

Journal of Visualized Experiments

An Optimized Approach to Co-stain the Blood Vessels and Neuronal Fibers in Adipose Tissue

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59266R2
Full Title:	An Optimized Approach to Co-stain the Blood Vessels and Neuronal Fibers in Adipose Tissue
Keywords:	Adipose tissues, Blood vessels, Angiogenesis, Neuronal fibers, Sympathetic innervation, White adipose tissue (WAT), Brown adipose tissue (BAT).
Corresponding Author:	Kai Sun University of Texas Health Science Center at Houston Houston, Texas UNITED STATES
Corresponding Author's Institution:	University of Texas Health Science Center at Houston
Corresponding Author E-Mail:	Kai.Sun@uth.tmc.edu
Order of Authors:	Xin Li Zhengmei Mao Li Yang Kai Sun
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Houston, Texas/USA

TITLE:

Co-staining Blood Vessels and Nerve Fibers in Adipose Tissue

AUTHORS AND AFFILIATIONS:

Xin Li¹, Zhengmei Mao², Li Yang¹, Kai Sun¹

¹Center for Metabolic and Degenerative Diseases, the Brown Foundation of Institute of Molecular Medicine, University of Texas Health Science Center at Houston. Houston, TX, USA

²Microscopy Core Facility Service Center, the Brown Foundation of Institute of Molecular Medicine, University of Texas Health Science Center at Houston. Houston, TX, USA

Corresponding Author:

Kai Sun (kai.sun@uth.tmc.edu)

Email Addresses of Co-authors:

Xin Li (Xin.Li.1@uth.tmc.edu)

Zhengmei Mao (Zhengmei.Mao@uth.tmc.edu)

Li Yang (Li. Yang@uth.tmc.edu)

KEYWORDS:

Adipose tissue, blood vessels, angiogenesis, nerve fibers, sympathetic innervation, white adipose tissue, WAT, brown adipose tissue, BAT

SUMMARY:

New blood vessel formation and sympathetic innervation play pivotal roles in adipose tissue remodeling. However, there remain technical issues in visualizing and quantitatively measuring adipose tissue. Here we present a protocol to successfully label and quantitatively compare the densities of blood vessels and nerve fibers in different adipose tissues.

ABSTRACT:

Recent studies have highlighted the critical role of angiogenesis and sympathetic innervation in adipose tissue remodeling during the development of obesity. Therefore, developing an easy and efficient method to document the dynamic changes in adipose tissue is necessary. Here, we describe a modified immunofluorescent approach that efficiently co-stains blood vessels and nerve fibers in adipose tissues. Compared to traditional and recently developed methods, our approach is relatively easy to follow and more efficient in labeling the blood vessels and nerve fibers with higher densities and less background. Moreover, the higher resolution of the images further allows us to accurately measure the area of the vessels, amount of branching, and length of the fibers by open source software. As a demonstration using our method, we show that brown adipose tissue (BAT) contains higher amounts of blood vessels and nerve fibers compared to white adipose tissue (WAT). We further find that among the WATs, subcutaneous WAT (sWAT) has more blood vessels and nerve fibers compared to epididymal WAT (eWAT). Our method thus provides a useful tool for investigating adipose tissue remodeling.

INTRODUCTION:

Adipose tissue has key metabolic and endocrine functions¹. It dynamically expands or shrinks in response to different nutrient stresses². The active tissue remodeling process consists of multiple physiological paths/steps including angiogenesis, fibrosis, and shaping of local inflammatory microenvironments²⁻⁴. Some physical stimuli, such as cold exposure and exercise, may trigger sympathetic activation, which ultimately leads to new blood vessel formation and sympathetic innervation in adipose tissues^{5,6}. These remodeling processes are linked tightly to systemic metabolic outcomes including insulin sensitivity, the hallmark of type 2 diabetes². Thus, visualization of these pathological changes is very important to understanding the healthy status of whole adipose tissues.

Angiogenesis is the process of new blood vessel formation. Since blood vessels provide oxygen, nutrients, hormones, and growth factors to tissue, angiogenesis has been considered a key step in adipose tissue remodeling, which has been documented with different techniques⁶⁻¹³. However, there remain questions about the resolution of the images, efficiency of immunostaining, and methods for quantification of vessel density. Compared to new blood vessel formation, innervation in adipose tissue has been underestimated for a long time. Recently, Zeng et al.¹⁴ used advanced intravital two-photon microscopy and demonstrated that adipocytes are surrounded by layers of nerve fibers¹⁴. Since then, researchers have started to appreciate the pivotal role of sympathetic innervation in regulation of adipose tissue physiology. Thus, developing an easy and practical approach to document adipose nerve innervation is important.

Here, we report an optimized method for the co-staining of blood vessels and nerve fibers based on our previous protocols. With this method, we can achieve clear images of blood vessels and nerve fibers without noisy background. Moreover, we obtain a resolution that is high enough for performing quantitative measurement of densities with open source software. By using this new approach, we can successfully compare the structures and densities of blood vessels and nerve fibers in different adipose depots.

PROTOCOL:

All procedures containing animal subjects have been approved by the Animal Welfare Committee of University of Texas Health Science Center at Houston (animal protocol number: AWC-18-0057).

1 Reagent preparation

1.1 1x phosphate-buffered saline (PBS, pH 7.4): to make 1 L of 1x PBS, dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ in 800 mL of distilled water. Adjust the pH to 7.4 and fill with distilled water to achieve a final volume of 1 L.

1.2 1% paraformaldehyde in 1x PBS (1% PFA, wt/vol): to achieve a final volume of 50 mL, add 3.125 mL of 16% PFA (see **Table of Materials**) to 46.875 mL of 1x PBS. Mix well and store at 4 °C for later use.

CAUTION: Paraformaldehyde is toxic. All procedures should be carried out under a fume hood to avoid inhalation and skin contact.

1.3 0.1% polysorbate 20 (see **Table of Materials**) in 1x PBS (1x PBST, pH 7.4, vol/vol): to achieve a final volume of 50 mL, add 0.05 mL of polysorbate 20 to 49.95 mL of 1x PBS. Vortex and store at 4 °C for up to 1 month.

1.4 1% octoxynol-9 (see **Table of Materials**) in 1x PBS (1x PBS-TX, pH 7.4, vol/vol): to achieve a final volume of 10 mL, add 0.1 mL of octoxynol-9 to 9.9 mL of 1x PBS. Vortex and store at 4 °C for up to 1 month.

1.5 2% sodium azide (wt/vol): to produce 10 mL of 2% sodium azide, add 0.2 g of sodium azide to 10 mL of 1x PBS. Mix well and store at 4 °C for later use.

1.6 90% glycerol in 1x PBS (vol/vol): to achieve a final volume of 10 mL, add 1 mL of 1x PBS to 9 mL of glycerol. Vortex and store at room temperature (RT) for later use.

2 Animal dissection and adipose tissue collection

2.1 Use 6-week-old C57BL/6J male mice. Anesthetize the mice with isoflurane (4%–5% for induction then 1%–2% for maintenance). Once anesthetized, perform a toe-pinch reflex to determine the anesthetic depth, judging by the lack of pedal reflexes. Once the mice are deeply anesthetized, dissect the mice following the protocol as previously published¹⁵.

2.2 Sterilize the blunt scissors and surgical area of the chest (no need to shave the hair). Open the chest by cutting the diaphragm and ribs along the lateral surface with a size of ~2 cm using blunt scissors. Clamp the chest flag with hemostat and reflect it by putting the hemostat over the head.

2.3 Make a small incision into the left ventricle (~0.5 cm) with iris scissors. Insert an olive-tipped perfusion needle through the incision site and clamp the needle tip in place with the hemostat. Attach the needle to a 50 mL syringe containing paraformaldehyde fixation solution (1% PFA, pH 7.4).

2.4 Open the right atrium by section cutting with iris scissors as the perfusion outlet. Start the perfusion by gently and continuously pushing the syringe at a perfusion rate close to 10 mL/min. Stop pushing when the fluid exiting the outlet is clear of any blood. The whole process should require ~5 min.

CAUTION: Perform these steps under a fume hood to prevent toxicity from PFA.

2.5 Dissect white and brown adipose tissues from the mice. Use scissors to break the tissue samples into small pieces of chunks approximately 5 mm x 5 mm x 3 mm. Transfer the small

chunks with the sterilized tweezers into the tissue embedding cassettes with proper labeling of the tissue samples on the cassettes.

2.6 Immerse the embedding cassettes containing tissue samples into the fixation solution (1% PFA in 1x PBS, pH 7.4) at 4 °C for 24–48 h.

NOTE: The fixation solution and fixation time vary based on sizes of the tissues¹⁶⁻¹⁸.

2.7 Wash the samples with fresh 1x PBS buffer three times (0.5 mL/wash).

CAUTION: Perform this step under a fume hood. The paraformaldehyde waste should be properly handled according to hazardous waste disposal procedures.

NOTE: The tissues can be stored in 1x PBS at 4 °C for further processing.

3 Antibody incubation

3.1 Cut the fixed tissue samples into approximately 2 mm³ cubes with the sterilized scissors. For permeabilization, transfer the samples into a 1.5 mL tube containing 1 mL of 1x PBS-TX (1% octoxynol-9 in 1x PBS, see **Table for Materials**, pH 7.4) and gently rotate the tubes at RT for 1 h at 18 rpm.

3.2 Carefully remove the 1x PBS-TX by aspiration. Wash the samples 3x by adding the 1x PBS directly into the same tubes (no need to change the tubes). During each wash, invert the tubes several times.

3.3 For blocking, add 0.5 mL of blocking buffer (**Table of Materials**) to the samples and incubate at RT for 2 h with gentle rotation.

3.4 To prepare 0.2 mL of primary antibody solution, dilute 1 µL of anti - endomucin (the marker of blood vessels)⁵ (1:200) and 1 µL of anti - tyrosine hydroxylase (TH, the marker of nerve fibers)⁵ (1:200, 1 µg/mL) antibodies (**Table of Materials** for antibody information) into 198 µL of blocking buffer. Vortex and spin down to recover the volume.

3.5 For the first antibody incubation, carefully remove the blocking buffer from the tissue chunks. Add 100 µL of primary antibody solution prepared in step 3.4 into tubes and incubate at 4 °C overnight.

NOTE: The antibody dilution and incubation time may vary among different antibodies. It is recommended to add 0.02% sodium azide to the antibody solution to prevent microbial growth. To prepare 0.2 mL of primary antibody solution with sodium azide, add 2 µL of antibodies (1 µL of each) and 2 µL of 2% sodium azide into 196 µL of blocking buffer.

3.6 Carefully collect the primary antibody solution for reuse if desired. Wash the samples with 1x PBST (pH 7.4, 100 μ L/wash) three times (30 min each) with gentle rotation at 18 rpm.

3.7 For the preparation of secondary antibody solution, dilute 1 μ L of fluorophore (wave length 495 nm) conjugated anti-goat IgG (1:200) and 1 μ L of fluorophore (wave length 650 nm) conjugated anti-rabbit IgG (1:200) (see **Table of Materials**) into 198 μ L of blocking buffer. Vortex and spin briefly to collect all the liquid.

NOTE: Protect the samples from light during the following procedures.

3.8 For the second antibody incubation, remove the last wash buffer from samples. Add 50 μ L of secondary antibody solution into tubes and incubate at RT for 2 h with gentle rotation at 18 rpm.

3.9 Carefully remove the secondary antibody solution. Wash the samples 3x with 1x PBST (pH 7.4, 30 min each) with gentle rotation at 18 rpm.

NOTE: Do not reuse this secondary antibody solution, as it may be contaminated with the trace amounts of primary antibodies brought by the tissues.

3.10 For optical clearance, immerse the samples in 1 mL of 90% glycerol and keep the samples at 4 $^{\circ}$ C in darkness until they become transparent.

NOTE: The immersion duration depends on the tissue type and size. Typically, a 2 mm³ cube of white adipose tissue needs overnight incubation, while the same size brown adipose tissue sample needs longer.

3.11 Adhere a silicone isolator to the slide to create a well for volume imaging. Alternatively, attach several layers of transparent tape to the slides and cut a square out of the middle to create a well (**Figure 2**).

NOTE: Adjust the well depth to fit the sample size. In this protocol, a 1 mm well depth is used.

3.12 Carefully transfer the samples into the well and fill it with the mounting medium (see **Table of Materials**). Lay a cover slip on the surface and seal the corners of the cover slip with high viscosity medium (see **Table of Materials**). Let the mounting medium cure for 24 h at RT in darkness.

NOTE: Ensure that no space is left between the sample and cover slip. Avoid bubbles under the cover slip. Avoid placing excessive pressure on the samples.

4 Image acquisition

4.1 Acquire Z-stack images (refer to steps 4.2–4.7) with the 20x objective of a confocal microscope/software.

4.2 Start the system. Select the **Configuration** tab and activate the argon laser and He Ne 633 laser. Select the **Acquire** tab followed by **Visible** to choose the laser line of 488 nm and 647 nm. Activate the PMTs by selecting the **Active** button, then choosing PMT1 for 488 nm and PMT2 for 647 nm.

4.3 Select **Live** to view an image of the samples. Use the z-Position knob to select a plane of focus within the XY region of interest. Adjust the brightness of the images by tuning the laser intensity, smart gain, and offset. For each experiment, set up the intensity by adjusting the smart gain and offset under the “QLUT” (quick look-up table) model.

NOTE: The numerical values of the setting may vary among each experiment.

4.4 While scanning, turn the Z-Position knob counterclockwise to move the scanner to the top of the region of interest, then select the **end** arrowhead to set the scanning end position. Turn the Z-Position knob clockwise to move the scanner through the specimen to the bottom of the region of interest, then select the **begin** arrowhead to set the scanning begin position.

NOTE: For the comparison between different samples, define the same Z-volume for different samples.

4.5 Adjust the z-step size to 3 μm . Change the image quality by selecting a format of 1024 x 1024, speed of 100 Hz, and line average of 2.

4.6 Select **Start** to initiate the z-stack image acquisition.

4.7 For reconstruction of the 3D images, select the **Process** tab then **Tool**, then select **3D Projection** and enter **Maximum** in the method list without changes to X, Y, and Z. Select **Apply**. The maximum intensity of the Z-volume will be stacked into a 2D image, which will be shown (**Figure 4A**).

5 Analysis of blood vessel and nerve fiber networks

5.1 Analysis of 2D images *via* open source software (see **Table of Materials**)¹⁹

5.1.1 Open the stacked images in the software. Adjust **Vessel diameter and intensity** until all the vessel structures can be properly selected (**Figure. 4B**).

5.1.2 Select **Run analysis** and export the data following guidance of the software.

5.2 Analysis of 3D images *via* the licensed software (see **Table of Materials**)

5.2.1 Open the raw data of images with the software. Adjust the threshold and other parameters until the vessel structures in each layer of the z-volume are properly selected (refer to the user guide for details in the software) (**Figure 4C**).

5.2.2 Analyze the selected segments characters and export the data following the guidance of the software.

REPRESENTATIVE RESULTS:

The distal region of the epididymal white adipose tissue (eWAT), medial region of the dorsolumbar subcutaneous white adipose tissue (sWAT), and medial region of the interscapular brown adipose tissue (BAT) were collected. The locations for collecting these tissues are indicated in **Figure 1**.

(insert **Figure 1** here)

After whole-mount staining, the tissue chunks were mounted in a well of 1 mm depth (**Figure 2**) and imaged with the confocal microscope. We first tested the effect of the clearing step with 90% glycerol incubation on quality of the images. We found that more blood vessels were positively stained with α -endomucin antibody in the glycerol-incubated sWAT, suggesting that the clearing step is critical for complete staining of the blood vessels (**Figure 3**, compare panels A and B).

(insert **Figure 2** here)

(insert **Figure 3** here)

However, the clearing step with glycerol did not affect the integrity or shape of the blood vessels (**Figure 3**). Given these beneficial effects, in the following experiments, the clearing step was performed.

We further compared the results of 2D and 3D analyses using different software (see **Table of Materials**). We found that, while the 3D images apparently provided more detailed structural information, the two analytic methods eventually did not show significant differences in terms of the length and branch number of the vessels (**Figure 4**).

(insert **Figure 4** here).

Previous publications have demonstrated that different adipose depots possess heterogeneous properties^{1,18}. Here we sought to determine whether blood vessels and nerve fibers exhibit different patterns among the depots using our newly developed method. To achieve this, we performed co-IF staining with α -endomucin (for blood vessels) and α -TH (for nerve fibers) antibodies in adipose tissue. Interestingly, results showed that there were significantly more blood vessels and nerve fibers in BAT compared to WATs (**Figure 5**, compare bottom lanes to top and middle lanes)

(insert **Figure 5** here)

Among the WATs, the sWAT exhibited higher blood vessel density than the eWAT (**Figure 5**, compare the middle to top lane). Of note, while the nerve fibers expanded in parallel with the blood vessels, they did not show significant co-localization (**Figure 5**, merged lanes). We further quantitatively measured the vessel area, number of junctions, and tube length with the 2D method and found similar results as described above (**Figure 6**).

(insert **Figure 6** here)

In summary, this approach successfully co-stained blood vessels and nerve fibers in different adipose tissues.

FIGURE LEGENDS:

Figure 1: Anatomy of subcutaneous white adipose tissue (sWAT), epididymal white adipose tissue (eWAT), and brown adipose tissue (BAT) indicates regions used for collecting the samples. (A) The regions outlined by white dashed lines represent sWAT, and the white asterisk highlighted location is the site for collecting sWAT. Regions outlined by black dashed lines are eWAT, and the black asterisk highlighted location is the site for collecting eWAT. **(B)** The regions outlined by black dashed lines are BAT, and the tissue collection region is highlighted by the asterisk.

Figure 2: Diagrams of the wells for volume imaging. (A) Diagram of a slide with a silicone isolator. **(B)** Diagram of a well with multiple layers of tape made by our lab.

Figure 3: Comparison of IF images of blood vessels acquired with or without optical clearing step with 90% glycerol. (A) Whole-mount immunofluorescence (IF) staining with anti-endomucin antibody (green) in sWAT. The sample was not subjected to the optical clearing step (step 3.10). **(B)** Whole-mount IF staining with anti-endomucin antibody in sWAT. The sample was subjected to the optical clearing step (step 3.10) before the mounting steps (steps 3.11 and 3.12).

Figure 4: Comparison of 2D and 3D analyses on vessel structure. (A) The effect of maximum projection on the images. **(B)** The analytic results by the 2D software (see **Table of Materials**). Particularly, the red lines indicate selected skeleton, while the blue dots indicate branching points. **(C)** The analytic results by the 3D software (see **Table of Materials**). The gray area is the selected region for analysis. The green line indicates the measured segments and green dots indicate the measured nodes. **(D)** The comparison of vessel length, segment numbers, branching node numbers, and terminal node numbers analyzed by 2D or 3D methods.

Figure 5: Comparison of blood vessels and nerve fibers acquired from different adipose depots. Whole-mount IF staining with anti-endomucin (green) and anti-tyrosine hydroxylase (TH) (red) antibodies in eWAT (**A**, **B**, merged in **C**), sWAT (**D**, **E**, merged in **F**) and BAT (**G**, **H**, merged in **I**).

Figure 6: Quantitative analysis of vascular and nerve fiber networks. (A) The percentage of blood vessel area in the samples of eWAT, sWAT, and BAT. (B) The number of junctions of the vascular networks in eWAT, sWAT, and BAT. (C) The total vessel length of vascular networks in eWAT, sWAT, and BAT. (D) The percentage of nerve fiber area in the samples of eWAT, sWAT and BAT. (E) The number of junctions of the nerve fibers in eWAT, sWAT, and BAT. (F) The total length of nerve fibers in eWAT, sWAT, and BAT. The analyses were performed with 2D software. The results were achieved from the images presented in **Figure 5**.

DISCUSSION:

Adipose tissue remodeling is directly linked to metabolic dysregulation during obesity development^{1,2}. Angiogenesis and sympathetic innervation are both essential for the dynamic remodeling process^{2,12}. Therefore, developing an applicable approach to visualize the new blood vessels as well as nerve fibers are of great importance. Previous methods have been reported for documenting angiogenesis in adipose tissue. However, some issues remain with these approaches, including low efficiency, fussy resolution, and noisy background. Meanwhile, sympathetic innervation has only been recognized recently as a critical step in adipose tissue pathology¹⁴. Even though the related findings are interesting, applied methods require advanced microscopy tools and are time-consuming. Here, we report an easy-to-follow approach modified from our previous protocol for co-staining blood vessels and sympathetic nerve fibers. Interestingly, we successfully stained the blood vessels and nerve fibers with high resolution, and the staining has qualities comparable to previously reported images¹⁸.

We previously stained the blood vessels in adipose tissues by immunohistochemistry or immunofluorescence with the α -CD31 antibody, a marker of endothelial cells, on paraffin-embedded slides^{8,13,20}. While the method allows us to distinguish blood vessel density between different groups, the microvascular vessels that were positively stained were not complete. Here, we chose a whole-mount method and performed IF staining with a α -endomucin antibody, another marker for blood vessels. With this optimized approach, we were able to achieve staining with more blood vessels, especially microblood vessels. Moreover, in this method, since we avoided steps such as paraffin embedding and formalin fixation, which have the potential to impair the integrity of blood vessels, we obtained images with higher resolution and more intact vessels. The unimpaired structure of the vessels further allowed us to measure and compare their lengths, branching, and areas. Of note, we added a clearing step with glycerol, so the tissue chunks could become more transparent, and this simple but critical step significantly enhanced efficiency of the staining^{18,21}.

Due to high levels of lipid contents in the adipose tissue, which might limit the accessibility of antibodies to the inner regions of the tissue, we cut the depots into small pieces to ensure adequate antibody binding to the target proteins. Therefore, our method successfully stains more blood vessels and nerve fibers than staining on whole tissues. However, it may lose spatial information and hence affect the integrity of the nerve fibers. To resolve this apparent issue and ensure the images are comparable among individual mice, it is suggested to collect the small chunks from the same regions in the adipose depots. If more spatial information is further needed, larger chunks should be obtained for staining. For larger samples, longer fixation times

with higher doses of FPA, higher concentrations of detergents for permeabilization, and longer periods of antibody incubation may be needed.

Adipose tissue innervation has been investigated recently. Multiple advanced techniques have been applied to visualize nerve fibers^{14,17}. These methods are either expensive or time-consuming. Here, we simply performed an IF stain with α -TH (a sympathetic nerve marker) and successfully stained the sympathetic nerve fibers at a high quality. More importantly, we performed the co-staining with α -endomucin and α -TH to investigate how the blood vessels interplay with sympathetic nerve fibers during adipose tissue remodeling.

Of note, we compared the results from 2D and 3D analyses using different software. It was found that, while the 3D images provided more detailed structural information, the two analytic methods did not show significant difference in terms of the length and branching of the vessels. Eventually, when analyzing with the 3D method, the software itself may detect some false signals that need to be manually adjusted. Given that the 2D software is purposely designed for vessel analysis, it is thus suggested to use this method for quantitatively measuring the structure of vessels.

With this method, blood vessels and nerve fibers in different adipose tissue were co-stained, and their densities, branching, and lengths were compared¹⁹. It was found that both blood vessels and nerve fibers are much thicker in BAT compared to WAT, suggesting that BAT is a more metabolically active adipose depot. Moreover, the blood vessel and nerve densities in sWAT were higher than in the eWAT, indicating that different WATs have different remodeling profiles.

In conclusion, this method efficiently co-stains blood vessels and nerve fibers in adipose tissue⁵. Furthermore, it serves as a useful tool for studying the dynamic changes in adipose tissue during obesity development.

ACKNOWLEDGMENTS:

This study was supported by the National Institute of Health (NIH) grant R01DK109001 (to K.S.).

DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

A



B

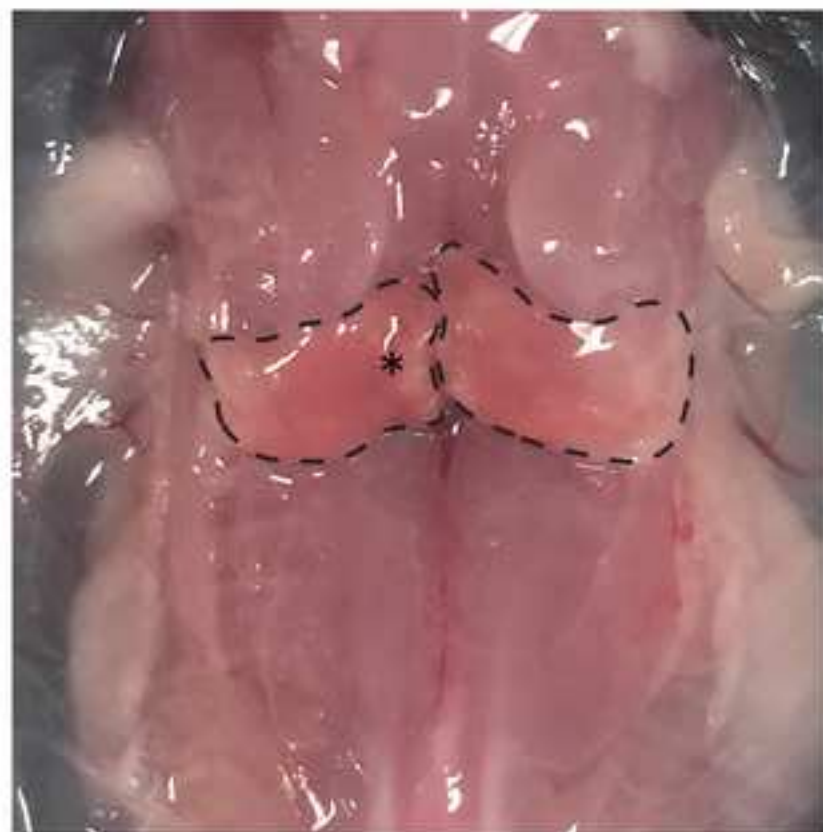


Figure 2



Figure 3

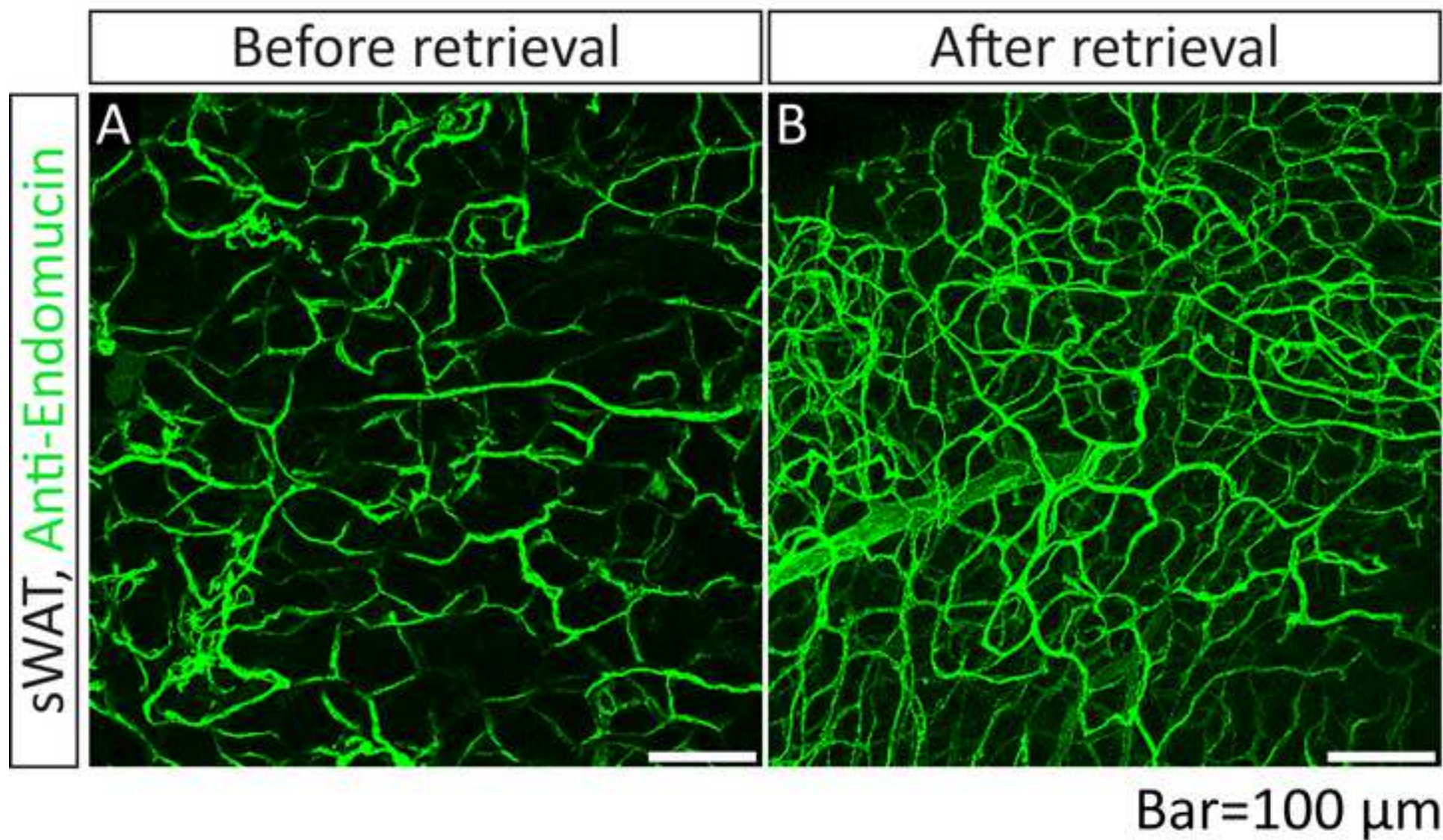
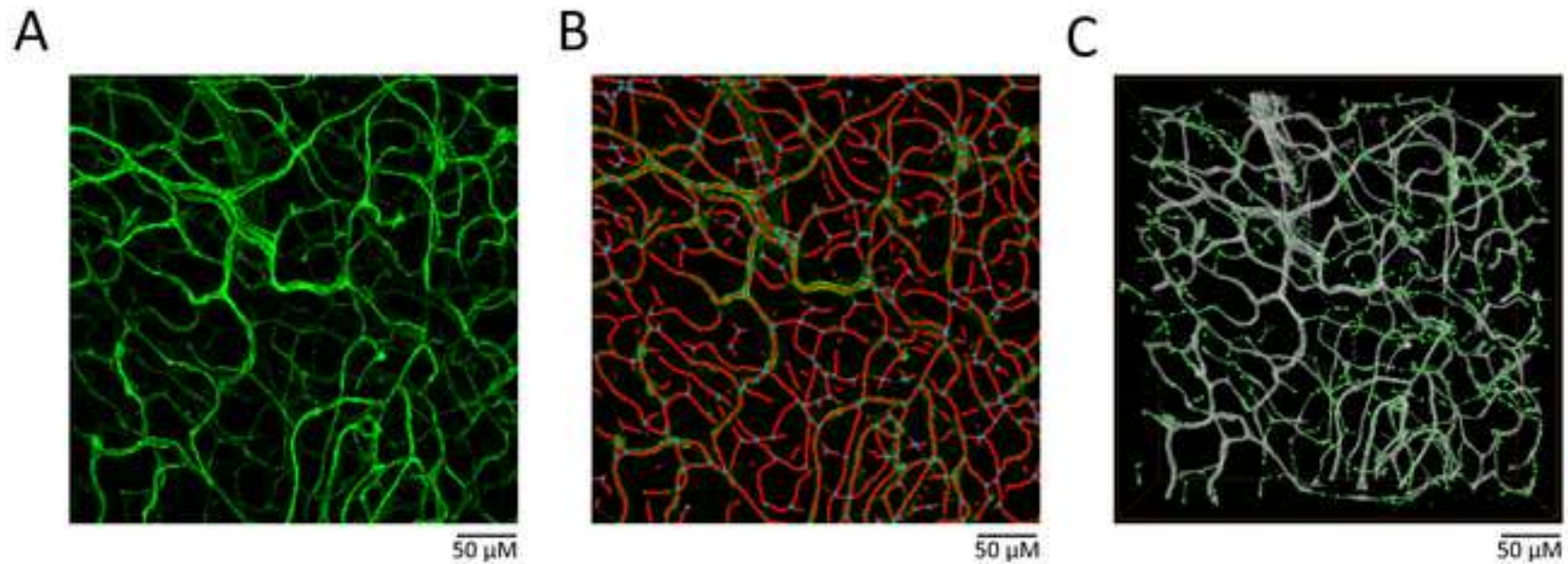


Figure 4

**D**

2-D software		3-D software	
Total Length (μ M)	10810.98	Total Length (μ M)	18211.94
Average Length (μ M)	80.08	Mean Length (μ M)	22.51
		Number of Segments	809
Total Number of Junctions	300	Branching Nodes	384
Total Number of End Points	457	Terminal Nodes	257

Figure 5

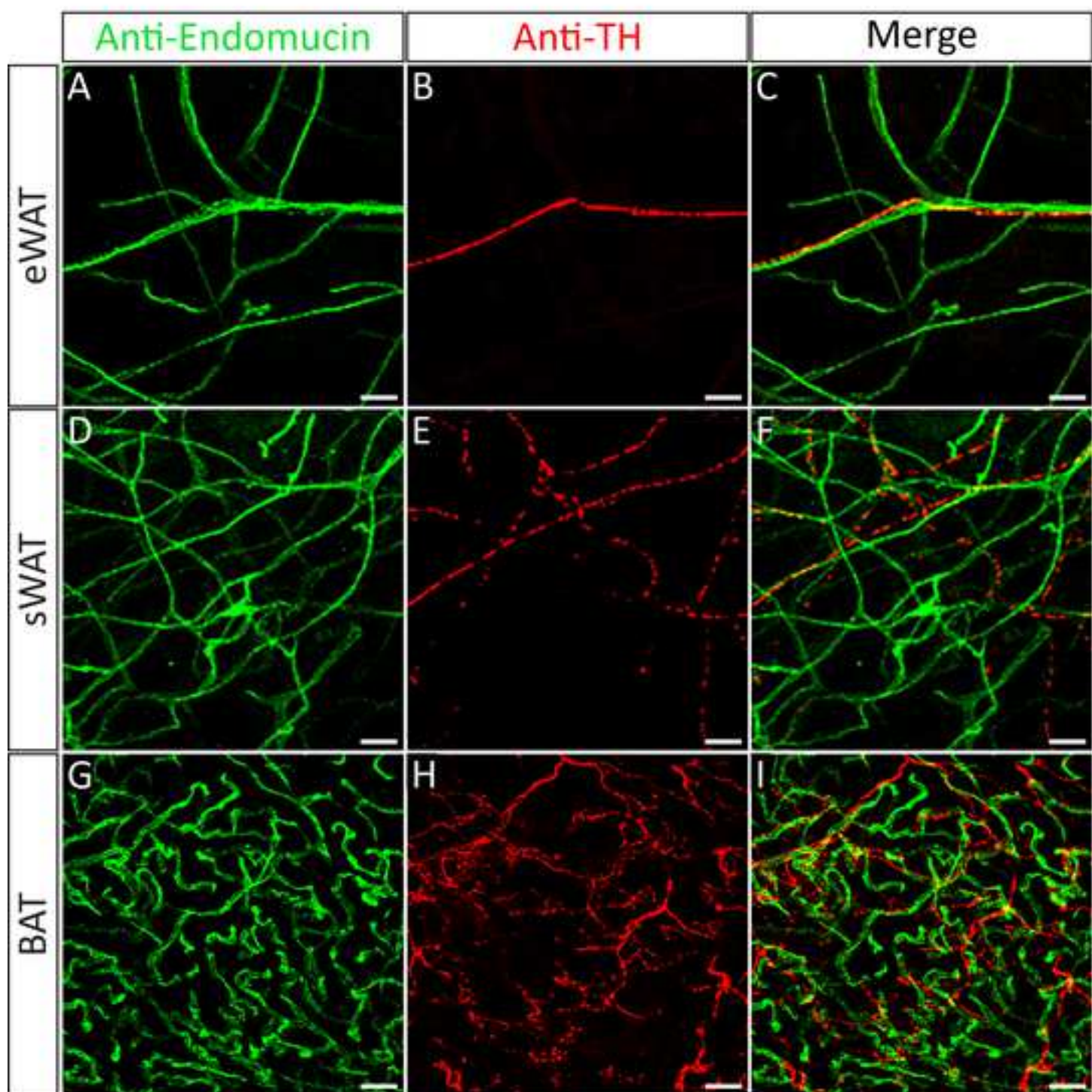
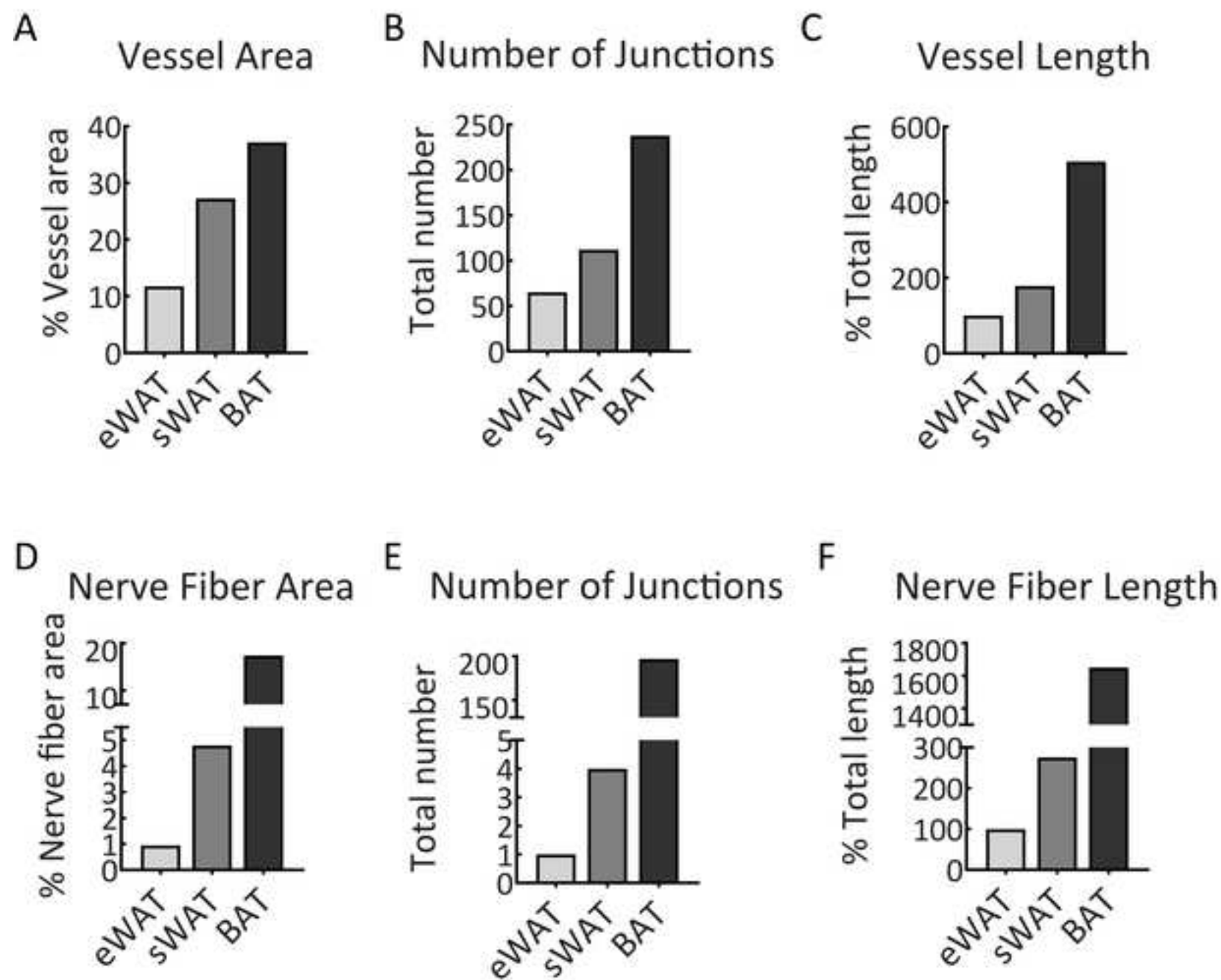
Bar=25 μ m

Figure 6



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Alexa Fluor 488 AffiniPure Bovine Anti-Goat IgG (H+L)	Jackson ImmunoResearch	805-545-180	Lot: 116969
Alexa Fluor 647 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	711-605-152	Lot: 121944
Amira 6.0	Thermo Fisher Scientific		Licensed software
Angio tool	National Institutes of Health		Open source software https://ccrod.cancer.gov/confluence/display/ROB2/Home
Anti-mouse endomucin antibody	R&D research system	AF4666	Lot: CAAS0115101
Anti-tyrosine hydroxylase antibody	Pel Freez Biologicals	P40101-150	Lot: aj01215y
Cover glasses high performance, D=0.17mm	Zeiss	474030-9020-000	
Cytoseal 280	Thermo Fisher Scientific	8311-4	High-viscosity medium
Glycerol	Fisher	G33-500	
Paraformaldehyde,16%	TED PELLA	170215	
Press-to-Seal Silicone Isolator with Adhesive, eight wells, 9 mm diameter, 1.0 mm deep	INVITROGEN	P24744	Silicone isolator
ProLong Diamond Antifade Mountant	Thermo Fisher Scientific	P36965	Mounting medium
SEA BLOCK Blocking Buffer	Thermo Fisher Scientific	37527X3	
Sodium azide	Sigma-Aldrich	S2002-100G	
Tissue Path IV Tissue Cassettes	Thermo Fisher Scientific	22-272416	
Triton X-100	Sigma-Aldrich	X100	Generic term: octoxynol-9
Tube rotator and rotisseries	VWR	10136-084	
Tween-20	Sigma-Aldrich	P1379	Generic term: Polysorbate 20



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

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Author(s):

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and Neuronal Fibers in Adipose Tissue
Xin Li, zhengmei Mao, Li Yang, KAI SUN

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CORRESPONDING AUTHOR

Name:

KAI SUN

Department:

Institute of Molecular Medicine

Institution:

University of Texas Health Science Center at Houston

Title:

Assistant professor

Signature:

Kai Sun

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Rebuttal Letter

Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to ..."

Here we report an optimized approach by which we have been able to successfully label and quantitatively compare the densities of blood vessels as well as nerve fibers in different adipose tissues.

Expand?

expand

Please remove this phrase "On the other hand"

It's already removed.

Need a one liner about what angiogenesis is and how it is a key step.

Angiogenesis is a process of new blood vessel formation. Since blood vessels provide oxygen, nutrients, hormones as well as growth factors to the tissues, angiogenesis has been considered to be a key step during adipose tissue remodeling.

Please use imperative tense and complete sentence throughout. Use 6week old C57BL/6 male mice.

Use 6 week old C57BL/6J male mice

Amount and concentration.

the mice with isoflurane (4 - 5% for induction then 1 – 2% for maintenance).

What do you look for? Please include – and look for the lack of pedal reflexes.

perform a toe-pinch reflex to determine the anesthetic depth judging by the lack of pedal reflexes.

Do you sterilize the surgical area before starting the surgery? Do you shave the hair? Do you use vet ointment for the eyes? Please provide every specific detail of performing your experiment. How big is the cut?

Sterilize the blunt scissors and the surgical area of the chest (no need to shave the hair). Open the chest by cutting diaphragm and ribs along the lateral surface with the size of ~ 2 cm using the blunt scissors. Clamp the chest flag with hemostat and reflect it by putting the hemostat over the head.

Commented [A1]: Amount and concentration.

Commented [A2]: What do you look for? Please include – and look for the lack of pedal reflexes.

Commented [A3]: Do you sterilize the surgical area before starting the surgery? Do you shave the hair? Do you use vet ointment for the eyes? Please provide every specific detail of performing your experiment. How big is the cut?

How small?

Make a small incision into the left ventricle (~0.5 cm) with iris scissors.

Commented [A4]: How small?

Open how? Please provide details.

Open the right atrium by section-cutting with iris scissors as the perfusion outlet.

Commented [A5]: Open how? Please provide details.

Transfer how?

Transfer the small chunks by the sterilized tweezers

Commented [A6]: Transfer how?

How is done,? Do you use OCT? What is meant by proper labeling?

into tissue embedding cassettes with proper labeling of the tissue samples on the cassettes.

Commented [A7]: How is this done? Do you use OCT ?

Commented [A8]: What is meant by proper labeling?

We cannot have paragraphs of text in the protocol section. Please consider moving some parts to the discussion.

The "Note a" following Step 2.5 is now moved to representative results.

"The distal region of the epididymal white adipose tissue (eWAT), the medial region of dorsolumbar - subcutaneous white adipose tissue (sWAT) as well as the medial region of interscapular brown adipose tissue (BAT) were collected. The locations for collecting these tissues are indicated in Figure 1."

The "Note b" following step 2.5 is now removed.

What volume?

1X PBS buffer three times (0.5 mL/wash).

Commented [A9]: What volume?

We cannot have paragraphs of text in the protocol section. Please consider making this an action step and/or a one liner note.

Note: The tissues can be stored in 1 X PBS at 4 °C for further process.

Cut how?

Cut the fixed tissue samples into approximately 2 mm³ cubes with the sterilized scissors.

Commented [A10]: Cut how?

Remove how by aspiration? Wash how? Do you add PBS directly to the same tube? Do you use different tube?

Carefully remove the 1x PBS-TX by aspiration. Wash the samples three times by adding the 1XPBS directly into the same tubes (no need to change the tubes). During each wash, invert the tubes several times.

Commented [A11]: Remove how by aspiration? Wash how? Do you add PBS directly to the same tube? Do you use different tube?

maker or marker? Needs citation

*(the marker of blood vessels)*⁵

Maker or marker please check. Needs citation here.

*the marker of nerve fibers*⁵

Is this correct? Please check. Not sure if 50 microlitres will cover the whole tissue?

Add 100 μ L of primary antibody solution prepared in step 3.4 into tubes

Volume?

Wash the samples with 1 X PBST (pH 7.4, 100 μ L/wash)

Please do not use commercial term in the manuscript and use generic term instead.

fluorophore (wave length 495 nm) conjugated anti-goat IgG (1:200) and 1 μ L of fluorophore (wave length 650 nm) conjugated anti-rabbit IgG (1:200) (see the Table of Materials)

Please consider making this an action step and rewrite in imperative tense. "Pause point: The mounted slides can be stored at 4 °C in darkness for about 2 weeks."

Now it is removed.

How do you acquire the image?

Acquire Z-stack images (refer to 4.2 to 4.7).

Please do not use commercial terms in the manuscript. Please use generic term instead.

confocal microscope/software.

How is this done?

Among the WATs, the sWAT exhibited more blood vessel density than the eWAT (Figure. 5, compare the middle to the top lane).

???

Changed to "collecting the samples".

Please remove the commercial term from the table in Figure 4.

Commented [A12]: maker or marker? Needs citation

Commented [A13]: Maker or marker please check. Needs citation here.

Commented [A14]: Is this correct? Please check. Not sure if 50 microlitres will cover the whole tissue?

Commented [A15]: Volume?

Commented [A16]: Please do not use commercial term in the manuscript and use generic term instead.

Commented [A17]: Please do not use commercial terms in the manuscript. Please use generic term instead.

Commented [A18]: How is this done?

Done!

