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Visualization of 3D white adipose tissue structure using whole-mount staining technique

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Re: JoVE manuscript submission

Dear Dr. Troyer,

We would like to submit our manuscript entitled 'Visualization of 3D white adipose tissue structure using whole-mount staining technique' into Journal of Visualized Experiments (JoVE). Please see below for some information about our manuscript.

Title	Visualization of 3D white adipose tissue structure using whole-mount staining technique
Author contributions	Y.J and J.L-H.Y: designed and performed experiments, demonstrate experimental procedures, wrote manuscript. J.-H.L:designed project, performed experiments, and wrote manuscript. P.-E.S: performed tissue clearing experiment. J.-R.K: supported and designed this project. H.-K.S: conceived and designed project, performed experiments, and wrote manuscript
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Adipose tissue is an important metabolic organ with high plasticity in response to environmental stimuli and nutrient status. Therefore, many advances have been made in the field of adipose biology during the last decade. Although various techniques have been developed in adipose tissue research, the conventional methods have some limitations in visualizing intact architecture and cellular component of adipose tissue. Hence, numerous studies attempt to develop a new method for tissue processing and tissue visualization.

Here, we present a new technique for adipose processing and immunostaining. This includes whole-mount adipose tissue staining and visualization by advanced microscopic imaging. We are confident that our protocol provides a method to study the

structures of the adipocytes and other cellular components without tissue distortion, achieving the most representative 3D visualization of the tissue. This protocol will contribute to further understanding of the development of adipose biology.

Thank you for your positive consideration.

Sincerely yours,

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TITLE:

Visualization of 3D White Adipose Tissue Structure Using Whole-Mount Staining

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KEYWORDS:

White adipose tissue, whole-mount staining, adipose visualization, immunolabeling, tissue clearing, immunolabeling-enabled three-dimensional imaging of solvent-cleared organs (iDISCO+)

SUMMARY:

The focus of the present study is to demonstrate the whole-mount immunostaining and visualization technique as an ideal method for 3D imaging of adipose tissue architecture and cellular component.

ABSTRACT:

Adipose tissue is an important metabolic organ with high plasticity and is responsive to environmental stimuli and nutrient status. As such, various techniques have been developed to study the morphology and biology of adipose tissue. However, conventional visualization methods are limited to studying the tissue in 2D sections, failing to capture the 3D architecture of the whole organ. Here we present whole-mount staining, an immunohistochemistry method that preserves intact adipose tissue morphology with minimal processing steps. Hence, the structures of adipocytes and other cellular components are maintained without distortion, achieving the most representative 3D visualization of the tissue. In addition, whole-mount staining can be combined with lineage tracing methods to determine cell fate decisions. However, this technique has some limitations to providing accurate information regarding deeper parts of adipose tissue. To overcome this limitation, whole-mount staining can be further combined with tissue clearing techniques to remove the opaqueness of tissue and allow for complete visualization of entire adipose tissue anatomy using light-sheet fluorescent microscopy. Therefore, a higher resolution and more accurate representation of adipose tissue structures can be captured with the combination of these techniques.

INTRODUCTION:

Adipose tissue is an essential organ for energy storage and is characterized by dynamic remodelling and nearly unlimited expansion[1]. In addition to energy homeostasis, adipose tissue also plays an essential role in hormone secretion of over 50 adipokines to modulate whole-body metabolic function[2]. Adipose tissue has a diverse architecture comprising of various cell types including mature adipocytes, fibroblasts, endothelial cells, immune cells, and adipocyte progenitor cells[3]. Recent studies have shown that obesity and other metabolic dysfunction can significantly alter adipose tissue function and its microenvironment, which includes but is not limited to enlargement of adipocytes, infiltration of inflammatory cells (*e.g.,* macrophages), and vascular dysfunction[3].

Conventional morphological techniques such as histology and cryosectioning demonstrate several limitations in studying adipose biology such as lengthy chemical processing steps, which can lead to tissue shrinkage and structure distortion[3,4]. Furthermore, these 2D techniques are insufficient to observe intercellular interactions exerted by different cell types, as the sections obtained are limited to smaller regions of the entire tissue[3]. Compared to conventional methods of fluorescent imaging, whole-mount staining does not require additional invasive steps, such as embedding, sectioning, and dehydration; thus, this avoids the problem of diminishing antibody specificity. As such, it is a simple and efficient method for imaging adipose tissue, with better preservation of adipocyte morphology and overall adipose tissue structure[5]. Therefore, whole-mount staining as a quick and inexpensive immunolabeling technique was established to preserve adipose tissue 3D architecture[1,6-8].

However, despite the preservation of adipose tissue morphology with use of whole-mount staining, this technique is still unable to visualize inner structures beneath the lipid surface of the tissue. Several recent studies[9,10] have established tissue clearing techniques combined with whole-mount immunolabeling[1,6] to allow for comprehensive 3D visualization within adipose tissue. In particular, dense neural and vasculature networks were visualized in recent studies[9-12]

with 3D volume imaging. Indeed, studying the neural and vascular plasticity of adipose tissue under different physiological conditions is essential to study its biology. Immunolabeling-enabled three-dimensional imaging of solvent-cleared organs (iDISCO+) tissue clearing is a process comprised of methanol pre-treatment, immunolabeling, and clearing of tissue opaqueness with organic chemical reagents dichloromethane (DCM) and dibenzyl ether (DBE)[13,14]. By making the adipose tissue entirely transparent, a more accurate representation of anatomy within the tissue such as blood vessels and neural fibers can be obtained[9,10]. IDISCO+ has advantages in that it is compatible with various antibodies and fluorescent reporters[11,14], and it has demonstrated success in multiple organs and even embryoes[14]. However, its main limitation is a long incubation time, in which 18 to 20 days are needed to complete the entire experiment.

Another important application of whole-mount staining is the visualization of cell fate in combination with a lineage tracing system. Lineage tracing is the labelling of a specific gene/marker in a cell that can be passed on to all daughter cells and is conserved over time[15]. As such, it is a powerful tool that can be used to determine the fate of a cell's progeny[15]. Since the 1990s, the Cre-LoxP recombinant system has become a powerful approach for lineage tracing in living organisms[15]. When a mouse line that expresses Cre, a DNA recombinase enzyme, is crossed with another mouse line expressing a reporter that is adjacent to a loxP-STOP-loxP sequence, the reporter protein is expressed[15].

For whole-mount staining, the use of fluorescent multicolor reporters is suitable for imaging of adipose tissue because it allows for minimal interference with intracellular activities of the adipocyte[16]. However, traditional reporters typically stain the cytoplasm, making it difficult to trace the lineage of white adipocytes, which have limited cytoplasmic content[17]. To overcome this problem, the use of membrane-bound fluorescent tdTomato/membrane eGFP (mT/mG) reporter marker is an ideal tool. Membrane-targeted tdTomato is expressed in Cre-negative cells[18]. Upon Cre excision, a switch to the expression of membrane-targeted eGFP occurs, making this reporter suitable for tracing the lineage of adipocyte progenitors[17,18] (**Supplementary Figure 1**).

The purpose of this paper is to provide a detailed protocol for whole-mount staining and show how it can be combined with other techniques to study the development and physiology of adipose tissue. Two examples of applications described in this protocol are its use with 1) multicolor reporter mouse lines to identify various origins of adipocytes and 2) tissue clearing to further visualize the neural arborization in white adipose tissue (WAT).

PROTOCOL:

All experimental animal protocols were approved by the Animal Care Committee of The Center for Phenogenomics (TCP) conformed to the standards of the Canadian Council on Animal Care. Mice were maintained on 12-hour light/dark cycles and provided with free access to water and food. 7-month old C57BL/6J male mice were used in the whole-mount staining experiment.

Note: Sections 1 to 2 are in chronological order, with section 3 being an optional step right after section 1. Section 4 can be performed to analyze adipocyte size and blood vessel density after the completion of section 2.

1. Materials Preparation and Tissue Isolation

1.1. Prepare fresh 1% paraformaldehyde (PFA) diluted in 1x phosphate buffered saline (PBS) for tissue fixation. Prepare 0.3% nonionic surfactant diluted in 1x PBS (hereafter referred to as PBS-0.3T) for subsequent tissue washing steps.

Note: For each tissue, prepare approximately 1.5 mL of 1% PFA to ensure complete immersion. Fixation volume may be increased or decreased depending on the tissue size.

CAUTION: This step is hazardous, as PFA is corrosive and toxic. Wear personal protective equipment (*e.g.,* nitrile gloves, lab coat, footwear, safety goggles) and handle in a fume hood.

1.2. Euthanize animals according to an approved procedure (*e.g.*, cervical dislocation and/or carbon dioxide asphyxiation). Without delay, dissect the desired adipose tissue depots (*e.g.*, inguinal white adipose tissue or perigonadal white adipose tissue)[18].

1.3. With dissection scissors, cut the tissue on a 100 x 15 mm Petri dish into pieces approximately 0.5-1 cm in size, and immerse them in microcentrifuge tubes filled with 1% PFA. Keep on ice.

2. Whole-Mount Staining of White Adipose Tissue

2.1. After dissection is complete, move the tissue samples in 1% PFA from the ice to room temperature (RT) for 1 hour, and then transfer the tissues to a 12- or 24-well cell culture plate for quicker washing.

2.2. Wash the tissues with PBS-0.3T, 3 times for 5 minutes each in RT on a shaker tilted at 22 degrees, with 20-25 tilts per minute as the speed.

Note: Use this tilt and speed for all subsequent steps involving the use of a shaker.

Note: If using a multicolor reporter mouse line such as mT/mG and additional antibody staining is not needed, the tissue is ready for microscopy after step 2.2.

2.3. Add 0.5-1 mL blocking buffer (5% animal serum diluted in PBS-0.3T). Put the plate on the shaker and incubate for 1 hour in RT.

2.4. Aspirate the blocking solution and add primary antibodies diluted in PBS-0.3T with 1% animal serum.

2.5. Place the plate on a shaker at 4 °C overnight.

2.6. The next day, wash the tissues with PBS-0.3T 3 times for 5 minutes each in RT.

2.7. Use appropriate secondary antibodies diluted in PBS-0.3T. Add 0.5-1 mL secondary antibody solutions to each well. Wrap the plate in aluminum foil and incubate it on a shaker for 1 hour at RT.

Note: Dilute the secondary antibody in the dark to prevent photobleaching.

2.8. After secondary antibody incubation, wash with PBS-0.3T twice for 5 minutes, each in RT. Image the samples if a neutral lipid stain is not desired. If visualization of lipid droplets is needed using the neutral lipid stain, wash with 1x PBS (without non-ionic surfactant) twice, for 5 min each.

2.9. After the washing steps, incubate with the neutral lipid stain diluted with 1:1500 dilution factor in 1x PBS for 30 min in RT. The tissues are now ready for microscopy. For future imaging, the tissues can be stored in this solution in 4 °C.

Note: Imaging quality decreases over time; hence, the best time for imaging is within 1 or 2 days.

2.10. Using forceps, lay the tissue flat on a 24 x 60mm glass coverslip and place it on an inverted confocal laser microscope system.

2.11. If staining of the nuclei with DAPI is desired, add 1-2 drops of mounting medium containing DAPI to completely submerge the tissue and prevent it from drying.

2.12. To obtain images of whole-mount stained tissues at multiple focal planes, perform Z-stacks of 100-150 µm in depth with 4-6 µm step-size at the desired magnification.

3. **Tissue Clearing and Immunolabeling Using iDISCO+**

Note: This protocol is based on previously published procedures[9,10,19].

3.1. Fixation and methanol pre-treatment

3.1.1. Incubate the tissues in 4% PFA diluted in 1x PBS at 4°C overnight in 2 mL microcentrifuge tubes.

Note: Leave the tissues in the 2 mL tubes for all the following treatments until imaging.

3.1.2. The next day, wash the tissues in 1x PBS three times, for 1 hour each on a shaker at RT.

Note: This step can be a pausing point to leave the sample overnight at RT or 4°C.

3.1.3. Dehydrate the tissues at RT in 20%, 40%, 60%, and 80% methanol, subsequently, for 1 hour

each. Dehydrate in 100% methanol at RT for 1 hour, then transfer the tissues to fresh 100% methanol and incubate at 4°C for 1 hour.

Note: Dilute methanol in distilled water. During methanol incubation, there is no need to put samples on a shaker as long as the tissue samples are immersed.

CAUTION: This step is hazardous, as methanol is toxic. It is highly flammable upon open flames. Wear personal protective equipment (*e.g.,* nitrile gloves, lab coat, safety goggles) and handle in a fume hood. Store the methanol away from ignition and in a flammable safety cabinet.

3.1.4. Bleach the tissues with 5% hydrogen peroxide (H_2O_2; 1 volume of 30% H_2O_2 diluted in 5 volumes of 100% methanol) overnight at 4 °C.

CAUTION: 30% hydrogen peroxide is very hazardous upon skin and eye contact. Wear personal protective equipment (*e.g.,* nitrile gloves, lab coat, safety goggles) and handle in a fume hood.

3.1.5. Rehydrate the tissues at RT in 80%, 60%, 40%, and 20% methanol and 1x PBS, subsequently, for 1 hour each.

3.1.6. Wash with 0.2% nonionic surfactant diluted in 1x PBS twice, for 1 hour each on a shaker at RT.

3.2. Immunolabeling

Note: Fill up the 2 mL tubes containing the tissue to the top of the tube with the solution used in each step to prevent tissue oxidation as soon as immunolabeling begins, until clearing is completed.

3.2.1. Permeabilize the tissues by incubating them in a solution of 1x PBS, 0.2% nonionic surfactant, 20% dimethyl sulfoxide (DMSO), and 0.3 M glycine at 37 °C on an incubated tabletop orbital shaker for 2 days.

Note: The maximum incubation time for 37 °C permeabilization step is 2 days.

3.2.2. Block the tissues in a solution of 1x PBS, 0.2% nonionic surfactant, 10% DMSO, 5% donkey serum, and 1% Fc block at 37 °C on an incubated tabletop orbital shaker for 2 days.

Note: The maximum incubation time for the blocking step is 2 days.

3.2.3. Incubate the tissues in primary antibodies of interest in a solution of 1x PBS, 0.2% polysorbate 20, 10 µg/mL heparin, 5% DMSO, and 5% donkey serum at 37 °C on an incubated tabletop orbital shaker for 4 days.

3.2.4. Wash with 1x PBS, 0.2% polysorbate 20, and 10 µg/mL heparin on a shaker at RT five times,

each for 1 hour.

Note: This step can be a pause point to leave samples overnight in RT.

3.2.5. Incubate tissues with secondary antibody in a solution of 1x PBS, 0.2% polysorbate 20, 10 µg/mL heparin, and 5% donkey serum at 37 °C on a tabletop orbital shaker for 4 days.

Note: From step 3.2.5, all samples need to be wrapped with aluminum foil to prevent photobleaching of secondary antibody.

3.2.6. Wash tissues in a solution of 1x PBS, 0.2% polysorbate 20, and 10 µg/mL heparin on a shaker in RT five times, for 2 hours each.

Note: This step can be a pausing point to leave the samples overnight at RT.

3.3. Tissue clearing of white adipose tissue and volume imaging

3.3.1. Dehydrate the tissues contained in 2 mL tubes by incubating in 20%, 40%, 60%, and 80% methanol, subsequently, each for 1 hour at RT. Then, dehydrate the samples in 100% methanol twice at RT.

Note: This step can be a pausing point to leave your samples in 100% methanol overnight at RT.

3.3.2. Incubate the tissues with a mixture of 2 volumes of DCM to 1 volume of methanol for 3 hours at RT on a shaker.

Note: DCM is volatile. Make sure the tubes are tightly sealed to prevent evaporation.

CAUTION: This step is hazardous. DCM is toxic upon inhalation. Prolonged exposure can potentially cause chemical burns. Wear personal protective equipment (*e.g.,* nitrile gloves, lab coat, footwear, safety goggles). Use a fume hood.

3.3.3. Incubate the tissues in 100% DCM twice, for 15 min each on a shaker at RT.

3.3.4. Incubate in 100% DBE in RT until imaging and for sample storage. Before imaging, invert the tubes several times to mix the solutions.

Note: Completely fill tubes with DBE to prevent oxidation, which can result in unsuccessful tissue clearing. Do not shake the tubes during DBE incubation.

CAUTION: This step is hazardous. DBE is toxic. It can cause irritation to the eyes and skin. Wear personal protective equipment (*e.g.,* nitrile gloves, lab coat, safety goggles) and handle in a fume hood.

3.3.5. Image the whole tissue sample with a light microscope that matches the refractive index of organic solvent DBE. Perform Z-stacking at a desired magnification and step-size for the whole tissue.

4. **Examples of Data Analysis from Whole-Mount Stained Tissue Images Using ImageJ**

Note: See https://imageJ.nih.gov/ij/download.html for download and installation instructions.

4.1. Quantification of blood vessel density (**Supplementary Figure 2**)

4.1.1. Import the saved images of only the channel with blood vessel immunostaining onto ImageJ.

Note: The images for quantification should be consistent in terms of staining procedure and image acquisition condition. Identical reagents should be used. The exposure time, intensity, and magnification should also be equivalent in the imaging process[20].

4.1.2. Convert the image color into black-and-white for blood vessel density quantification. To do so, under "Image" tab, select the "Adjust" command, then the "Color Threshold" option. In the "Threshold Color" display box, choose "Dark Background"[20] and select "B&W" under "Threshold Color".

4.1.3. To measure the percentage of area of blood vessel signal against background, select the "Analyze" tab, then the "Analyze Particles" command. Under the display of "Analyzed Particles", select the "Clear Results" and "Summarize" options. Click "OK".

4.1.4. Copy and paste the value of the percentage area under the summary tab into a spreadsheet for analysis. The percentage of area will indicate the blood vessel density. Repeat steps 4.3.1-4.3.4 for each individual image.

4.2. Quantification of adipocyte size[21] (**Supplementary Figure 3**)

4.2.1. Import the saved images of the adipocytes onto ImageJ.

4.2.2. To set the scale of the image, measure the length of the scale in pixels by tracing a line to the scale with a known distance on the image using the straight-line selection tool. Under the "Analyze" tab, select the "Set Scale" command. The distance of the line that was traced before will be automatically calculated in pixels.

4.2.3. The "Set Scale" display box will appear. Enter the known distance and unit of length. Select "Global" to apply the scale setting for all imported images and click "OK".

4.2.4. To choose area as the method of measurement, under the "Analyze" tab select the "Set Measurements" command. A list of different options for measurements will appear. Select the

"Area" option and click "OK".

4.2.5. Using the freehand selection tool, trace the perimeter of each adipocyte of interest. Under the "Analyze" tab, select the "Measure (Ctrl + M)" command, and the area of the adipocyte will appear. Repeat this procedure for other adipocytes in the image.

Note: To ensure accurate measurements, use multiple images for quantification.

4.2.6. Copy and paste the area measurements into a spreadsheet for further data analysis.

REPRESENTATIVE RESULTS:

Due to the fragility of adipose tissue, methods involving multiple processing steps and sectioning can lead to disfigurement of adipose tissue morphology[3] (**Figure 1A**). However, whole-mount staining can preserve the morphology of adipocytes, ensuring accurate interpretation of results (**Figure 1B**).

Over-fixation of adipose tissue leads to fixative-induced autofluorescence. As shown in **Figure 2A**, green and red channels staining for tyrosine hydroxylase (TH) and PECAM-1 signals, respectively, overlap in identical regions of the tissue, indicating that autofluorescence may have occurred due to over-fixation in PFA for 3 days. In contrast, **Figure 2B** shows a representative image of whole-mount staining when proper fixation is performed, as the signal for TH staining occurs in different areas relative to PECAM-1 signal, demonstrating that this signal is not autofluorescence and is in fact a positive signal.

Whole-mount staining is an important visualization tool for Cre-loxP-based lineage tracing of adipocytes[15], with mT/mG being the ideal reporter system[18]. Ng2, a marker for adipocyte progenitor cell population, is a plasma membrane proteoglycan. In this system, Ng2-Cre-positive cells express m-GFP, whereas m-Tomato is expressed in Ng2-Cre-negative cells (**Figure 3**).

Adipose tissue is an incredibly dynamic organ, capable of expanding and shrinking under different physiological conditions and demands[20]. Imaging whole-mount stained adipose tissue allows for quantification of imorphological changes under different experimental conditions. In particular, adipose tissue is highly vascularized, which is important in mediating metabolic homeostasis upon rapid changes in energy level[1]. For instance, C57BL/6J mice that undergo 24 hours of fasting display significantly smaller adipocyte size (**Figure 4**), indicating lipolysis, and a trend in elevated blood vessel density compared to continuously fed mice (**Figure 5**). ImageJ software was utilized to quantify the size of the adipocytes and blood vessel density, as described above.

Tissue clearing is a relatively new technique developed to remove the opaqueness of adipose tissue to allow visualization deep within the tissue volume[9,10] (**Figure 6**). Whole-mount staining on uncleared IWAT using confocal microscopy only showed sparse sympathetic innervation, since nerve fibers underneath the surface of the tissue could not be visualized (**Figure 7A**). However, dense neural arborization could be observed after tissue clearing and immunolabeling with the use of iDISCO+ as well as the use of light-sheet fluorescent microscopy (LSFM) (**Figure 7B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Comparison of adipose tissue morphology with conventional morphological techniques and whole-mount staining technique. (A) H&E stained adipose tissue on a paraffin-embedded section (left), with black arrowheads indicating distorted regions of adipocytes. Lectin (carbohydrate binding protein, white arrows) fluorescent dye injection, immunofluorescent staining of F4/80 (macrophage marker, yellow arrowheads), and DAPI nuclei staining of adipose tissue on cryosection (right). (B) White adipose tissue visualization using whole-mount staining with a step-size of 5 μm. The total Z-stack depth captured is around 100 μm. Adipocyte lipid droplets were stained with neutral lipid stain (grey), and blood vessels were stained with PECAM-1. Image was captured with microscopy with a step-size of 5 μm for Z-stacking (red).

Figure 2: **Visualization of neural fibers and blood vessels using whole-mount stained adipose tissue.** (A) Representative microscopic images of undesirable results from whole-mount stained PWAT due to over-fixation in PFA for 3 days. Overlapping signals are indicated by white arrowheads. (B) Representative microscopic images of a positive result from whole-mount stained IWAT from control mouse. The images were captured at 100X magnification.

Figure 3: Lineage tracing using mT/mG system in the adipose tissue. Representative images Cre-positive (mG) and Cre-negative (mT) cells in IWAT of a Ng2-Cre; mT/mG mouse. The images were captured at 200X magnification.

Figure 4: Visualization and quantification of adipocyte size. (A) Representative images of adipocytes in PWAT of fed and 24-hour fasted C57BL/6J mice using neutral lipid staining. The images were captured at 200x magnification. (B) Adipocyte size comparison between fed and 24-hour fasted C57BL/6J mice using ImageJ software. Values are expressed as mean ± SEM; 2-tailed unpaired Student's *t*-test; ***$p < 0.001$.

Figure 5: Visualization and quantification of blood vessel density. (A) Representative images of blood vessels in fed and 24-hour fasted C57BL/6J mice using PECAM-1 antibody. (B) Comparison of blood vessel density between fed and 24-hour fasted C57BL/6J mice using ImageJ software. Values are expressed as mean ± SEM; 2-tailed unpaired Student's *t*-test.

Figure 6: Clearing of adipose tissue using the iDISCO+ method. Prior to clearing, the tissue is opaque. The tissue becomes completely transparent at the end of the tissue clearing steps.

Figure 7: Whole-mount stained IWAT compared with tissue-cleared IWAT using iDISCO+ method. (A) Visualization of neural fibers using TH antibody (1:500) in whole-mount stained IWAT at 100x magnification with confocal microscopy with a step-size of 5 μm. The total Z-stack depth captured is around 100 μm. (B) Visualization of neural fibers using TH antibody (1:200) in whole-mount stained IWAT with iDISCO+ protocol at 1.6X magnification using LSFM with a step-size of 4 μm. The total Z-stack depth captured is around 8 mm.

Supplementary Figure 1: Schematic diagram for mT/mG lineage tracing system. A dual

fluorescent system that employs membrane-targeted eGFP and membrane-targeted tdTomato. Before Cre recombination, the mT is globally expressed. When the cell expresses Cre, the mT cassette is excised, and mG is expressed permanently. pA represents polyadenylation sequences after the stop codon.

Supplementary Figure 2: Application of ImageJ software to quantify percentage area of blood vessel density. (A) "Image" tab commands and options to convert an image into a black-and-white threshold color. (B) Summary measurement of percentage area of vessel density using the "Analyze Particles" command.

Supplementary Figure 3: Application of ImageJ software to quantify adipocyte area. "Analyze" tab: "Set Scale" and "Measurement" commands to measure the area of the adipocyte(s). Results display shows the area of each adipocyte measured.

DISCUSSION:

Although conventional techniques such as histology and cryosection offer benefits for observing intracellular structure, whole-mount staining provides a different perspective in adipose tissue research, which enables 3D visualization of cellular architecture of minimally processed tissue.

In order to successfully perform whole-mount staining, the following suggestions should be taken into consideration. Different adipose tissue depots can yield various immunostaining results; thus, the type of adipose tissue depot used should be determined first. For instance, brown adipose tissue (BAT) is denser relative to white adipose tissue (WAT) due to smaller, multilocular adipocytes[21]. This increased density of BAT makes it difficult for antibodies to permeate through the tissue. In addition, the size of the adipose tissue is also imperative for proper antibody staining, since too large/thick tissues can also result in insufficient antibody penetration. Hence, when obtaining the adipose tissue during animal dissection, the distal portion of the perigonadal fat tissue should be used, since this is the thinnest region and can allow for sufficient antibody penetration and consistent data. Alternatively, for denser tissues, use of a fluorescent reporter mouse line, such as mT/mG, may allow for better visualization of the marker of interest, since this avoids the issue of insufficient antibody penetration. Subsequently, 1% PFA is used for tissue fixation to preserve the natural distribution of proteins and ensure tissue permeabilization and antibody penetration[22,23]. However, over-fixation can decrease antigen recognition and produce autofluorescence[24]. This is due to the reaction between aldehyde groups on PFA and other aldehyde-containing fixative and tissue components, which creates fluorescent compounds[24]. To prevent the need for an antigen-retrieval step, it is recommended to leave tissues in a fixative for only an hour at room temperature and begin the next steps as soon as fixation is completed[7]. Like many other immunolabeling techniques, titrating the antibody concentration is an essential troubleshooting step to ensure the desired signal.

Indeed, there are several limitations of whole-mount staining despite the aforementioned significance and advantages. Whole-mount staining has stringent requirements regarding tissue type and size, because insufficient antibody penetration and uneven staining can occur in thicker tissues such as muscle and liver. Also, whole-mount staining is not the most accurate

representation of certain antibodies such as tyrosine hydroxylase (TH), a marker for the sympathetic nervous system, as its signal is often masked by dense lipid content in adipose tissue (**Figure 7**). In addition, the uneven surface of adipose tissue also posed a challenge for confocal imaging, since signals located at different layers of the adipose tissue cannot be easily captured on a single image. Therefore, the quantification of signal intensity in images obtained from whole-mount staining yield inconsistent results for whole-nerve fiber arborization. The distal portion of PWAT is usually the least lipid-dense; hence, better images can be obtained from this area. However, whether this area is representative of the entire tissue for immunostaining in all types of antibodies will require further investigation.

By making tissue optically transparent, a clearing method reduces light scattering, enabling visualization of the deep structure[25]. iDISCO+ is an inexpensive protocol recently developed that combines whole-mount immunolabeling with volume imaging of various large cleared tissues[9]. Modified iDISCO+ methods with LSFM demonstrated by recent studies[9,10] observed dense dendritic arborization on WAT that cannot be seen with conventional immunolabeling and confocal microscopy. Conventional confocal imaging uses an imaging beam system and pinhole for laser scanning. The scanning speed and penetrating depth are limitations of the microscope; thus, 3D tissue dynamics are often missed. In contrast, light-sheet microscopy has apparent advantages in that it uses sheet-scanning and only illuminates one optical section at a time, capturing all the fluorescence molecules within that section. Moreover, fluorophores in other sections are not excited, preventing photo-bleaching and phototoxic effects[26]. Thus, the amount of nerve innervation within the adipose tissue can be more accurately assessed with iDISCO+ and LSFM. Despite this, there are some limitations to the use of iDISCO+ and LSFM. For instance, users should note that only channels in the red and far-red channel are compatible with iDISCO+, because longer wavelengths of light are better able penetrate the sample[27]. Additionally, autofluorescence in the blue-green spectrum is quite high in large tissue samples, so imaging in the red and far-red spectra will help reduce any autofluorescence that occurs[14]. In regard to transgenic fluorescent reporter mice, iDISCO+ can be used to visualize reporter proteins. However, immunolabeling of the fluorescent reporter with a secondary antibody should be conducted, as the endogenous signal may fade during the tissue clearing process[14]. Nonetheless, this technique is extremely valuable for studying sympathetic nervous system-adipose tissue interactions and for investigating adipose plasticity under different physiological and metabolic conditions.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

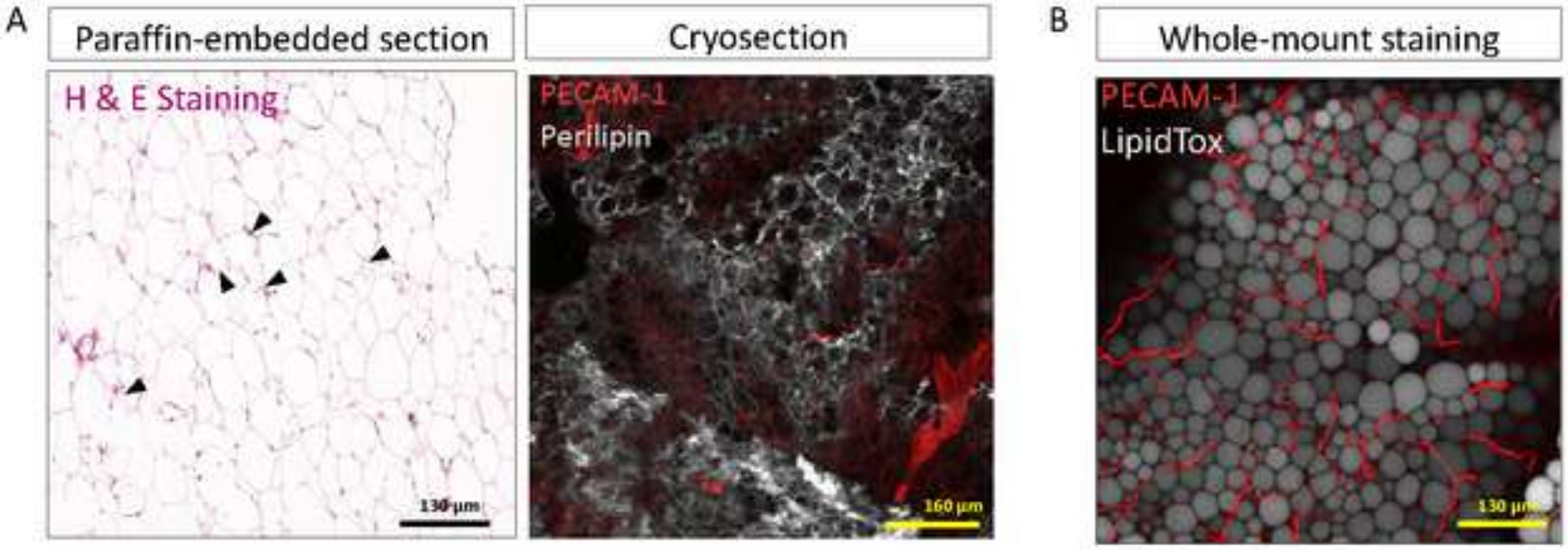

Figure 2

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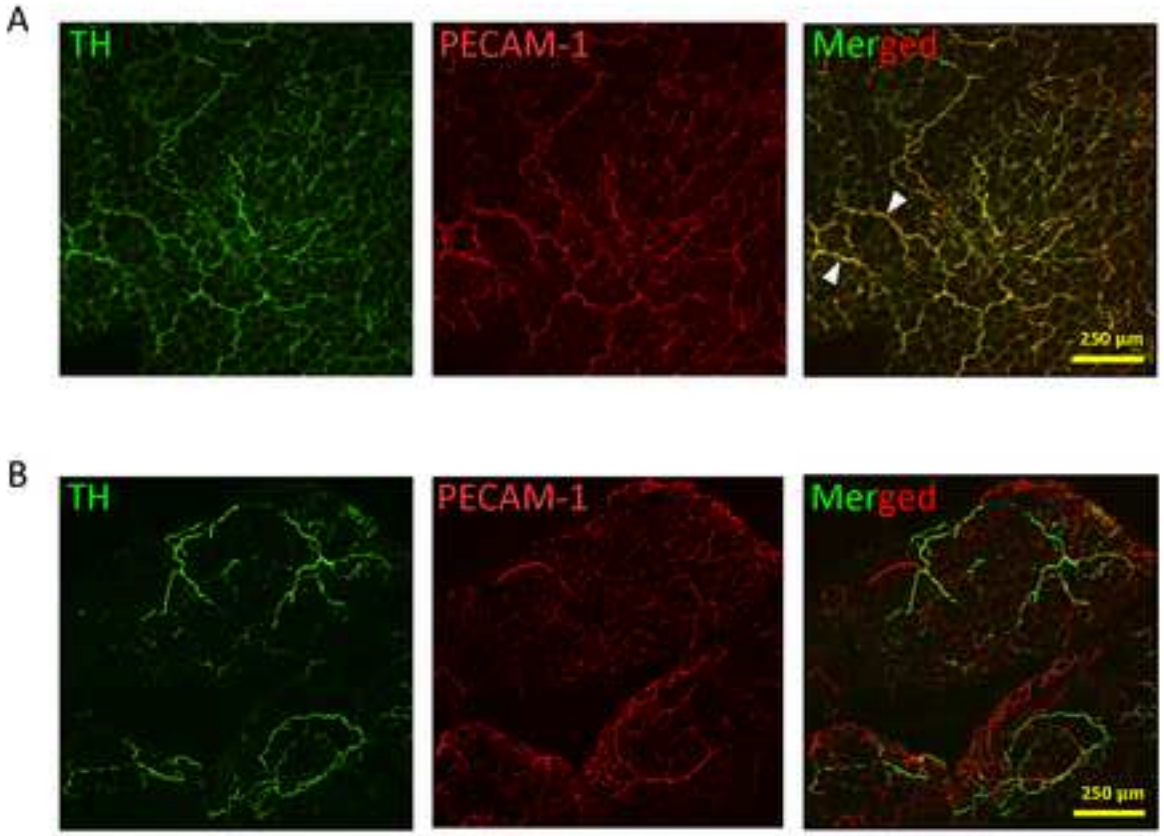

Figure 3

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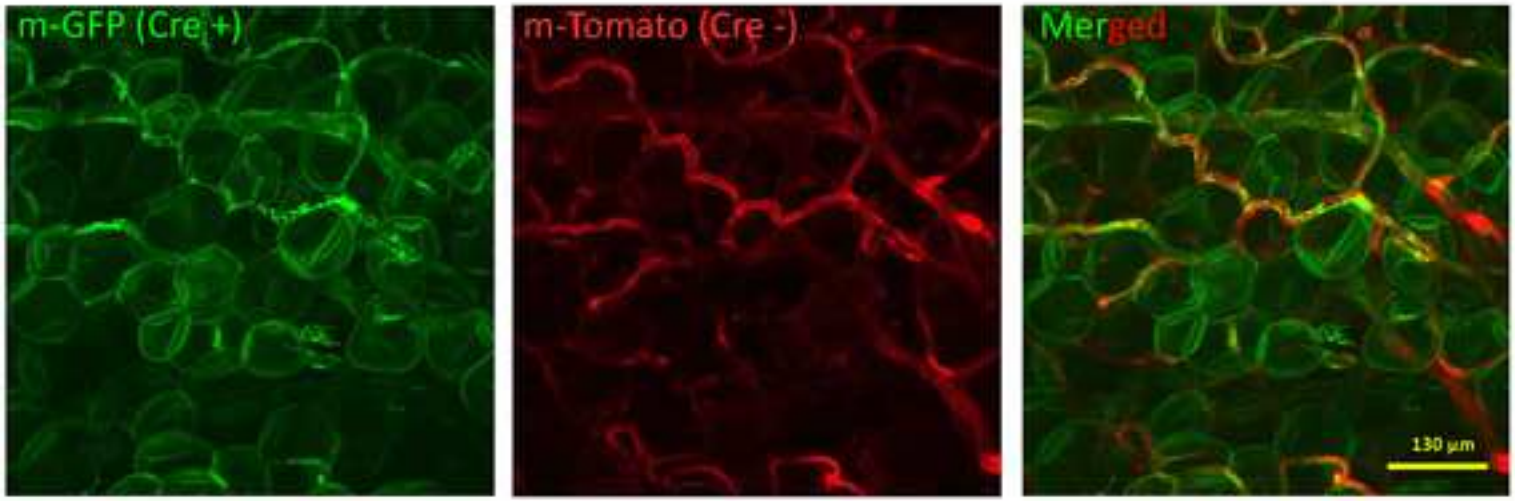


Figure 4

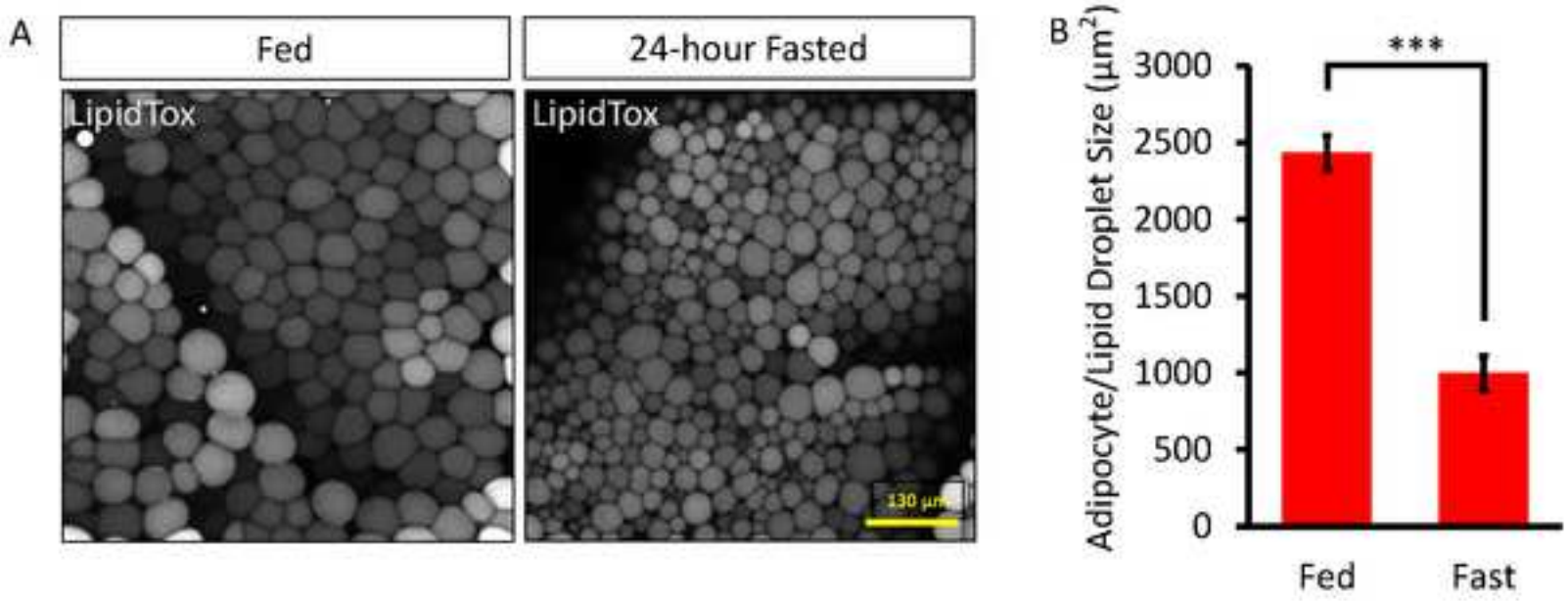


Figure 5

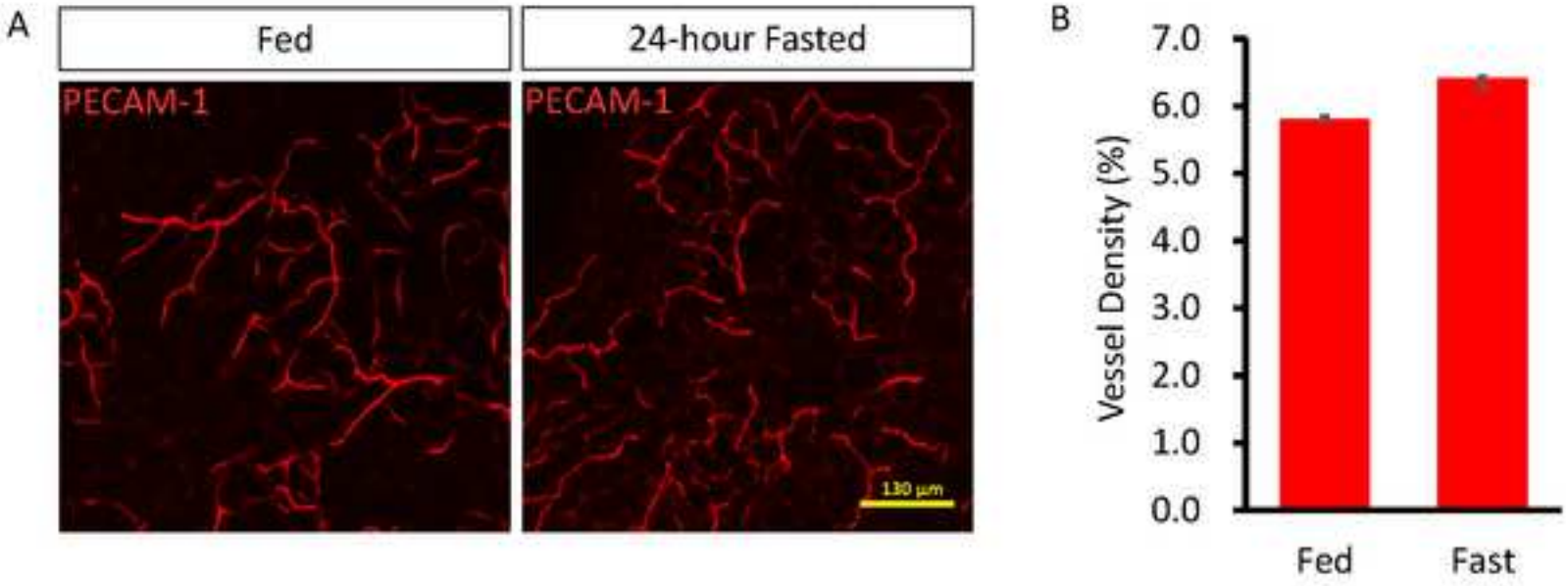
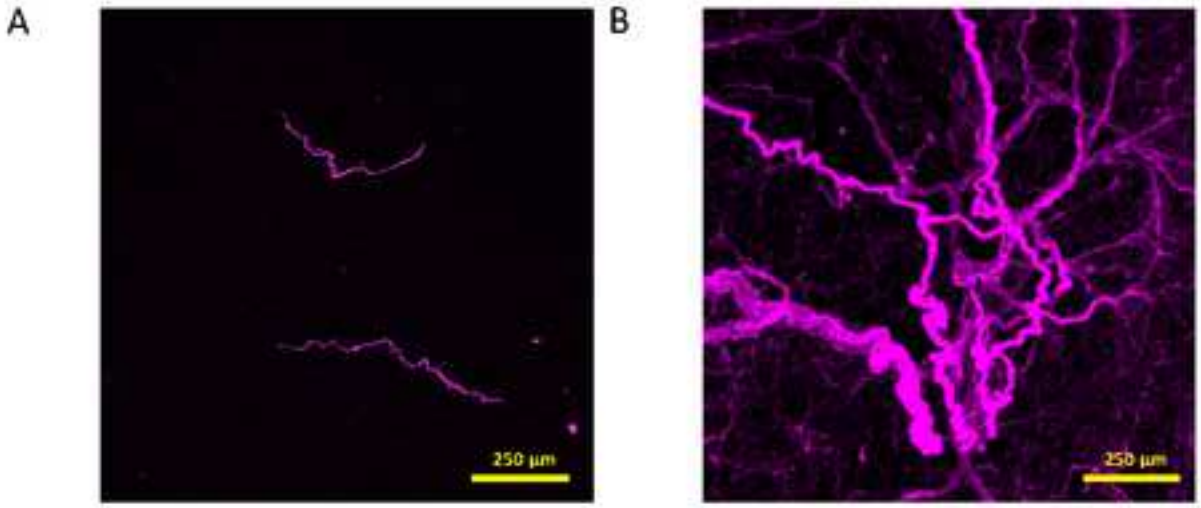


Figure 6

Figure 7



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
LipidTox	Life Technologies	H34477	
PECAM-1 primary antibody	Millipore	MAB1398Z(CH)	
TH (tyrosine hydroxylase) primary antibody	Millipore	AB152, AB1542	
DAPI stain	BD Pharmingen	564907	
Nikon A1R confocal microscope	Nikon		Confocal microscope
Ultramicroscope I	LaVision BioTec Jackson		Light sheet image fluorescent microscope
Alexa Fluor secondary antibodies	ImmunoResearch		Wavelengths 488, 594 and 647 used
Purified Rat Anti-Mouse CD16/CD32	BioSciences	553141	
Dichloromethane	Sigma-Aldrich	270997	
Dibenzyl-ether	Sigma-Aldrich	33630	
Methanol	Fisher Chemical	A452-1	
	BIO BASIC		
30% Hydrogen Peroxide	CANADA INC	HC4060	
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650	
Glycine	Sigma-Aldrich	J7126	
Heparin	Sigma-Aldrich	H3393	
	VECTOR		
Lectin kit I, fluorescein labeled	LABORATORIES	FLK-2100	
F4/80	Bio-Rad	MCA497GA	
VECTASHIELD Hard Set Mounting	VECTOR		
Medium with DAPI	LABORATORIES	H-1500	
Paraformaldehyde (PFA)			
Phosphate Buffer Saline (PBS)			
Triton-X			

Tween
Animal serum (goat, donkey)

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Dear Dr. Steindel,

We thank you and the reviewers for the thorough review and many helpful suggestions for our study. In the revised manuscript, we have carefully addressed all the concerns raised by the reviewers. As detailed in the rebuttal, we have addressed all reviewers' comments point-by-point and described additional experimental result that strengthen our methodology.

In the revised manuscript, new data were incorporated in the Main Figures, and the text has been revised and clarified for better description and discussion. We wish that you and reviewers would be satisfied with our revised version. Should you need additional information, please let us know.

Thank you again for your kind consideration of our manuscript.

Sincerely,

Hoon-Ki Sung

Reviewer #1

GENERAL COMMENTS

In this manuscript Sung and collaborators describe a protocol for 3D analysis of adipose tissue and an original tissue clearing technique. The technique has some interest considering that it allows for tridimensional examination of adipose tissue architecture. One advantage is the fact that with appropriate fixation, the immunofluorescence signals do not overlap in the visualization of blood vessels. The technique has the potential to be applied for many other outcome measurements in adipose tissue. Specific comments are provided below.

We thank the reviewer for the valuable comments.

1. *On lines 288-291 the Authors imply that the whole-mount technique preserves the morphology of adipocytes better than methods involving multiple processing steps. This is repeated in the discussion. However, the study does not include formal proof that this is the case. A representative image is hardly sufficient to ascertain that this is the case.*

   We thank the reviewers for pointing out the repetition of our statements within the manuscript. We realize our previous image of cryosection in Figure 1A is not intuitive enough to show the limitations of conventional tissue process and imaging techniques due to the differences in magnification and antibody staining between the two images. Hence, we've changed Figure 1A to include an image of immunostaining of PECAM (1:1000) and perilipin (1:1000) on a cryosection of IWAT with comparable magnification (100x) and antibody staining (PECAM and LipidTox) to Figure 1B, which depicts the whole mount stained sample imaged with confocal microscopy. As shown in the revised Figure 1A (new data), structure of the blood vessels on frozen sections are not as intact as the case in whole mount staining of Figure 1B. In addition, the adipocyte structure is distorted during sectioning, whereas in whole mount staining, the structure of the adipocytes remains its original shape, due to the minimum tissue processing procedure. In addition, because whole mount staining does not require sectioning of the tissue, fibrous and connective structures such as blood vessels remain intact for observation. We understand that these conventional methods are still widely used for adipose biology, and the method works for the effects they desire. We would like to emphasize that different techniques can be exploited for different study purposes. While traditional histology and cryosection methods with immunolabeling provide valuable information about intracellular study, whole mount staining would be better for observing intercellular interactions within adipose tissue such as communication between adipocytes, the vascular system, neural projections and macrophages. We've replaced the repetition in discussion with the above reasoning, in order to provide the readers clear instructions about when to use whole mount staining.

2. *The time needed for the tissue clearing technique and immunolabeling using*

iDISCO+ is apparently quite long (17 days as estimated from the protocol). Duration must be stated formally and perhaps addressed as a limitation in the discussion.

We thank the reviewer for the important comment about the limitation of tissue clearing. In our revised version, we stated this limitation of iDISCO+ in the introduction after mentioning the advantages of this method. We've also included shorter versions of iDISCO+ that have been previously proposed in other studies [1,2]. As well, future studies are needed for optimizing the length of time required to complete tissue clearing.

2. *Some parts of the protocol are needlessly specific and detailed while others would require more description. For example, the description of the adipose tissue sampling technique is highly detailed but is likely to vary as a function of the species examined (e.g. humans), or operating procedures in any given research center. How these details affect the outcome of the protocol is uncertain (e.g. cardiac perfusion). On the other hand, some parts of the protocol would benefit from more details including shaker and centrifugation speed, for example*

   We thank the reviewers for commenting on the detail handling in our protocol. In our revised version, we agree with this comment about adipose tissue sampling techniques being highly detailed and may vary between research facilities and types of model organisms used. Since sampling techniques are not the focus in our paper, we've shortened this section in the revised version, and provided a reference regarding adipose tissue sampling techniques in mice. Cardiac perfusion was mentioned in many tissue clearing protocols[1,2], however it is not an absolutely required step for us. We've done tissue clearing without perfusion and still achieved high quality images without high autofluorescence(Figure 7B). In case of any confusion that may arise, we decide to remove cardiac perfusion step, but performing this step may help to further reduce autofluorescence in the samples. We also added more information about the shaker speed during incubation in our revised protocol as a suggestion. However, the desired speed might vary depending on different machines. Thus, the user's choice can be flexible regarding the shaker speed.

4. In the discussion section (lines 393-394 and 416), the description of the tissue sampling technique is unclear. What is referred to when mentioning the 'tip' of perigonadal adipose tissue? It is expected that adipose tissue from other compartments will have a different morphological aspect and no tip. How critical is the selection of this particular region of this fat depot in terms of success of the method?

We thank the reviewer for raising the important questions regarding our sampling techniques. The 'tip' of perigonadal adipose tissue (PWAT) refers to the distal portion of PWAT[3]. This region is used in whole mount staining for the following reasons: 1). The 'tip' of PWAT is the thinnest region to ensure proper

fixation and easy antibody penetration; 2). The morphology of this region is better preserved without physical disturbance by surgical tools during dissection. 3). Since this region is considered as active adipogenic niche, it provides significant biological and cellular information[3]. Other parts of the adipose tissue can be used for whole mount staining as well, but because those regions are more lipid and connective tissue dense region, structures can be hidden in different depth of the tissues, which posed a challenge for fair comparison of structures within same depth captured. To avoid unfair comparison, we choose the distal portion of PWAT throughout our experiments. As a suggestion, we believe this may be helpful for the readers to successfully replicate our methodology. Lipid and connective tissue dense region can be better visualized through tissue clearing technique.

5.The numbering of the protocol sections is somewhat misleading. The reader at first may assume that the step numbers are in chronological order. However, section 5 may be performed after section 2 as an optional step.

We thank the reviewer for the important comment. Section 5 was moved to section 3, as suggested by the reviewer. Please note that we were not able to arrange all the sections in chronological order, since some sections are independent with each other, therefore other people can skip the sections that are not needed for their own experimental procedures. For example, section 1 to 2 should be in chronological order of whole mount staining procedure. Section 3 tissue clearing with immunolabeling can be performed as an additional step if specific structures are in interests[1,2]. After section 2, section 4 can also be performed as an additional step for analyzing parameters associated with adipose biology. Understanding that this order can be potentially misleading, we added a note in the beginning of our protocol to denote how we organize the protocol.

6- Only one application of the tissue clearing technique is shown. Other applications could have been interesting.

We thank the reviewer for this important comment. We have added a brief introduction regarding other applications of tissue clearing, specifically for iDISCO+ tissue clearing technique. We talked about iDISCO+ being compatible with various antibodies and even some of the fluorescent reporter systems. Moreover, iDISCO+ can be used in a wide variety of organs, making it a useful tool to gain a deep understanding of intracellular activities within organs. Fat tissue is the major focus in our lab, therefore we mainly focused on white adipose tissue clearing and staining. Describing other applications of tissue clearing technique is beyond our scope, and future studies are needed to demonstrate other interesting applications of tissue clearing.

7- In the reference section, please make sure that the websites are cited according

to Journal policies.

We thank the reviewer for this comment about our website reference style. We have contacted journal editor and confirmed that we are following the right format.

Reviewer #2:

Manuscript Summary:

This manuscript provided a detailed description of the procedure for the newly developed whole-mount staining of white adipose tissues. The authors demonstrated the procedure using a few examples, e.g. blood vessel staining and nerve staining. They also compared it with cryosections and paraffin sections. The protocols were well written to demonstrate the strength of the staining and imaging method. Troubleshootings and cautions were also raised and discussed in the description. Overall, publication of this manuscript will benefit the community studying adipose tissue biology.

We thank the reviewer for the valuable comments.

Minor Concerns:

More recently, imaging of blood vessels in white adipose tissues using antibodies against PECAM and other makers was reported (Cao, Molecular Metabolism, Volume 14, Aug 2018), so the introduction part is suggested to reflect the recent progress.

We thank the reviewer for this important suggestion. We've included a short description regarding the recent progress of iDISCO+ and volume imaging on vasculature in the introduction. We agree with the reviewer that adding this information will give readers more information regarding iDISCO+ application.

Reviewer #3:

Manuscript Summary:

The authors describe how to whole-mount stain and image adipose tissue in mice and how to improve this protocol by a step of tissue clearing.

We thank the reviewer for the valuable comments.

Major Concerns:

1- The manuscript will be strongly improved if the final aim was more precisely defined. It is not clear whether the manuscript focus on whole-mount staining protocol or on the combination of whole-mount staining with tissue clearing techniques. In the abstract, the authors mention the limit of whole-mount staining to provide accurate information for the deeper parts of adipose tissue and propose

to combine it with i) tissue clearing techniques and ii) light sheet fluorescent microscopy. The advantage of combining tissue clearing and whole-mount staining is not enough demonstrated and the protocol of adipose tissue imaging does not mentioned light sheet microscope which is yet used in figure 7... There is no discussion about confocal vs light sheet microscopy.

We thank the reviewer for their valuable suggestion. Indeed, our manuscript lacked clear direction as to our main focus, which is the combination of whole mount staining with tissue clearing techniques. Based on this comment, we have removed unnecessary details in our protocol (such as dissection procedure) that do not focus on the two techniques and provided additional information of the advantages of combining tissue clearing with whole mount staining in the discussion. With regards to lack of mention of light sheet fluorescent microscopy (LSFM) procedure in the manuscript, we would like to emphasize that we focus on whole mount staining and tissue clearing procedures, not on the imaging techniques. Hence, considering the limitation of the manuscript length, LSFM use is not the main objective of the manuscript and readers should look into other references to learn LSFM procedure[4]. As such, we have also removed unnecessary details within our protocol regarding confocal microscopy to keep the aim of the manuscript clear. We apologize that there is no discussion of the pros and cons of both confocal and light sheet microscopy and appreciate the reviewer for pointing this out. Our revision has included a short comparison in the discussion between both microscopic techniques to improve the quality of the manuscript.

2- Two different protocols of adipose tissue have been used (1% PFA, 1 hour at room temperature vs 4% PFA, overnight at 4°C) without explanation. Did the authors try more than two fixation protocols (3 days vs 1 hour in figure 2)? 1 hour fixation seems very short for a tissue even cut into cubes. Did the authors verify that there was no fixation gradient within their samples? Finally, in the discussion section, the authors say that "it is recommended to leave the tissues in fixative for only 1 hour in room temperature", without any references citation.

We thank the reviewer for their insightful comment. Indeed, 1% PFA in room temperature is used solely for whole mount staining, whereas 4% PFA in 4ºC overnight is used for the combination of immunolabelling with tissue clearing. There are a number of reasons for the different fixation procedures. For one, our lab and other previous publications have used 1% PFA in 1 hour at room temperature and have received optimal imaging results (no background autofluorescence), compared to when 4% PFA is used which induced background autofluoresence. Fixation at room temperature occurs at a faster rate than at 4 ºC, hence a shorter incubation time, 1 hour, is used for whole mount staining[3]. 4% PFA at 4ºC is the standard fixation condition for tissue clearing as reported by multiple articles on various tissue clearing techniques[1,2,4]. In addition, the reason for using

a higher percentage of fixative solution, compared to whole mount staining is, to preserve the tissue structure better in order to withstand harsh clearing conditions[5], whereas little disturbance of the tissue occurs with whole mount staining other than antibody incubation. With regards to figure 2, we believe the reviewer may have misunderstood the purpose of these images. Figure 2A and 2B are not depicting two different fixation protocols. Rather, Figure 2A demonstrates that high autofluorescence occurs with accidental overfixation of the sample with 3 days of incubation in PFA, whereas Figure 2B shows an optimal imaging result with no autofluorescence when proper fixation is conducted (1% PFA for 1 hour at room temperature), thus confirming the importance of following the proper fixation protocol.

3- Concerning figure 7: to better appreciate the gain of tissue clearing technique, authors should show disappearance of TH labelling in the depth of the tissue when only whole-mount staining is applied. Why magnification is different between figures 7A and 7B? The thickness of the Z-stack should be mentioned for the two images. This immunostaining is done on iWAT that presents a clear regionalization of sympathetic innervation (Chi J et al. 2018). Authors should thus specify where images have been acquired within the inguinal fat pad.

We thank the reviewer for raising these critical points. we would like to first address the reason as to why different magnifications were used in the images for figure 7. In addition, we apologize for the lack of certain details in our figures such as the Z-stack and region of the tissue imaged and have revised according to the reviewer's comments. Figure 7A was taken by Nikon A1R confocal microscopy which has the minimal magnification of 100X (objective×zoom). However, the maximum magnification of the light-sheet microscope (LaVision BioTec, Ultramicroscope I) is 12.6× (objective×zoom). Figure 7B was captured at magnification of 1.6 (objective× zoom) to gain an appreciation of whole volume imaging of fat tissue. Understanding that it is not fair to compare the two images at different magnifications, we cropped LSFM image to make a comparable magnification to that of Figure 7A in Figure 7B. With these images, now we provide more convincing evidence that iDISCO+ and LSFM is an excellent method to visualize deep structure within white adipose tissue. We also appreciate the reviewer for suggesting our paper to specify the region of fat pad we used for the figure. For Figure 7A of whole mount staining experiment, the inguinal region of subcutaneous white adipose tissue was used, which has been reported to exhibit higher neurite density[1]. Figure 7B is a representative result from iDISCO+ and light-sheet imaging, which shows much higher TH density than 7A from conventional whole mount staining. Notably, the region used for 7B is dorsolumbar region from subcutaneous fat pad, which has been reported to have less neurite density compared to that of inguinal region. Even with the consideration of regional difference, tissue clearing still offers apparent advantages, and if we had used the inguinal region for both experiments, we might expect higher significance in terms

of neurite density resulted from tissue clearing.

Minor Concerns:
1- Figure 1: Scale bars are missing in figure 1A. Does the figure 1B result from a Z-stack? In this case, the size of the Z-stack of this image should be mentioned.

We thank reviewer for raising this important point to us. We've added scale bar to Figure 1A, and addressed the Z-stack information in Figure legend for Figure 1B.

2- In figure 3 (right panel), orthogonal views (X-Z and Y-Z) and higher magnification should be helpful to discriminate Ng2-positive cells (green labelled) and Ng2-negative cells (red labelled). Indeed, some vessels appeared double labelled which may be due to pericytes (i.e. Ng2 positive cells). Authors should also illustrate that TH fibers or immune cells are not green labelled.

We thank the reviewer for this comment. We understand that with the higher magnification, it should be more obvious that it is not double labelling, but rather overlap of the Ng2+ (green labelled) and Ng2- (red labelled) signals.
Although, we appreciate this comment, we do not believe it is necessary to illustrate that TH fibers or immune cells are not green labelled. The purpose of this figure is to solely demonstrate the application of lineage tracing with whole mount staining, not to demonstrate experimental results of Ng2 expression in the white adipose tissue.

*3- Figure 4B: It is the size of lipid droplet which is quantified, not the size of adipocyt*e.

We thank the reviewer for this critical comment. In our experiments, we have used perilipin antibody and LipidTox to stain for lipid droplet, which outlines the membrane of adipocytes. Since we mentioned whole mount staining retains the original shape of the adipocytes, it provides accurate measurement of the size of the lipid droplet as well. The two terms can be used interchangeable and we have revised Figure 4B according to this comment.

4- Does the GFP fluorescence of mT/mG mice is maintained after clearing procedure? It would be important to verify this point in order to apply whole-mount adipose tissue clearing and imaging, to reporter mice.

We thank reviewers for this important comment about the compatibility of fluorescent reporter mice system with our tissue clearing procedure. Previous organic solvent based tissue clearing procedures rapidly reduce signal from fluorescent proteins and fluorescent reporters[5]. Therefore, IDISCO+ protocol [4] was specifically designed to address this limitation and is compatible with

immunolabeling. Although we haven't tried this method on reporter mice, it has been reported by several studies[4,6] that endogenous fluorescent reporters such as GFP and tdTomato can be visualized with immunolabeling of a secondary antibody. Without immunolabeling on the reporters, endogenous fluorescent signal may fade, but exactly how long can the signal be maintained is currently beyond our scope to answer.

Reference

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6 Cao, Y., Wang, H., Wang, Q., Han, X. & Zeng, W. Three-dimensional volume fluorescence-imaging of vascular plasticity in adipose tissues. *Mol Metab.* 10.1016/j.molmet.2018.06.004, (2018).

Supplemental Figure 1

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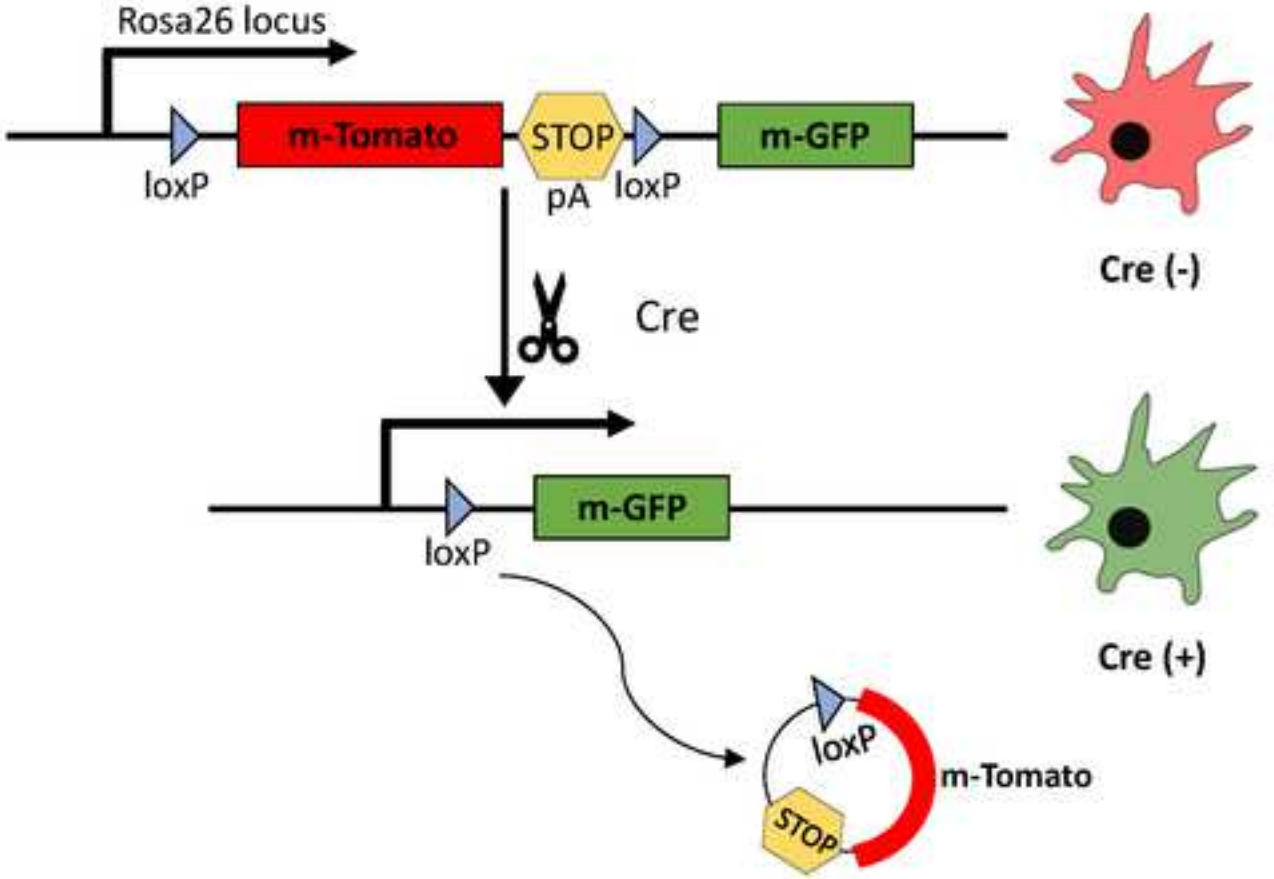

Supplemental Figure 2

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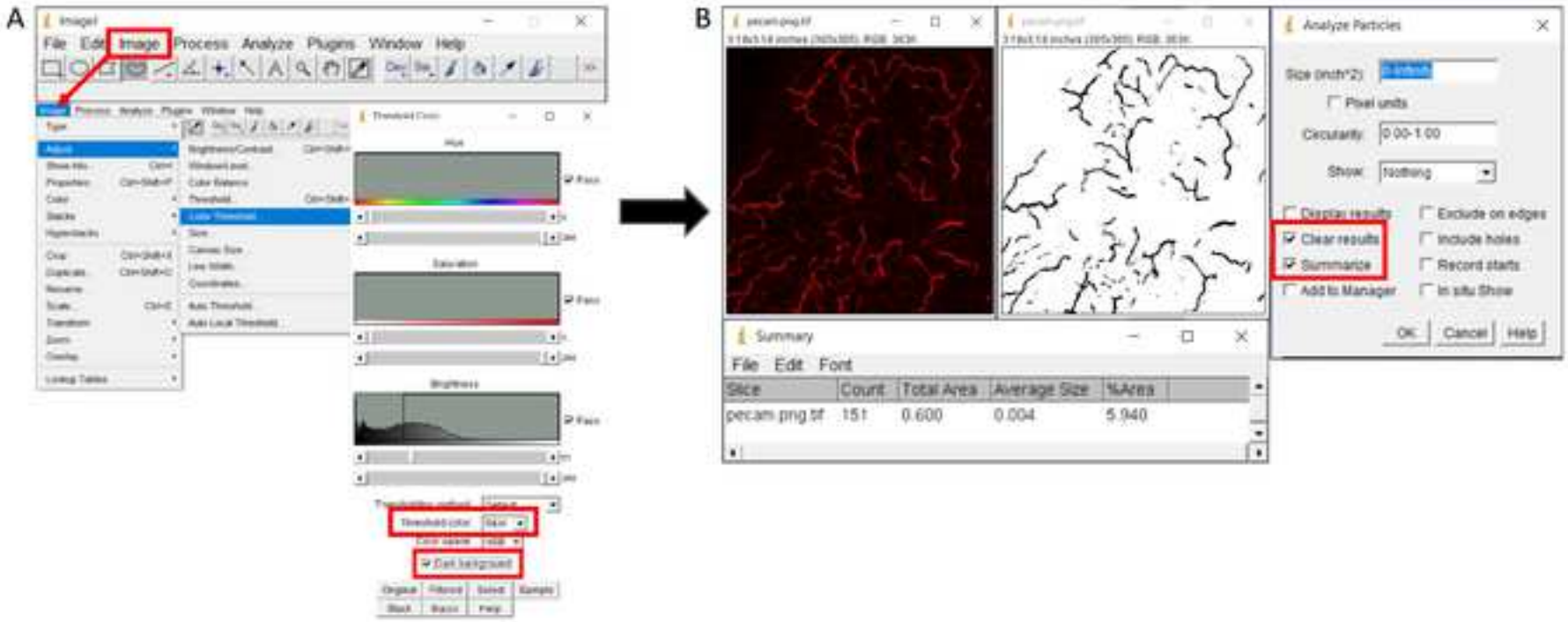

Supplemental Figure 3

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