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.
Protocol
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] | Abcam | Cat# ab108986 |

(Continued on next page)
### 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 [EP1570Y], 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 552, 1:1000**
Thermo Fisher Scientific Cat# A-32733

**Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568, 1:1000**
Thermo Fisher Scientific Cat# A-11005

**Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594, 1:1000**
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# 51813

**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-B4 From Griffonia simplicifolia, Alexa Fluor 568 Conjugate, 5 μg/mL**
Thermo Fisher Scientific Cat# I21411

**Isolectin GS-B4 From Griffonia simplicifolia, Alexa Fluor 594 Conjugate, 5 μg/mL**
Thermo Fisher Scientific Cat# I21413

**Isolectin GS-B4 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)/Pnos/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/ktownsendlab/willows_et_al-2020 (Continued on next page)
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   |
|--------------------------|---------------------|----------|
| 1x PBS                   | 1x                  | 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.

1x PBS w/Heparin

| Reagent       | Final concentration | Amount   |
|---------------|---------------------|----------|
| 1x PBS        | 1x                  | 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 H2O        | 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% H₂O₂ in 20% DMSO/MeOH

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| 20% DMSO/MeOH            | n/a                 | 90 mL    |
| 50% H₂O₂                 | 5% v/v              | 10 mL    |
| **Total**                | **5% v/v**          | **100 L**|

Make and use fresh.

### HEPES Buffer

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Milli-Q H₂O              | 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-IB₄ Working Solution

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| HEPES Buffer             | n/a                 | 100 mL   |
| Isolectin-IB₄ 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 H₂O              | 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 H₂O              | 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

 grote; 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., CO₂ 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.  ≤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.
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 approaches.

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

Option 2 – TrueBlack: incubate tissues in 1/3 (0.1%) TrueBlack® Lipofuscin Autofluorescence Quencher for 10 min at ~20°C followed by washing the tissue in 1X 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.

Option 3 – H2O2 Bleaching: stepwise dehydration in MeOH diluted in 1/3 PBS (50% MeOH, 1 h; 80% MeOH, 1 h; 100% MeOH, 2 h, replaced fresh after 1 h) at ~20°C Bleach in 5% H2O2 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; 1X 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 1X 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.
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-IB4, 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-IB4 Vascular Staining
    a. If desired, label tissue vasculature using an Isolectin GS-IB4 Alexa Fluor conjugate. See validated conjugates in the key resources table.
    b. Start by making Isolectin-IB4 working solution. This can be made up in advance and frozen until time of use. Recipe described in materials and equipment.
    c. Allow Isolectin-IB4 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-IB4 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):
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
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). Alter-
natively, 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. Addi-
tionally, 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 Stellars 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 ful-
filled 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 imag-
ing. 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 refer-
ence 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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