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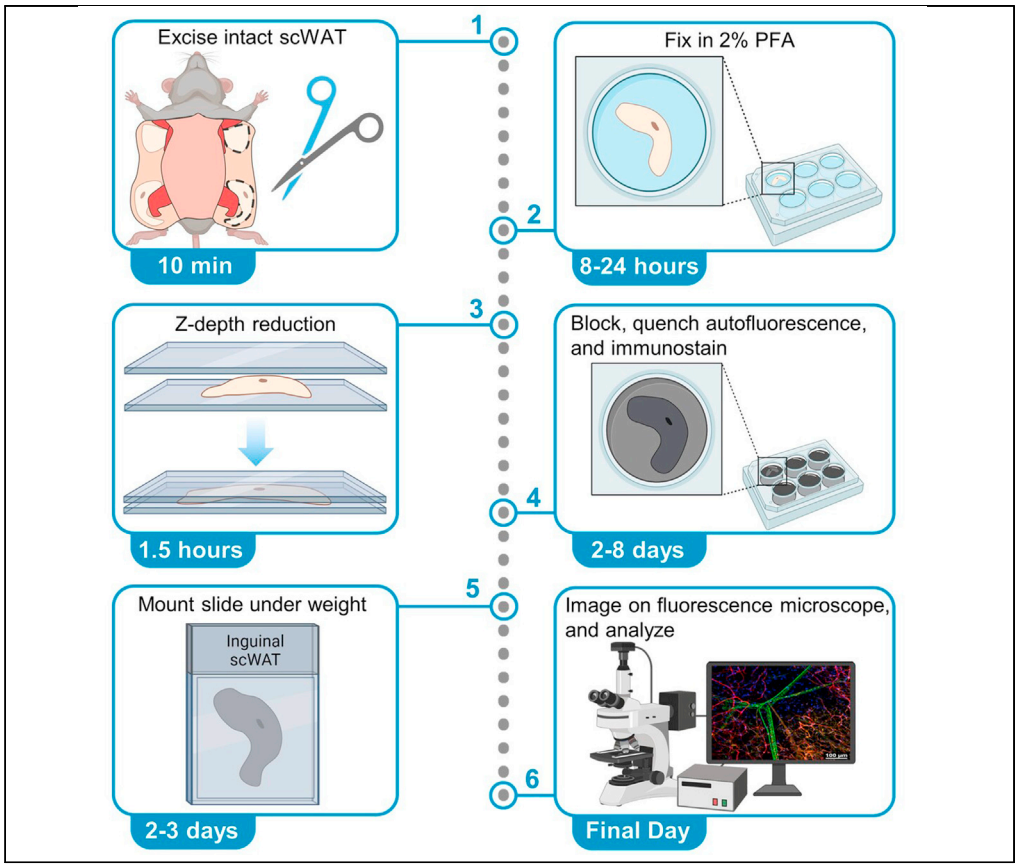
Protocol

A clearing-free protocol for imaging intact whole adipose tissue innervation in mice



Here we provide a clearing-free protocol for processing intact, whole mount subcutaneous white adipose tissue (scWAT) for immunofluorescence as an alternative to current clearing-based approaches. We use a combination of Z-depth reduction and autofluorescence quenching techniques to fluorescently label, image, and quantify adipose tissue innervation effectively throughout intact mouse tissues without the need for optical clearing or light sheet microscopy. This protocol has been optimized and validated for adipose neurovascular labeling.

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Highlights

Whole mount adipose tissue processing for immunofluorescence staining and imaging

Clearing-free protocol that uses Z-depth reduction and autofluorescence quenching

Tissues are imaged with widefield and confocal microscopes

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A clearing-free protocol for imaging intact whole adipose tissue innervation in mice

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SUMMARY

Here we provide a clearing-free protocol for processing intact, whole mount subcutaneous white adipose tissue (scWAT) for immunofluorescence as an alternative to current clearing-based approaches. We use a combination of Z-depth reduction and autofluorescence quenching techniques to fluorescently label, image, and quantify adipose tissue innervation effectively throughout intact mouse tissues without the need for optical clearing or light sheet microscopy. This protocol has been optimized and validated for adipose neurovascular labeling.
For complete details on the use and execution of this protocol, please refer to Willows et al. (2021).

BEFORE YOU BEGIN

Prepare 6-well plates and tools

Timing: 5 min

1. Label each well of a 6-well plate with mouse and tissue identifiers.

   Note: You will be excising 2 axillary and 2 inguinal depots per mouse. Each tissue will be placed in a separate well for the entirety of this protocol.

   Note: The use of a 6-well plate is recommended for all incubations and washes to give tissues room to move and prevent tissue folding or damage. Throughout this protocol enough reagent will need to be added to each well to fully cover the tissue; 3–5 mL depending on tissue size. Plan accordingly when making reagents.

2. Prepare tools and equipment as described below.

   Optional: It is recommended to make up reagents in advance (unless stated otherwise). Alternatively, the protocol has several long incubation steps which also provide ample time to make reagents.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Monoclonal Recombinant Anti-PGP9.5 [EPR4118], 1:200	Abcam	Cat# ab108986

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit Polyclonal Anti-Myelin Protein Zero, 1:250	Abcam	Cat# ab31851
Rabbit Polyclonal Anti-Tyrosine Hydroxylase, 1:500	Merck Millipore	Cat# AB152
Mouse Monoclonal Anti-beta III Tubulin [2G10] Alexa Fluor 488, 1:200	Abcam	Cat# ab195879
Rabbit Monoclonal Recombinant Anti-S100 beta [EP1576Y] Alexa Fluor 647, 1:200	Abcam	Cat# ab196175
Rabbit Anti-GFP, Alexa Fluor 488, 1:500	Thermo Fisher Scientific	Cat# A-21311
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, 1:500	Thermo Fisher Scientific	Cat# A-11008
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 532, 1:500	Thermo Fisher Scientific	Cat# A-11009
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555, 1:1000	Thermo Fisher Scientific	Cat# A-21428
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568, 1:1000	Thermo Fisher Scientific	Cat# A-21428
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594, 1:1000	Thermo Fisher Scientific	Cat# A-11005
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647, 1:500	Thermo Fisher Scientific	Cat# A-11012
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 647, 1:250	Thermo Fisher Scientific	Cat# A-32733
Chemicals, peptides, and recombinant proteins		
Paraformaldehyde	Sigma-Aldrich	Cat# P6148
10× PBS Solution	Teknova	Cat# P0496
Sodium Azide	Sigma-Aldrich	Cat# S2002
Bovine Serum Albumin	Sigma-Aldrich	Cat# A4503
Triton X-100	Bio-Rad Laboratories	Cat# 1610407
Typogen Black / Sudan Black B	Sigma-Aldrich	Cat# 199664
TrueBlack Lipofuscin Autofluorescence Quencher	Biotium	Cat# 23007
Hydrogen Peroxide Solution	Sigma-Aldrich	Cat# 516813
Dimethyl Sulfoxide	Sigma-Aldrich	Cat# D8418
Methanol	Fisher Chemical	Cat# A452
HEPES	Sigma-Aldrich	Cat# H7523
Sodium Chloride	Sigma-Aldrich	Cat# S3014
Calcium Chloride Dihydrate	Sigma-Aldrich	Cat# C3306
Isolectin GS-IB4 From Griffonia simplicifolia, Alexa Fluor 568 Conjugate, 5 µg/mL	Thermo Fisher Scientific	Cat# I21411
Isolectin GS-IB4 From Griffonia simplicifolia, Alexa Fluor 594 Conjugate, 5 µg/mL	Thermo Fisher Scientific	Cat# I21413
Isolectin GS-IB4 From Griffonia simplicifolia, Alexa Fluor 647 Conjugate, 5 µg/mL	Thermo Fisher Scientific	Cat# I32450
DAPI, dilactate, 100 ng/mL	Sigma-Aldrich	Cat# D9564
Heparin Sodium Salt from Porcine Mucosa	Sigma-Aldrich	Cat# H3393
EMS Glycerol Mounting Medium With DABCO	Electron Microscopy Sciences	Cat# 17989-5
Experimental models: organisms/strains		
Mouse: C57BL/6J	The Jackson Laboratory	Cat# 000664
Mouse: C57BL/6-Tg(Uchl1-EGFP)G1Phoz/J	The Jackson Laboratory	Cat# 022476
Software and algorithms		
Code generated for complementary quantification has been made publicly available on GitHub	This paper, GitHub.com	https://github.com/ktownsendlab/willows_et_al-2020

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nikon Elements Basic Research Package	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements/niselements-advanced-research
LAS X	Leica Microsystems	https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/
Other		
75 × 51 mm Glass Slide, 1.2 mm thick	Electron Microscopy Sciences	Cat# 71862-01
5 cm Wide Binder Clips	ACCO	Cat# 72102
Cover glass, 48 × 60 mm, 1.5 thick	Brain Research Laboratories	Cat# 4860-1.5D
6 well plate	VWR	Cat# 10861-696
Narrow Pattern Forceps	Fine Science Tools	Cat# 11002-12
Fine Scissors	Fine Science Tools	Cat# 14061-09
Orbital Shaker	VWR	Cat# 89032-100
Aspirator	Southern Labware	Cat# 305-2001-FLS
Eclipse E400 Epifluorescence Microscope	Nikon Instruments	N/A
SP8 Confocal Microscope	Leica Microsystems	N/A
A1R Confocal Microscope	Nikon Instruments	N/A

MATERIALS AND EQUIPMENT

1. You will need access to a cold room (4°C) and an orbital shaker.
2. It is recommended to have an aspirator setup to remove buffers from wells between washes/incubations.
3. A pair of forceps and dissecting scissors.
4. Access to an epifluorescence and/or confocal microscope.

2% PFA		
Reagent	Final concentration	Amount
1× PBS	1×	1000 mL
Paraformaldehyde	2% wt/v	20.0 g
Filter and adjust pH to 7.4	n/a	n/a
Total	**2% wt/v**	**1000 mL**
Store aliquots at −20°C for up to 6 months. Thaw at time of use and keep on ice		

1× PBS w/Heparin		
Reagent	Final concentration	Amount
1× PBS	1×	1000 mL
Heparin	10 U/mL	0.02 g
Total	**10 U/mL**	**1000 mL**
Store up to 3 months at ~20°C.		

Blocking Solution		
Reagent	Final concentration	Amount
Milli-Q H_2O	n/a	99 mL
Bovine Serum Albumin	2.5% v/v	2.50 g
Triton X-100	1.0% v/v	1 mL
Total	**n/a**	**100 mL**
Store up to 2 weeks at 4°C.		

20% DMSO/MeOH		
Reagent	Final concentration	Amount
MeOH	80% v/v	80 mL
DMSO	20% v/v	20 mL
Total	**20% v/v**	**100 mL**
Store up to 2 weeks at ~20°C.		

5% H_2O_2 in 20% DMSO/MeOH		
Reagent	Final concentration	Amount
20% DMSO/MeOH	n/a	90 mL
50% H_2O_2	5% v/v	10 mL
Total	**5% v/v**	**100 L**
Make and use fresh.		

HEPES Buffer		
Reagent	Final concentration	Amount
Milli-Q H_2O	n/a	500 mL
Calcium Chloride Dihydrate	1 mM	74.0 mg
Sodium Chloride	154 mOsm/L of Na^+	4.50 g
HEPES	25 mM	2.979 g
Adjust pH to 7.4	n/a	n/a
Total	**n/a**	**500 mL**
Stored at ~20°C for up to a year.		

Isolectin-IB_4 Working Solution		
Reagent	Final concentration	Amount
HEPES Buffer	n/a	100 mL
Isolectin-IB_4 Alexa Fluor Conjugate	5 µg/mL	500 µg
Total	**5 µg/mL**	**100 mL**
Stored at 4°C for up to 3 months, or at −20°C for longer.		

0.5 mg/mL DAPI Stock Solution		
Reagent	Final concentration	Amount
Milli-Q H_2O	n/a	10 mL
DAPI (10 mg/mL)	0.5 mg/mL	500 µL
Total	0.5 mg/mL	**10 mL**
Stored at 4°C for up to a year.		

100 ng/mL DAPI Working Solution		
Reagent	Final concentration	Amount
Milli-Q H_2O	n/a	100 mL
DAPI Stock Solution	100 ng/mL	20 µL
Total	100 ng/mL	**100 mL**
Make fresh at time of use.		

STEP-BY-STEP METHOD DETAILS

Excising intact subcutaneous adipose tissue from mice

Timing: Dissection, 10 min; Fixation, 8–16 h

In this step you will remove intact axillary and inguinal subcutaneous white adipose tissues from mice for immunostaining. For a demonstration of an axillary and inguinal subcutaneous white adipose tissue (scWAT) dissection see Methods video S1.

1. Euthanize mouse
   a. Follow general euthanasia protocol (i.e., CO_2 euthanasia followed by cervical dislocation.

   Note: This protocol is performed without perfusion. Any potential benefits or drawbacks of perfusion with this protocol have not been tested thoroughly at this time.

   ⚠ CRITICAL: Maintain inguinal scWAT orientation for all future steps, up to and including mounting. Most importantly, make note of whether the tissue was taken from mouse-left or mouse-right as the tissue will always curve towards the body when placed medial side up (congruent to tissue positioning during dissection, Figure 1). Additionally, the axillary end of the tissue will be wider than the posterior end. With these notes in mind, if it is known which side of the mouse the depot was excised from, it can always be returned to appropriate orientation when needed.

   ⚠ CRITICAL: Maintain axillary scWAT orientation for all future steps, up to and including mounting. The Axillary depot is more difficult to orientate than the inguinal depot as there are no obvious features for orientation. The most important step is to excise the tissue and place it medial side up into a well noting mouse-left or mouse-right. The axillary end of each tissue will have characteristic browning (in most cases) which can help orientate further, but this will be obscured with the addition of autofluorescence quenching stains described later.

2. Excise inguinal scWAT depot.
   a. Place mouse ventral side up, spray with 70% EtOH.
   b. Make a central incision from the base of the tail extending to the top of the sternum, being sure not to penetrate the underlying peritoneal cavity.
   c. Gently use forceps and thumbs to pull ventral skin away from both flanks, pulling slowly with even pressure, making sure not to damage the underlying adipose tissue. The skin can be pinned back (as demonstrated in Methods video S1) to better expose the adipose depots.
   d. Identify the inguinal scWAT depot against the skin besides each hip (Figure 1 – boundaries of each depot). Use scissors to cut the large vasculature leading to the depot and then trace the edges of the tissue, cutting along the way.
   e. Use forceps to gently roll the adipose away from the skin – cutting the anchoring connective tissue as you go. Continue until the entire depot is free from the skin.
   f. Weigh the depot and place it into a 6-well plate with 3–5 mL ice-cold 2% PFA and fix at 4°C. Fix the tissue without agitation to prevent the tissue from folding onto itself during the fixation process.
      i. $\leq$0.08g ; fix for 8 h.
      ii. >0.08g ; fix for 16–24 h.

   Note: Once tissues are adequately fixed without agitation, they are unlikely to fold during incubations performed on an orbital shaker as utilized in later steps.

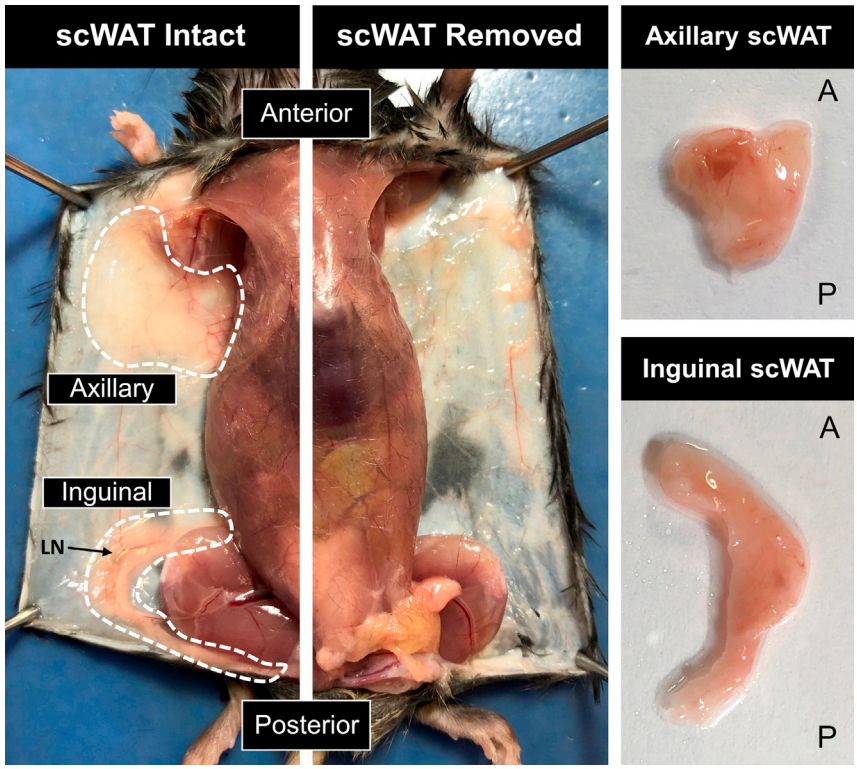

Figure 1. Excising intact subcutaneous white adipose tissue (scWAT) depots from mice

A male C57BL/6J mouse had skin pinned back to display inguinal and axillary scWAT depots before and after excision. White dashed lines are used to accentuate tissue boundaries. Tissues were kept medial side up (as in the intact scWAT image) and directionality was maintained; anterior (A, towards head) and posterior (P, towards tail).

3. Excise axillary scWAT depot.
   a. Identify axillary depot underneath both forelimbs (Figure 1).
   b. Use forceps to pull away a thin muscle layer enveloping the dorsolateral portion of each axillary depot.
   c. Use scissors to trace the edges of the tissue, cutting along the way.
   d. Use forceps to gently roll the adipose tissue away from the skin – cutting the anchoring connective tissue as you go. Continue until the entire depot is free from the skin.
   e. Weigh the depot and place it into a 6-well plate with 3–5 mL ice-cold 2% PFA to fix at 4°C. Fix the tissue without agitation.
      i. ≤0.08g; fix for 8 h.
      ii. >0.08g; fix for 16–24 h.

Reducing tissue thickness and autofluorescence

Timing: Z-depth reduction, 1 h 30 min; permeabilization, 12 h; autofluorescence quenching, 4 h-1 day

Adipose tissue is notoriously difficult to image due to its size and inherent autofluorescence. In this step we will be using a combination of Z-depth reduction and various autofluorescence quenching techniques to greatly reduce tissue background autofluorescence. It should be noted that hypertrophic adipocytes, and human adipocytes, may be more prone to cell lysis on the surface of the tissue with this technique. In some instances, whole tissue imaging is not required, and instead smaller

representative tissue pieces can be used. We have also outlined this alternative approach as an optional step below.

Note: All washes and incubations are performed on an orbital shaker.

4. Z-depth Reduction.
   a. Following fixation, aspirate PFA from each well of the 6-well plate and add 1× PBS w/heparin. Wash tissues for an hour at 4°C, replacing 1× PBS w/heparin after 30 min. Heparin is used here to help remove highly autofluorescent blood cells from the vasculature.

   ⏸ **Pause point:** The conclusion of any series of 1× PBS washes throughout this protocol marks a good pause point if desired (e.g. washes following fixation, autofluorescence quenching, or antibody staining.) Tissues can remain in 1× PBS for several days with no observed detriment to staining outcome. It is prudent to do antibody incubations immediately prior to imaging, for best results.

   b. Use forceps to take the tissue out of PBS and lay it flat onto the center of a large (75 × 51 mm) glass slide.
   c. Use a transfer pipette to add 2–3 drops of PBS onto the tissue to prevent the tissue from drying out.
   d. Take a second large glass slide and place it on top of the tissue; sandwiching the tissue betwixt.
   e. Take two large (5 cm wide) binder clips and clamp them onto the slides (Figure 2).
   f. Allow tissues to remain compressed for 1 h 30 min at 4°C.
   g. Remove binder clips and gently separate the two slides without damaging the tissue. See Figure 3 for an example of what tissues should look like after Z-depth reduction.

   Optional: To entirely avoid compressing tissues, Z-depth reduction can be removed from this protocol by cutting each adipose tissue into 4–5 smaller pieces and following through with the remaining steps until mounting. Mount each piece of tissue onto a concave slide (0.8–1.9 mm deep, depending on tissue thickness). We do not recommend this alternative approach unless it is determined that tissue compression must be completely avoided. Only the most superficial structures will be visible. See Willows et al. (2021) for a comparison of both appropaches.

5. Blocking and Permeabilization.
   a. Once the slides are separated place the tissue back into a 6-well plate with 3–5 mL blocking buffer at 4°C for 16–24 h to reduce non-specific binding of antibodies.

   Note: Here, bovine serum albumin is used as the blocking agent with great effect. Alternatively, using secondary-matched sera as a blocking agent is common with many other immunostaining protocols, but has not yet been thoroughly evaluated for use with this protocol.

   Note: For larger tissues (>0.30 g) extend the blocking & permeabilization period to 3 days. Exchange fresh blocking buffer daily.

6. Autofluorescence Quenching.
   a. There are four approaches (used separately) that can be taken to mitigate tissue autofluorescence, each with their own pros and cons summarized here and in greater detail in Willows et al. (2021).
   b. Option 1 – typogen black: Incubate tissues in 0.1% typogen black for 20 min at ~20°C followed by washing the tissue in 1× PBS for 4 h at ~20°C , replacing PBS every 1 h. See Figure 3 for an example of what tissues should look like after incubation in typogen black.
      i. Pro – a good balance between blocking autofluorescence and maintaining fluorophore signal intensity.

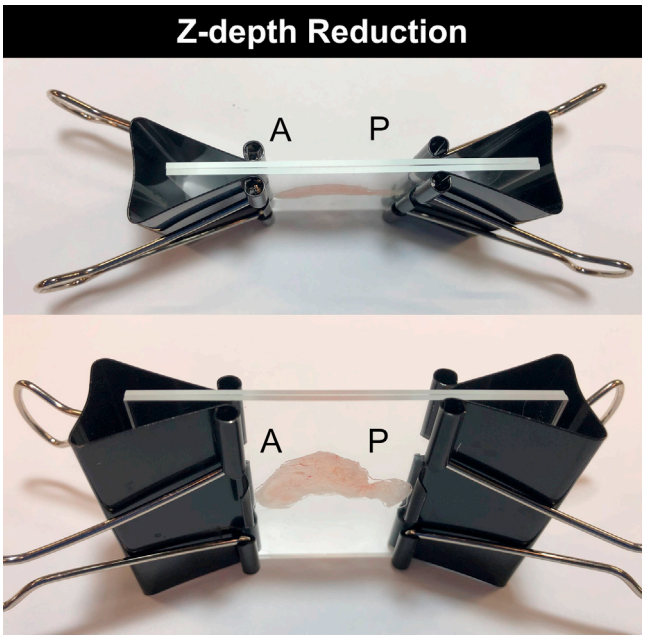

Figure 2. Z-depth reduction of whole scWAT
Inguinal scWAT depot placed between two large glass slides held together with binder clips. Shown at two angles.

ii. Con – typogen black staining fluoresces with far-red (647 nm) excitation.

c. Option 2 – TrueBlack: incubate tissues in 1× (0.1%) TrueBlack® Lipofuscin Autofluorescence Quencher for 10 min at ~20°C followed by washing the tissue in 1× PBS for 4 h at 4°C, replacing PBS every 1 h.
   i. Pro – does not fluoresce in far-red wavelength.
   ii. Con – can diminish endogenous and immuno-fluorescence intensity by overly masking areas in black haze. This can hide low-intensity structures such as small neurites.

d. Option 3 – H_2O_2 Bleaching: stepwise dehydration in MeOH diluted in 1× PBS (50% MeOH, 1 h; 80% MeOH, 1 h; 100% MeOH, 2 h, replaced fresh after 1 h) at ~20°C Bleach in 5% H_2O_2 in 20% DMSO/MeOH (recipe in materials and equipment) for 16–24 h at 4°C. This is then followed by rehydration (100% MeOH, 2 h, replaced fresh after 1 h; 20% DMSO/MeOH (recipe in materials and equipment), 1 h; 80% MeOH, 1 h; 50% MeOH, 1 h; 1× PBS, 2 h, replaced fresh after 1 h) at ~20°C. Adapted from Renier et al. (2014).
   i. Pro – reduces autofluorescence without affecting fluorophore intensity.
   ii. Con – de/rehydration steps add several hours, and bleaching adds an additional day to the protocol. MeOH preparation has been demonstrated not to be compatible with some antibodies as it can denature epitopes (Renier et al., 2014) and should be tested and validated on a case by case basis.

e. Option 4 – Room Temperature: doing all future steps at ~20°C (even when recommended at 4°C) will reduce tissue autofluorescence, but only slightly.
   i. Pro – shortens protocol by at least 4 h while still offering some level of autofluorescence reduction.
   ii. Con – the least effective means of reducing autofluorescence and performing all future steps at ~20°C can result in increased non-specific binding of antibodies.

Optional: Washes following autofluorescence quenching techniques can be performed in 1× PBS w/ heparin to further reduce vascular autofluorescence. Additionally, sodium azide can be added to PBS at a concentration of 0.02% for all future washes to prevent microbial growth.

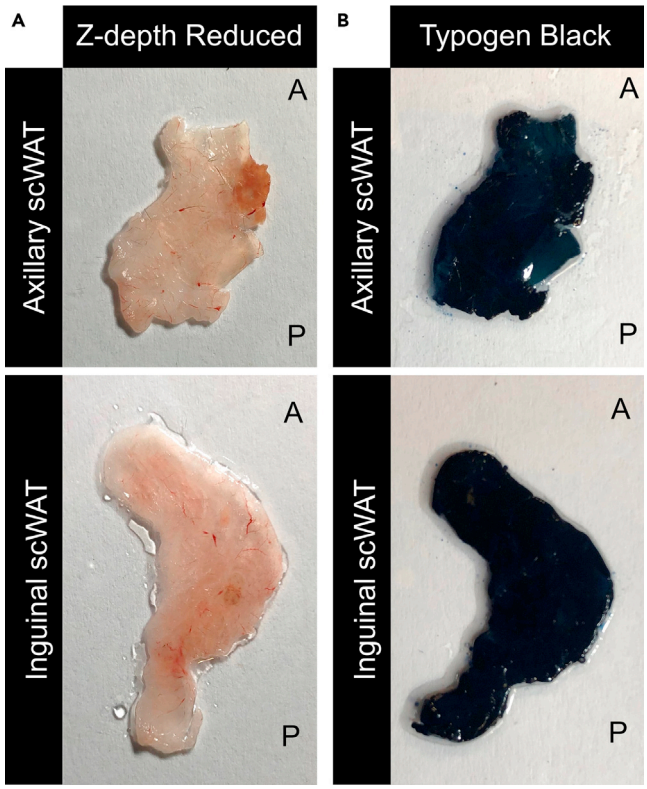

Figure 3. Quenching scWAT autofluorescence

(A and B) Intact axillary and inguinal scWAT depots immediately following Z-depth reduction (A) and subsequent autofluorescence quenching with typogen black (B).

⚠ CRITICAL: The addition of non-ionic detergents (Triton X-100, Tween 20) into wash buffers is common to many immunostaining protocols, but if using typogen black or TrueBlack autofluorescence quenching, avoid the addition of these detergents as they can wash out these stains from the tissue, greatly reducing their quenching potential. The use of detergents has not been tested with the other quenching techniques.

Fluorescent labeling

Timing: 1–7 days

The following steps outline how to fluorescently label Z-depth reduced adipose tissues or tissue blocks with primary antibodies, secondary antibodies, isolectin-IB_4, and DAPI. It is important that, to reduce non-specific binding and cross reactivity, co-staining follows the order outlined below.

Note: All washes and incubations are performed on an orbital shaker.

7. Using a Direct Reporter with Endogenous Fluorescence
   a. For mouse reporters with high signal intensity you can proceed directly to step 12 or if co-labeling is desired, proceed to step 8.
   b. For mouse reporters with low signal intensity it is recommended to boost the endogenous signal with a fluorophore conjugated antibody raised against the fluorescent protein of interest (i.e., anti-GFP conjugated Alexa488). This should be conducted immediately after step 9 is completed.

8. Primary Antibodies.
   a. To make primary antibody solution, you will dilute desired primary antibody in 1× PBS. Dilutions are antibody specific but typically range from 1:100 to 1:500. For antibodies not validated in this protocol, we recommend performing an antibody titration to find what works best. When titrating antibodies for this protocol, it is best to start with what works well for thin sections as this can provide decent enough staining. However, concentrating this further by two-fold typically results in the best outcomes. Concentrations for antibodies that we have validated with this protocol can be found in the key resources table.

   ⚠ CRITICAL: Non-ionic detergents are commonly added to antibody solutions to increase antibody permeation and reduce non-specific binding, however, as mentioned previously, detergents can reduce the effectiveness of typogen black and TrueBlack, and has not been tested with the other quenching techniques.

   b. You will need 3–5 mL of antibody solution per tissue, 3 mL being the absolute minimum if using a 6-well plate (see "troubleshooting".)
   c. Incubate tissues in primary antibody solution for 2 days at 4°C.
   d. After incubation, wash tissues in 1× PBS for 4 h at 4°C, replacing PBS every 1 h.

   Note: Perform sequential staining of antibodies when co-staining.

   Note: Only validated antibodies, tested in a Western Blot for specificity, and ideally with knock-out tissues and proper tissue controls, should be used for this procedure.

9. Secondary Antibodies
   a. If not using a directly conjugated primary antibody, dilute the desired secondary antibody in 1× PBS. Again, dilutions will be antibody and fluorophore specific, and should be optimized by titration. Secondary antibody concentrations tend to be identical to that used for immunostaining thin sections as recommended by the vendor. Only Alexa Fluor conjugated antibodies have been validated for this protocol. See validated secondary antibody concentrations in the key resources table.
   b. Incubate tissues in secondary antibody solution for 16–24 h at 4°C.
   c. After, wash tissues in 1× PBS for 4 h at 4°C, replacing PBS every 1 h.
   d. If co-staining with additional primary antibodies go back to step 8. If not, continue to step 10.

   ⚠ CRITICAL: We recommend avoiding the use of anti-mouse secondary antibodies. In most cases, non-specific binding has been unavoidable even with mouse-on-mouse blocking solutions. We recommend using antibodies raised in mice that are directly conjugated to a fluorophore, or preferably, using primary antibodies raised in alternative species such as rabbits.

10. Isolectin-IB_4 Vascular Staining
    a. If desired, label tissue vasculature using an Isolectin GS-IB_4 Alexa Fluor conjugate. See validated conjugates in the key resources table.
    b. Start by making Isolectin-IB_4 working solution. This can be made up in advance and frozen until time of use. Recipe described in materials and equipment.
    c. Allow Isolectin-IB_4 working solution to warm to ~20°C if not already. Add 3–5 mL to each tissue and incubate 16–24 h at ~20°C.
    d. Wash tissues in 1× PBS for 2 h at ~20°C, replacing PBS every 1 h.

    Optional: 5 μg/mL Isolectin-IB_4 working solution can be further diluted to 1 μg/mL to save on reagents with only a slight decrease in fluorescence intensity.

11. DAPI Nuclear Staining

a. If nuclear labeling is desired, start by making DAPI working solution from stock (described in materials and equipment), to be used fresh.
b. Incubate tissues in 3–5 mL DAPI working solution for 1 h at ~20°C.
c. Wash in 1× PBS for 2 h at ~20°C, replacing PBS after 1 h.

Mounting tissues

Timing: 3 days

A benefit of our protocol is the ability to permanently mount entire subcutaneous adipose tissues onto glass slides. Below we outline the steps for mounting.

12. Mounting tissues.
    a. Label the top of a large glass slide with mouse and tissue identifying information.
    b. Remove tissue from 6-well plate and place it at the center of the large glass slide.
    c. Add a generous amount (4–6 drops) of glycerol based mounting fluid to the tissue and place a large (48 × 60 mm) glass coverslip over the tissue.

Note: Glycerol-based mountants are more compatible with lipid laden tissue, and the added viscosity aids in adhering the coverslip to the slide. Incomplete coverslip adherence will result in tissues drying out, tissue oxidation, and decreased fluorophore lifespan.

    d. Apply gentle pressure to the coverslip to help it adhere to the tissue and slide. This is required for the next step to prevent the coverslip from sliding off the tissue.
    e. Place the coverslipped slides underneath uniform weight (15–20 kg) for 3 days. Ten textbooks placed over a series of slides works very well.
    f. After 3 days, remove the slide from underneath the textbooks and seal the edges with nail polish (Figure 4).
    g. Let dry for 5 min and proceed to imaging.

Note: For best outcome we recommend imaging within a week after mounting is complete. However, tissues are typically viable for imaging up to several weeks after mounting, and in some cases even several months.

Imaging

Timing: 1 min-40 h

Unique to this tissue processing approach is the ability to image whole adipose tissues effectively on standard widefield epifluorescence microscopes as well as confocal microscopes. Below are descriptions for best utilizing both methods of imaging.

13. Widefield Epifluorescence Microscopy
    a. Application:
        i. Suitable for single channel imaging, most qualitative analysis, and answering 'yes or no' questions through representative images. Fast time to image with only minutes needed to set up and capture several images.
    b. Magnifications:
        i. Images of adipose tissue can be captured on widefield microscopes easily using up to 40× objectives. Higher magnifications are possible but the associated decrease in working distance will make it a challenge to capture an in-focus image of large tissues.
    c. Extended Depth of Field (EDF):

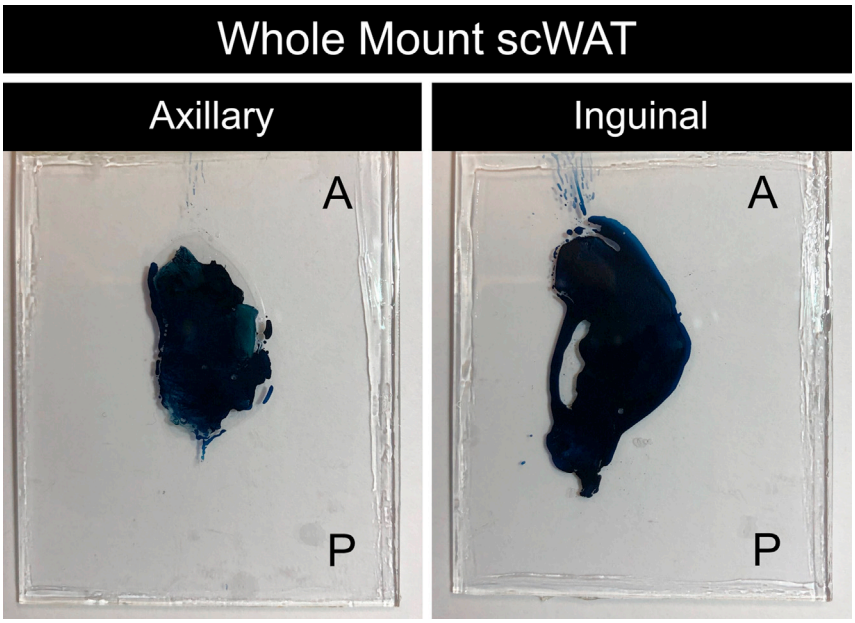

Figure 4. Mounting whole scWAT onto glass slides

Whole axillary and inguinal scWAT depots were fully mounted and sealed following immunostaining. Anatomical orientation was maintained by placing the tissues medial side up with the anterior portion of the tissue closest to the label.

i. After Z-depth reduction, tissue thickness will still range 40–200 μm. It can be difficult to capture images with the entire field of view in focus. A widefield microscope equipped with software capable of EDF can generate images with more of the field of view in focus. However, if EDF is pushed passed reasonable limits it can create artefact in your image.

d. Background Autofluorescence:

i. Even with autofluorescence quenching, background will still be present on epifluorescence microscopes (see "troubleshooting"). Strong staining and bright fluorophores can help overcome this, but confocal microscopy will further help to reduce background.

14. Confocal Microscopy

a. Application:

i. Suitable for quantification, demonstrating colocalization, and generating 3D images of specific regions or entire tissues to capture total neurovascular structure.

b. Magnifications:

i. Confocal microscopy is capable of imaging whole adipose tissues with up to 63× objectives without difficulty – further increased with confocal zoom. We have not tried higher objective magnifications at this time.

c. Tiled Z-stacks:

i. Entire adipose depots can be imaged in a series of Z-stacks extending through the entire tissue thickness. These can be tiled together to create one large 3D image. These can then be maximum intensity projected to create a 2D representation of the 3D data. These tiled z-stacks of whole tissues can be very time consuming to capture; taking anywhere from 8 to 40 h depending on the size of the tissue, number of fluorophores being captured, and the desired XY and Z resolutions.

EXPECTED OUTCOMES

With this protocol you will be able to fix and immunostain entire subcutaneous adipose depots and effectively image them without optical clearing on widefield and confocal microscopes. Below we

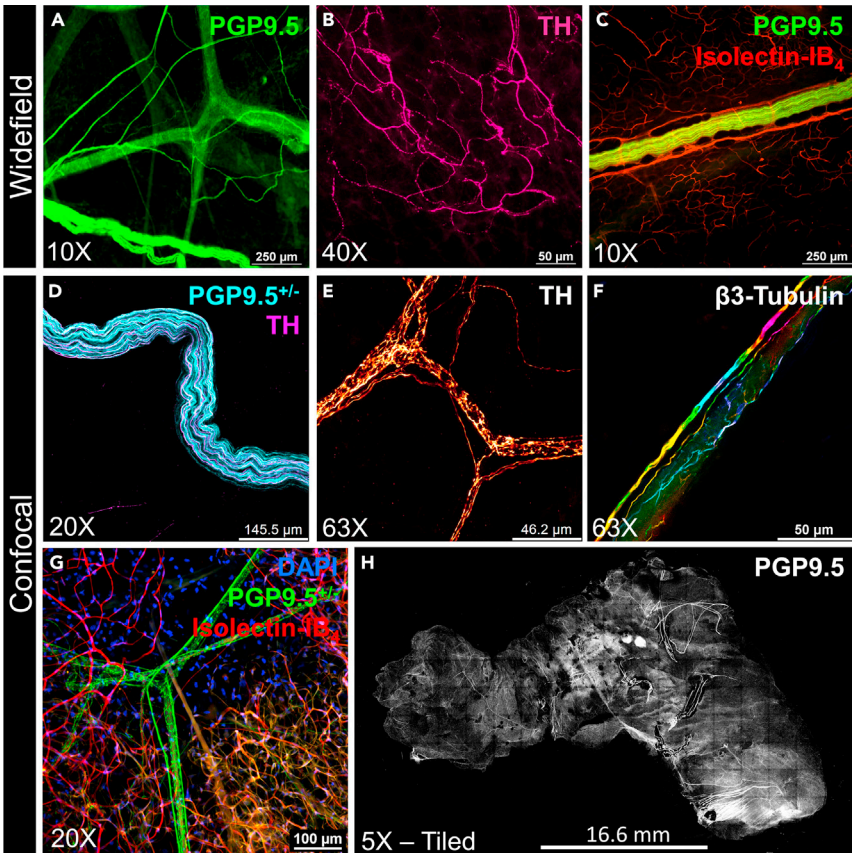

Figure 5. Imaging whole mount scWAT

(A–H) A series of images captured of whole mount scWAT display various combinations of the immunostaining and imaging described in this protocol. Captured on Nikon E400 (A–C), Leica SP8 (D–F, H), or Nikon A1R (G). Peripheral nerves (green) around autofluorescent lymphatic vessels. Widefield, 10× objective, 250 µm scale bar, BL6 mouse, anti-PGP9.5 (A). Sympathetic nerves (magenta). Widefield, 40× objective, 50 µm scale bar, BL6 mouse, anti-Tyrosine Hydroxylase (B). Peripheral nerve bundle (green) and blood vessels (red). Widefield, 40× objective, 250 µm scale bar, BL6 mouse, anti-PGP9.5 and Isolectin-IB4 (C). Peripheral nerve bundle (cyan) co-stained for sympathetic nerves (magenta). Confocal Z_{max} projection, 20× objective, 145.5 µm scale bar, PGP9.5-EGFP mouse, anti-Tyrosine Hydroxylase (D). Sympathetic nerves (orange) around a blood vessel (not stained). Confocal Z_{max} projection, 63× objective, 46.2 µm scale bar BL6 mouse, anti-Tyrosine Hydroxylase (E). Peripheral nerves (height-color coded) around a blood vessel (not stained). Confocal Z_{max} projection, 63× objective, 50 µm scale bar, BL6 mouse, anti-β3-Tubulin (F). Peripheral nerves (green), capillaries (red), nuclei (blue). Confocal Z_{max} projection, 20× objective, 100 µm scale bar PGP9.5-EGFP mouse, DAPI and Isolectin (G). Intact inguinal scWAT depot innervation (grey). Confocal Z_{max} projection, 5× objective, 16.6 mm scale bar, tiled whole depot, BL6 mouse, anti-PGP9.5 (H).

have provided examples of images captured using this protocol (Figure 5). For additional examples, see previous publications using this protocol (Blaszkiewicz et al., 2019, 2020; Willows et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

Additionally, we have developed a means to quantify whole tissue innervation that is complementary to the tissue processing and imaging techniques described here. For further details see Willows et al. (2021) and access the code at https://github.com/ktownsendlab/willows_et_al-2020.

LIMITATIONS

This protocol was developed with adipose nerve and neurovascular immunostaining in mind. We strongly believe that it is applicable for any number of research goals but will need to be validated

for uses outside of the scope of this protocol. The Z-depth reduction approach can potentially alter tissue morphology, so we recommend researchers implementing this technique conduct their own observational analysis to ensure that Z-depth reduction does not create artefact in the structures they analyze. Z-depth reduction, by definition, compresses tissue in the Z-axis and results in reduced Z-axis spatial resolution. (Willows et al., 2021) provides comparisons of adipocyte size with and without this technique, for example.

TROUBLESHOOTING

Problem 1

Autofluorescence quenching with typogen black or TrueBlack reduces signal intensity and hides fluorescing structures.

Potential solution

Try using more dilute concentrations of these quenching agents as they can mask fluorescence signal. We have demonstrated the use of a few different concentrations (Willows et al., 2021). Alternatively, using hydrogen peroxide bleaching or negating autofluorescence quenching entirely may be preferable in certain circumstances.

Problem 2

Tissues don't compress sufficiently when coverslip is added during mounting.

Potential solution

Reduce fixation time. Overly fixed tissues will be a bit more rigid and tend to 'slide around' when gentle pressure is applied with the coverslip.

Problem 3

High background autofluorescence even after quenching.

Potential solution

The use of very bright fluorophores at high concentrations (anti-rabbit IgG Alex Fluor 647 Plus, 1:200) will overcome most background signal if imaged on a confocal microscope.

Problem 4

Low intensity fluorescence signal.

Potential solution

Increase the volume of antibody solutions to 5 mL and avoid conjugated primary antibodies if possible. Indirectly labeling with secondary antibodies will provide greater signal intensity. Additionally, increasing incubation times may also help.

Problem 5

Imaging entire whole depot innervation is taking 'too long.'

Potential solution

Adjust image acquisition settings. Optimizing image capture settings for a tiled Z_{max} projection of an entire tissue can greatly reduce acquisition time. Settings will vary between confocal systems, but here are what we recommend for a Leica SP8 and Leica Stellaris 5 systems: Resolution 720 × 720 (can be reduced further if needed), scan speed 600 Hz, pinhole 1AU, scanned bi-directionally, 2-line average, Z-step size of 10–16 μm, tiled with a 10% tile overlap. Increase laser intensity and gain as needed. DAPI and Alexa647 can be imaged simultaneously, but all other fluorophores must be imaged sequentially by line (or by frame if using DAPI; long depletion time can cause bleed through.) Using a microscope that can 'lasso' areas of interest for tiling instead of imaging entire rectangles will prevent wasting hours on imaging empty space.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, [Kristy L. Townsend (kristy.townsend@osumc.edu)].

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all imaging datasets generated or analyzed by this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.101109.

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AUTHOR CONTRIBUTIONS

J.W.W. and M.B. developed, optimized, and wrote the protocol, and provided fluorescence imaging. K.L.T. conceived the study and wrote the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list. The author list of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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