±0.67 µm^2) when compared to the sham group (36.39±1.12µm^2). 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



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



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 migroangiography (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:

List of reagents needed:

reagent / tool needed	company / Cat No.	price
low melting temperature agarose	Lonza / #50081	139$
0.02µm FluoSpheres sulfate	Invitrogen / #F8845	264$
sodium chloride 0.9%	Baxter / # 2F7122	4.50$
heparin	Sagent Pharmaceuticals NDC25021-400-01	≈8$/vial
1x PBS (500ml)	Life Technologies / #10010-023	17.62$
3ml syringes	various	
5ml syringes	various	
10ml syringes	various	
20ml Glass Scintillation Vials	Fisher Scientific / #03-337-14	≈0.5$ each
20 gauge needles	various	
27 gauge butterfly catheter	Exel Corp. / #26709	50x / 11.85$
3M KCL	various	
Pentobarbital (Nembutal™)	Oak Pharmaceuticals / NDC #76478-501-20	
Buprenorphine (Buprenex™)	Reckitt Benckiser /NDC #12496-0757-1	
4% paraformaldehyde	various / Electron microscopy sciences / #15713	104$
30% sucrose	various / Fisher Scientific #S5500	500g/100$
OCT	Sakura Finetek / # 4583	72$
cryo embedding molds	Polysciences / #18986	288x/197$
Superfrost Plus Slides	Fisher Scientific / #12-550-15	≈1$/ slide
Prolong Gold Antifade reagent	Life Technologies / # P36930	10ml/ 139$
water bath (42°C)	various	
microwave	various	
surgical heating pad	various	
scissors / tweezers	various	
MATLAB sofware	MathWorks (check for cheaper campus license)	≈500$
Image J	NIH download http://rsbweb.nih.gov/ij/download.html	free
confocal microscope	NikonC1 eclipse / various others	

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. Anesthesize 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 trough 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 surroutweended 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 over night.
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 over night in the dark at roomtemperature.

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 µm^2) and perimeter (in µm) without any cut off value (cut off values can be choosen within the excel sheet or by analyzing the pictures with script #2:

```
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 μm^2) and perimeter (in μm) with a cut off value of individual capillary area (>4.9μm^2 and < 100μm^2) to automatically exclude arterioles, arteries, veins, venules and glomerular capillary convolutes:

```
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
```

```
%Converts 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, it 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
```

```
csvwrite('PerimQuant1.csv', SP) %Writes data to perimeter 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 4.9μm^2 and larger than 100μm^2.
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 μm^2 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 perimter.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.