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Protocol for assessing *ex vivo* lipolysis of murine adipose tissue

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Highlights

Detailed protocol to quantitatively measure *ex vivo* adipose tissue lipolysis in mice

Steps to dissect adipose tissue and detect glycerol and fatty acids

Measures adipose tissue function under normal and disease/ trauma conditions

Here, we provide a detailed protocol for assessing *ex vivo* lipolysis of subcutaneous and visceral white adipose tissue. We describe a robust approach to detect depot-specific changes in lipolytic potential under basal and beta-adrenergic receptor-stimulated conditions. Given that adipose tissue plays a critical role in systemic metabolic health, this experimental protocol can be used to determine changes in adipose tissue function in health and disease.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Protocol for assessing *ex vivo* lipolysis of murine adipose tissue

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SUMMARY

Here, we provide a detailed protocol for assessing *ex vivo* lipolysis of subcutaneous and visceral white adipose tissue. We describe a robust approach to detect depot-specific changes in lipolytic potential under basal and beta-adrenergic receptor-stimulated conditions. Given that adipose tissue plays a critical role in systemic metabolic health, this experimental protocol can be used to determine changes in adipose tissue function in health and disease.

BEFORE YOU BEGIN

Before beginning work with mice, be sure the required institutional approvals are in place for your lab.

Institutional permissions

⏱ Timing: 30 min

Animal experiments were performed in accordance with and with the approval of the Institutional Animal Care and Use Committee at The Ohio State University.

1. Prepare Krebs Ringer Bicarbonate Hepes (KRBH) buffer, pH 7.4, one day prior to the protocol. One hour before harvesting the tissue, prepare KRBH-BSA buffer, pH 7.4, with 2% fatty acid-free BSA, and incubate at 37°C in a lab oven or incubator.

   Note: KRBH buffer can be stored for up to 3 months at 20°C–25°C. As KRBH is very stable at 37°C with low pH fluctuations, CO_2 incubator is optional. This protocol can be carried out in a regular laboratory oven or a shaker incubator with temperature adjustment controls.

2. Label 24-well plate(s) for respective adipose tissue depots and treatment specifications. Add 1 mL KRBH buffer into each well of the 24-well plate(s) and incubate along with the bottle or vial of KRBH.

   Note: Use 6-well plate(s) instead of 24-well plate(s) if the entire tissue depot is used for the assay. For a 6-well plate, adjust the volume to 2.5–3 mL KRBH per well to sufficiently submerge the tissue.

3. Disinfect surgical tools required for excising adipose tissue using 70% ethanol.
4. Disinfect a dissecting tray using 70% ethanol.
5. Label 0.5 mL tubes for media collection, or glycerol and fatty acid estimation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Phospho-Akt (Ser473) (D9E) XP, 1:1000	Cell Signaling Technology	Cat #4060, RRID: AB_2315049
Rabbit Akt (pan) (C67E7) Rabbit mAb, 1:1000	Cell Signaling Technology	Cat #4691, RRID: AB_915783
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	Cat #7074, RRID: AB_2099233
Donkey Anti-Rabbit IgG Antibody, HRP conjugate	Sigma-Aldrich	Cat #AP182P, RRID: AB_92591
Chemicals, peptides, and recombinant proteins		
Bovine Serum Albumin, Fatty Acid Free	Sigma-Aldrich	Cat #A7030
Hepes	Sigma-Aldrich	Cat #H4034
Sodium Bicarbonate	Thermo Fisher Scientific	Cat #S233500
Sodium Chloride	Thermo Fisher Scientific	Cat #S271-3
Magnesium Sulphate	Sigma-Aldrich	Cat #M7506
Calcium Chloride	MP Biomedicals	Cat #153502
Potassium Phosphate Dibasic	Sigma-Aldrich	Cat #P3786
Insulin (Humulin R)	Thermo Fisher Scientific	Cat #NC1415864
Isoprenaline hydrochloride	Abcam	Cat #ab146724
Tris-HCl	Sigma-Aldrich	Cat # 10812846001
Sodium Deoxycholate	Sigma-Aldrich	Cat #D6750
NP-40 (IGEPAL CA-630)	Thermo Fisher Scientific	Cat #J61055
Sodium Dodecyl Sulphate (SDS)	Bio-Rad	Cat #1610302
0.5 M Ethelene-Di-Amine-Tetraacetic Acid (EDTA)	Thermo Fisher Scientific	Cat #AM9260G
Ponceau S Solution	Sigma-Aldrich	Cat #P7170
Blotting Grade Blocker Non Fat Dry Milk	Bio-Rad	Cat #1706404XTU
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	Cat #04693132001
PhosSTOP™	Sigma-Aldrich	Cat #4906845001
Critical commercial assays		
Glycerol Assay Kit (Cell Based)	Abcam	Cat #ab133130
Free Fatty Acid Quantitation Kit	Sigma-Aldrich	Cat #MAK044
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	Cat #23225
Glycerol Standard Solution	Sigma-Aldrich	Cat #G7793
Experimental models: Organisms/strains		
Mouse: C57BL/6J (8–9 weeks old, male and female)	The Jackson Laboratory	Cat #000664
Software and algorithms		
GraphPad Prism 8.0	GraphPad	https://www.graphpad.com/
Gen 3.10	Agilent BioTEK	https://www.agilent.com/
Other		
BIOTEK Synergy H1 microplate reader	Agilent BioTEK	Cat #11-120-535
Surgical Scissors	Fine Science Tools	Cat #14501-14
Dumont #2 Forcep	Fine Science Tools	Cat #11223-20
Anatomical Forceps	Fine Science Tools	Cat #91100-12
24-Well Plate (Clear)	Sigma-Aldrich	Cat #SIAL0524
24-Well Plate (Black)	Sigma-Aldrich	Cat #CLS3925
Lysing Matrix D (RNAse-DNAse Free)	MP Biomedicals	Cat #116913050-CF
Bullet Blender®Homogenizer	Next Advance	Cat #BBX24
Sonicator, Model 120	Thermo Fisher Scientific	Cat #FB120110
Heating Block	Thermo Fisher Scientific	Cat #88860021
Isotemp Oven (Model 516G)	Thermo Fisher Scientific	Cat #13246516G
Gel Loading Tips (5–200 μL)	Electron Microscopy Sciences	Cat #72447-01
Nitrocellulose Membrane Roll, 0.2 μm	Bio-Rad	Cat #1620112

MATERIALS AND EQUIPMENT

Krebs-Ringer HEPES buffer, pH 7.4		
Reagent	Final concentration	Amount
HEPES (1 M)	30 mM	15 mL
Sodium bicarbonate (1 M)	10 mM	5 mL
Potassium phosphate (1 M)	4 mM	2 mL
Sodium chloride (4 M)	120 mM	15 mL
Magnesium sulphate (1 M)	1 mM	500 µL
Calcium chloride (1 M)	1 mM	500 µL
Milli-Q H_2O	n/a	462 mL
Adjust pH to 7.4	n/a	n/a
Filter sterilize	n/a	n/a
Total	n/a	500 mL

Store up to 3 months at 20°C–25°C.

Adipose tissue lysis buffer, pH 7.5		
Reagent	Final concentration	Amount
Tris-HCl (1 M)	50 mM, pH 7.5	2.5 mL
Sodium chloride (4 M)	150 mM	1.875 mL
IGEPAL CA-630 (10%)	1%	5 mL
Triton X-100 (10%)	1%	5 mL
Sodium dodecyl sulphate (SDS) (10%)	0.1%	500 µL
Sodium deoxycholate (10%)	0.5%	2.5 mL
EDTA (0.5 M)	2 mM	200 µL
Milli-Q H_2O	n/a	32.425 mL
Adjust pH to 7.5	n/a	n/a
Filter sterilize	n/a	n/a
Total	n/a	50 mL

Add one tablet of cOmplete™, EDTA-free protease inhibitor cocktail and PhosSTOP™ phosphatase inhibitors to 10 mL of lysis buffer. Store up to 12 months at −20°C in aliquots of 10 mL.

STEP-BY-STEP METHOD DETAILS
Dissection of subcutaneous (iWAT) and visceral (eWAT) adipose tissue

© Timing: 10 min/animal

In this section, we will excise subcutaneous (i.e., inguinal) and visceral (i.e., perigonadal) white fat pads from adult (8–9 weeks old) C57BL/6J mice. Careful dissection of these fat depots is critical for the procedure. Follow Figures 1A–1E for detailed identification of the fat pads.

1. Euthanize mouse – Follow general CO_2 euthanasia procedure in accordance with the approval of the Institutional Animal Care and Use Committee.
2. Excise subcutaneous white adipose tissue depot.
   a. Place 8–9 week-old C57BL/6J mouse (male or female) ventral side up and spray with 70% ethanol.
   b. Make a longitudinal midline incision from the base of the tail to top of the sternum, as shown in Figure 1A.

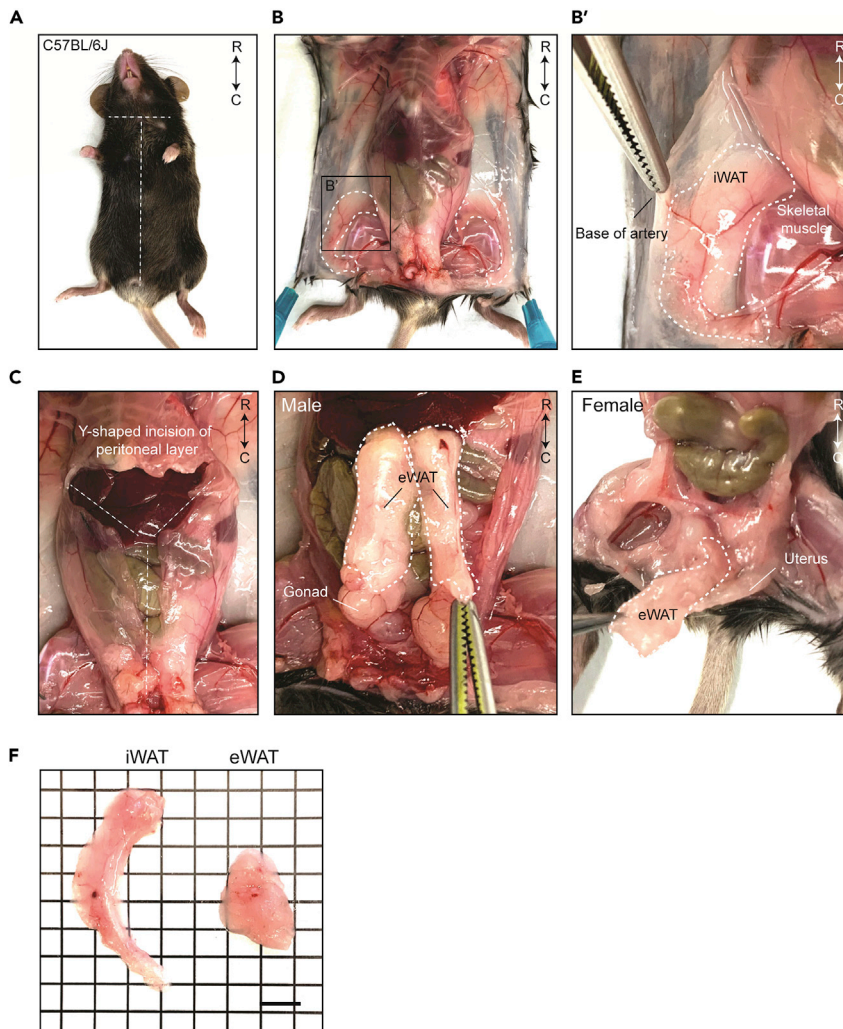   △ CRITICAL: Be careful not to dissect the underlying peritoneal cavity at this point.

Figure 1. Adipose tissue dissection from adult mice

(A) Schematic of adipose tissue dissection in C57BL/6J mice (8–9 weeks old). Mouse is positioned ventral side up, with major white adipose tissue located caudal to the rib cage (R: rostral, C: caudal). White dotted lines represent the dissection scheme.

(B) Subcutaneous adipose tissue depots (iWAT) are exposed after making skin incision and are bilaterally positioned beside the skeletal muscle (outlined by white dotted line).

(B') Closer view of iWAT with base of the major artery connecting the adipose tissue labeled to show where to make superficial cut into tissue.

(C) Schematic of dissection of peritoneal layer of C57BL/6J mouse for eWAT extraction. White dotted lines represent the dissection scheme.

(D) eWAT of male C57BL/6J mouse with gonads labeled for reference of location.

(E) eWAT of female C57BL/6J mouse with uterus labeled for reference of location.

(F) Isolated iWAT and eWAT from an adult male C57BL/6J mouse. Scale bar, 0.5 mm.

c. Make a lateral incision to either side of the midline at the top of the sternum and use anatomical forceps or thumb to pull the skin apart.

d. Once the adipose tissue is exposed as shown in the Figure 1B, pin the skin to the dissection base.

Note: Subcutaneous adipose tissue is bilaterally positioned around the skeletal muscle as shown in Figure 1B'.

   e. Use a pair of anatomical forceps to hold the edge of the depot (Figure 1B'). Make a superficial cut at the base of the large vessel that joins the tissue, and cut along the tissue boundaries, gently pulling the tissue away from the skin.

   f. Once the tissue is completely detached from the skin, tap it on a Kimwipe to drain any interstitial fluid. Weigh the fat depot using a precision scale (0.2–3 grams range).

3. Excise visceral white adipose tissue depot.

   a. Make a midline Y-shaped incision in the peritoneal cavity to expose the visceral organs (Figure 1C).

Note: The perigonadal adipose tissue is located just below the rostral starting point of the inguinal adipose tissue and can be visualized once the peritoneal cavity is cut open. For male mice, it is around the gonadal sac (Figure 1D); for female mice, it is around the uterus (Figure 1E).

⚠ CRITICAL: Pay attention not to dissect parts of the uterus or gonads.

   b. Using a pair of anatomical forceps, hold and cut the tissue along the anterior edge of the gonads or uterus. After dissection, repeat step 2f. Following dissection, adipose tissue should appear as shown in Figure 1F.

Setting up plate for glycerol and fatty acid estimation

⏱ Timing: 30 min

Following tissue dissections, set up the assay plate for glycerol and fatty acid release (Figure 2A). Beta-adrenergic receptor stimulation is known to activate adipose tissue lipolysis (Greenberg et al., 2001). Thus, we used the β_3-adrenoreceptor agonist isoproterenol to stimulate lipolysis in adipose tissue explants.

4. Tissue transfers.

   a. Immediately after dissection and weighing, transfer whole iWAT (~350–500 mg) or eWAT (~150–400 mg) adipose tissue into 15-mL centrifuge tubes containing 5 mL of pre-warmed KRBH buffer (w/o 2% BSA), pH 7.4.

   b. Incubate the tube in a laboratory oven or CO_2 incubator as described in step 1 of 'before you begin'.

   c. Repeat steps 1–3 for each biological replicate.

   d. After dissecting the last animal, the next step is tissue trimming and setting up a 24-well plate for the assay.

5. Plate set up and time course for glycerol and fatty acid release.

   a. Transfer the content of the 15 mL tube (step 4) into a sterile 15 cm tissue culture plate. Using a sterile scissor, cut 50 mg (approximately 0.5 mm³) of adipose tissue, and place it into one well of a 24-well plate containing 1 mL of pre-warmed KRBH buffer with 2% fatty acid free BSA (KRBH-BSA buffer).

   b. While trimming adipose tissue replicates, incubate the plate in laboratory oven (37°C).

   c. Following plate set up, aspirate medium from each well of the plate and replenish with KRBH-BSA buffer containing either vehicle (CTR) or isoproterenol (10 μM) (representative plate layout in Figure 2A).

   d. After 5 min of setting up the plate, collect 100 μL of medium (i.e., KRBH-BSA buffer) as a baseline. Replenish with 100 μL of the respective medium (with vehicle or isoproterenol). Incubate for 2 h at 37°C and collect 1 mL aliquot of the incubation medium.

   e. Incubate baseline and 2 h media aliquots at 65°C for 10 min to overcome residual enzymatic action in the released media.
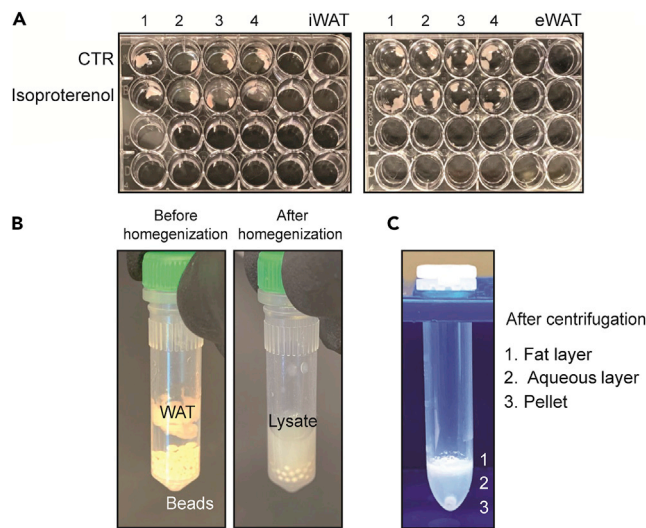
Figure 2. Plate layout for lipolysis assay and protein extraction

(A) 24-well plate layout containing dissected iWAT (n=4 biological replicates) and eWAT (n=4 biological replicates) for glycerol and fatty acid estimation. Each well contains 50 mg of either iWAT or eWAT.

(B) Before and after homogenization of 50 mg of adipose tissue in Lysing Matrix D tubes (2 mL) containing 1.4 mm diameter zirconium beads and 300 μL of adipose-lysis buffer.

(C) Distribution of the fat layer, aqueous layer (lysate), and pellet in 1.5-mL tube after centrifugation of homogenized adipose tissue.

> **Note:** It is recommended to set up the reaction plate close to the incubator or oven to minimize time loss during the dissection and incubation.

   f. Wash the tissue chunks in the 24-well plate using 1 mL of 1× PBS, and store the tissue at −80°C after discarding PBS.

⏸ **Pause point:** Samples can be assayed for glycerol and fatty acid right away or later. In the case of assaying later, samples should be stored at −80°C.

Glycerol estimation

⏱ Timing: 1.5–2 h

Glycerol released in the media is estimated using a glycerol assay kit (Abcam) by a colorimetric method.

> **Alternatives:** Free glycerol reagent (Sigma, Cat #F6428) or free glycerol assay kit (Abcam, Cat #ab174092) can be used for colorimetric or fluorometric detection, respectively.

> ⚠ CRITICAL: Assay reagents may be hazardous (may cause eye irritation and asthma if inhaled). Refer to the safety data sheet for use. Wear protective eyewear and gloves at all times while handling the reagents.

6. Thaw samples stored at −80°C and glycerol reagent stored at −20°C at 20°C–25°C for 30 min protected from light.
7. Prepare a range of glycerol standards (e.g., 125, 62.5, 31.25, 15.6, 7.81, 3.90, 1.95 and 0 μg/mL) in distilled (d)H_2O.

Note: Glycerol standards can be prepared in either PBS or dH$_2$O. Before testing all the samples, run a trial assay to determine if the glycerol levels in the media are within the standard range of the assay. Adjust the standard range if required.

8. In a clear 96-well plate, add 50 μL of either sample or standard plus 100 μL of the glycerol reagent in duplicates. Mix well by shaking on a horizontal shaker for 15 s. Cover the plate using aluminum foil.
9. Incubate for 15 min at 20°C–25°C.
10. Collect absorbance at 540 nm using a standard UV-visible spectrophotometric plate reader capable of end-point absorbance analysis.

Note: It is highly recommended to continue with the fatty acid estimation after this to avoid freeze-thaw cycles of the glycerol-fatty acid sample. If a break is unavoidable, samples should be stored at 4°C protected from light, and fatty acids can be assayed the following day.

Free fatty acids estimation

⏱ Timing: 2 h

Released free fatty acids in the media are estimated using a free fatty acid kit (Sigma) by a fluorescence-based method.

Alternatives: WAKO-NEFA-HR(2) free fatty acid kit (Fujifilm, Cat# 999-34691 and 999-34891) can be used for colorimetric detection.

⚠ CRITICAL: Assay reagents may be hazardous (may cause eye irritation and asthma if inhaled). Please refer the safety data sheet for use. Wear protective eyewear and gloves at all times while handling the reagents.

11. Sample and standard preparation.
    a. Thaw samples following step 6.
    b. Prepare a range of standards.

    Prepare a 0.1 nmol/μL palmitic acid standard solution by diluting the stock solution to 10 times using the assay buffer provided in the kit in a 1.5-mL centrifuge tube. In a black solid bottom 96-well plate add 0, 2, 4, 6, 8 and 10 μL of the diluted standard solution generating a 0.2–1 nmol/well concentration.

    ⚠ CRITICAL: Wrap the tube using aluminum foil to protect it from light at all times.

    c. Dilute samples 1:1 using the assay buffer (e.g., 50 μL sample + 50 μL assay buffer). Add 5 μL of the diluted sample to the plate.
    d. For standards, add 50, 48, 46, 44, 42 and 40 μL of assay buffer; for samples, add 45 μL of assay buffer to bring the final volume to 50 μL.
    e. Add 2 μL of ACS reagent (Acetyl CoA Synthetase enzyme mix) to each well of sample and standard solutions and incubate for 30 min at 37°C.
    f. Mix well by shaking on a horizontal shaker for 15 s. Cover the plate using aluminum foil.
12. Assay.
    a. Master reaction mix composition is provided below for each reaction (Table 1). Calculate the amount of master mix required based on sample number and technical replicates and prepare it accordingly.

Table 1. Master-mix composition of FFA assay

Reagent	Volume (µL)
Fatty Acid Assay buffer	44
Fatty Acid Probe	2
Enzyme mix	2
Enhancer	2

    b. To 50 µL of either sample or standard, add 50 µL of the master mix in duplicate. Cover the plate using aluminum foil to prevent light exposure. Mix well by shaking on a horizontal rocker for 15 s.

    ⚠ CRITICAL: It is critical to use black plates with a solid bottom for this assay if using a micro-plate reader that has top detection capability. Otherwise, use black plates with a clear bottom.

13. Incubate for 30 min at 37°C.
14. Measure fluorescence intensity at an excitation maximum of 535 nm and an emission maximum of 590 nm using a multimode plate reader that can measure fluorescence intensity under standard conditions.

Protein extraction and estimation from adipose tissue

🕐 Timing: 2.5–3 h

15. Tissue homogenization.
    a. Thaw 24-well plate containing adipose tissue samples on ice for 30 min.
    b. Add tissue samples into labeled and pre-chilled DNAse-RNAse free Lysing Matrix D tubes (2-mL) that contain zirconium beads of 1.4 mm diameter.

Alternatives: Safe-Lock tubes (2.0 mL, Eppendorf, Cat #022363352; 1.5 mL, Eppendorf, Cat #022363204), Stainless Steel Beads, 5 mm (Qiagen, Cat #69989). If using the Qiagen beads, one 5 mm bead/2-mL tube is sufficient (An et al., 2017).

    c. Add six volumes of ice-cold lysis buffer (e.g., for 100 mg of tissue, add 600 µL of lysis buffer), supplemented with protease and phosphate inhibitor for each sample. Lysis buffer can be stored up to 12 months at −20°C in aliquots of 10 mL.
    d. Homogenize the samples in a bullet blender homogenizer for 5 min at maximum speed.

Note: The sample will appear milky at the end of homogenization (Figure 2B).

    e. Transfer the tissue homogenate to clean pre-chilled 1.5-mL tubes.
    f. Centrifuge the samples at 13,523 g for 15 min at 4°C.
    g. After centrifugation three different layers are observed, as shown in Figure 2C.

16. Lysis and separation.
    a. Using a 23-gauge needle or a gel-loading fine tip, carefully penetrate the fat layer (Figure 2C), collect the aqueous supernatant, and transfer into clean pre-chilled 1.5-mL tubes.
    b. Incubate on ice for 30 min and sonicate for 10 s using a hand-held sonicator set at 80% amplitude.
    c. Centrifuge at 13,523 g for 15 min at 4°C.
    d. Transfer the aqueous supernatant into clean pre-chilled 1.5-mL tubes.
    e. Repeat steps 16c and 16d two more times to maximize extraction of the lipid content.
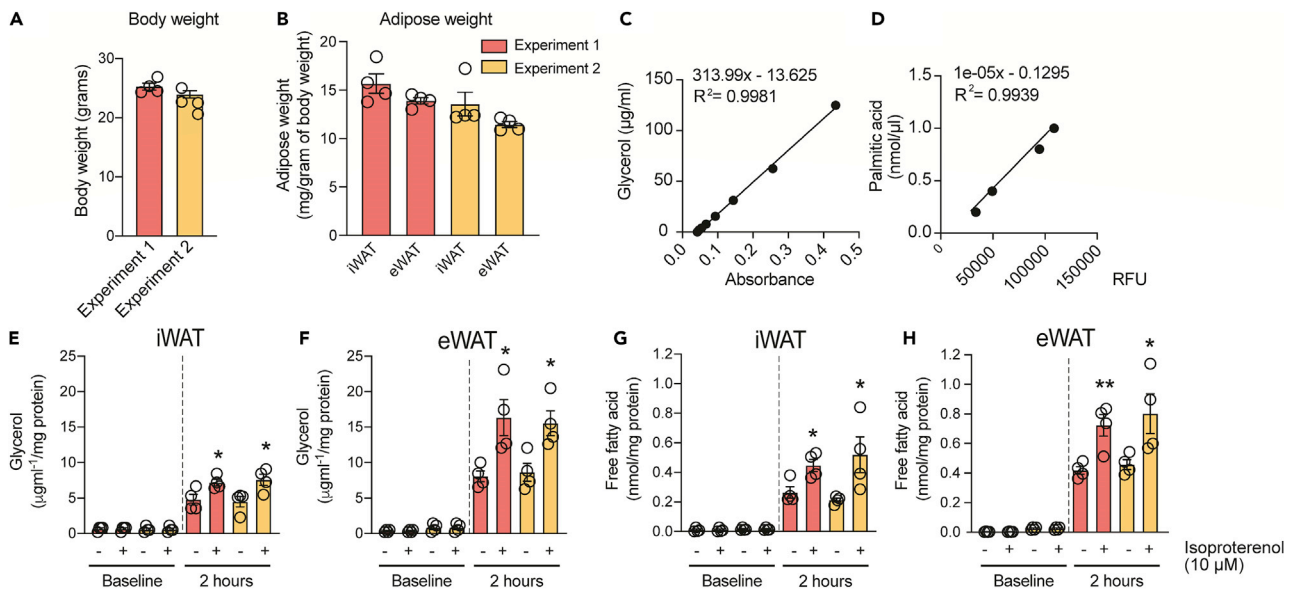
Figure 3. Assessing basal and isoproterenol induced adipose tissue lipolysis

(A) Mouse (8–9 weeks old male C57BL/6J) body weight. Mean and SEM (n=4 biological replicates/experiment, 2 independent experiments).

(B) Adipose tissue weight. Mean and SEM (n=4 biological replicates/experiment, 2 independent experiments).

(C and D) Glycerol (C) and fatty acid (D) standard linear plot (average of two measures/data point).

(E and F) Released glycerol content from iWAT (E) and eWAT (F) after 2 h of treatment with either vehicle (dH$_2$O) or isoproterenol (10 μM). Mean and SEM (unpaired 2-tailed Student's t-test *p<0.05, n=4 biological replicates/experiment, 2 independent experiments).

(G and H) Released free fatty acids from iWAT (G) and eWAT (H) after 2 h of treatment with either vehicle (dH$_2$O) or isoproterenol (10 μM). Mean and SEM (unpaired 2-tailed Student's t-test *p<0.05, **p<0.01, n=4 biological replicates/experiment, 2 independent experiments).

    f. Store the samples at −80°C until protein estimation.

17. Protein estimation.
    a. Thaw samples on ice for 20 min.
    b. Dilute samples to 10 times using dH$_2$O.
    c. Add 5 μL of samples and 200 μL of BCA reagent mix prepared freshly (1 part of Reagent A: 50 parts of Reagent B, e.g., to 5-mL of Reagent A, add 100 μL of Reagent B; mix well by vortex before use).
    d. Incubate the samples at 37°C for 30 min.
    e. Measure absorbance at 562 nm.
    f. Calculate the appropriate concentration of protein in the lysate against a BSA standard (0–2 mg/mL).

Glycerol and fatty acid quantitation and normalization

18. For glycerol (Figures 3C, 3E, and 3F):
    a. Evaluate glycerol levels in the samples by measuring absorbance at 540 nm using a standard UV-visible spectrophotometric plate reader. Extrapolate the glycerol concentration using the standard plot that was generated in step 10.
    b. Normalize the value to the respective protein concentration of the sample.
    c. Glycerol levels will be expressed as μg/mL glycerol per mg of protein.

19. For free fatty acids (Figures 3D, 3G, and 3H):
    a. Evaluate fatty acid levels in the samples by measuring fluorescence intensity at an excitation maximum of 535 nm and an emission maximum of 590 nm using a multimode plate reader that can measure fluorescence intensity under standard conditions. Extrapolate the fatty acid concentration using the standard plot that was generated in step 14.

    b. Multiply it by two (dilution factor, as the sample was originally diluted to half).

    c. Divide the value by five (5 μL is sample volume/well.).

    d. Normalize the value in step 20c to the respective protein concentration of the sample esti-
       mated earlier.

    e. Free fatty acid levels will be expressed as nmol/mg protein.

Alternatives: Glycerol and fatty acids can also be normalized using the dry weight of the sam-
ple or by total lipid content of the sample (Girousse et al., 2013).

⚠ CRITICAL: We recommend to confirm functional viability of the adipose tissue under the
experimental conditions described above. To do so, treat additional iWAT (50 mg) or
eWAT with vehicle (CTR) or insulin (100 nM) in a 24-well plate containing KRBH-BSA buffer
while running steps 5a–e and incubate at 37°C for 2 h. At the end of the incubation period,
collect the adipose tissue and homogenize it as described in steps 15 and 16. Fractionate
a portion of the lysate (e.g., 20 μg total protein) by SDS-polyacrylamide gel electrophoresis.
Transfer (300 mAmp for 3 h) the separated proteins to a nitrocellulose membrane (0.2 μm,
Bio-Rad). To confirm equal loading and transfer of the samples, stain the membrane with Pon-
ceau S (MilliporeSigma). After blocking at 20°C–25°C with 5% nonfat milk (Bio-Rad) in TBST
for 1 h, probe the membrane with a rabbit monoclonal anti-phospho-Akt (Ser473) (1:1000).
Depending on the phospho-antibody manufacturer recommendations, 5% (w/v) BSA in
TBST may be used as an alternative blocking solution. Insulin activates the Akt pathway in
a biochemically active adipose tissue as shown in Figure 4. Use a rabbit monoclonal anti-
Akt (pan) (1:1000) for protein-loading control. Densitometry analysis can be performed using
ImageJ (National Institute of Health). After subtracting the background, measure the inten-
sity of phospho-Akt bands at 60 kDa and normalize it to the loading control (e.g., total-Akt).
We recommend using three to four biological replicates for each experimental condition.

EXPECTED OUTCOMES

Completion of this experimental protocol will allow users to correctly assess lipolytic potential by esti-
mating glycerol and fatty acid content released from adipose tissue explants under basal and beta-
adrenergic receptor stimulation. It is expected that basal glycerol and fatty acid levels will increase
over the 2-h incubation period. Given that lipolytic machinery genes (i.e., pHSL and Atgl) are differen-
tially expressed in subcutaneous and visceral fat depots, it is expected that fat depots from distinct
anatomical regions differ in lipolytic potential (Lee et al., 2013). At a minimum, users are expected to
see a 1.5 fold increase of both glycerol and fatty acid levels over basal levels with isoproterenol induc-
tion. If the experimental condition (e.g., obesity, type 2 diabetes etc.) alters the expression of the adi-
pose tissue lipolytic machinery, changes in basal and stimulated lipolysis can be easily detected.

QUANTIFICATION AND STATISTICAL ANALYSIS

ImageJ software was used to quantify band intensities from immunoblots (https://imagej.nih.gov/ij/).
Statistical analysis was performed using Prism (v. 8.0, GraphPad Prism). Data shown in this study are
presented as the mean ± standard error of the mean (SEM) of four biological replicates. Statistical
significance between groups was evaluated using 2-tailed Student's t-test. Significance was defined
as *$p < 0.05$, and **$p < 0.01$.

LIMITATIONS

Here, we have described a protocol to measure *ex vivo* the lipolytic potential of white adipose tissue.
The same method can be adapted to measure lipolytic potential of brown adipose tissue. Although
this technique can reliably detect endogenous lipolytic potential of adipose tissue as a functional
readout of metabolic homeostasis in health and disease (Roy and Tedeschi, 2021), it remains an
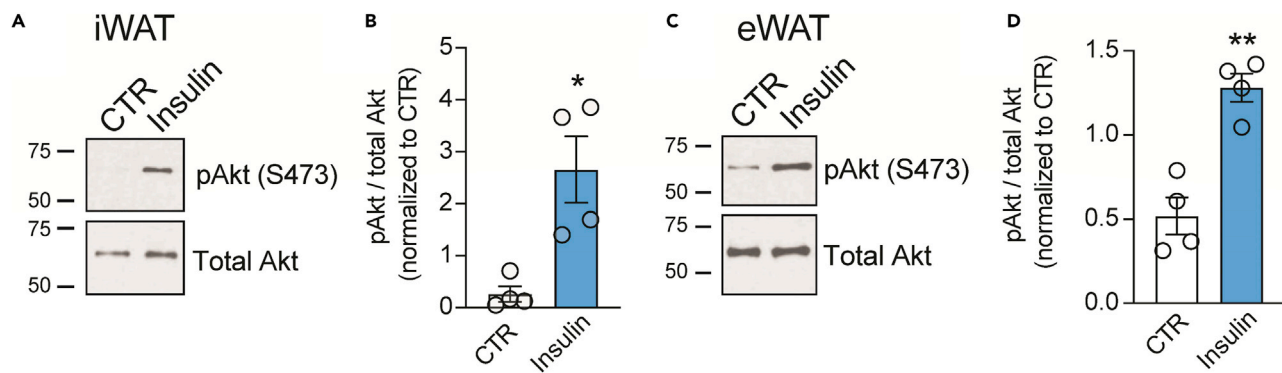end point assessment. When continuous assessment is necessary, in-situ microdialysis of adipose

Figure 4. Biochemical and functional characterization of adipose tissue explants

(A) Immunoblot shows phospho-Akt (Ser473) expression in iWAT treated with either vehicle (CTR) or 100 nM Insulin for 2 h. Total-Akt is used as loading control.

(B) Quantification of (A). Mean and SEM (unpaired 2-tailed Student's *t*-test *p<0.05, n=4 biological replicates/condition).

(C) Immunoblot shows phospho-Akt (Ser473) expression in eWAT treated with either vehicle (CTR) or 100 nM Insulin for 2 h. Total-Akt is used as loading control.

(D) Quantification of (C). Mean and SEM (unpaired 2-tailed Student's *t*-test **p<0.01, n=4 biological replicates/condition).

tissue should be considered as it enables real-time detection of lipolysis in response to an external stimuli like cold exposure (Arner et al., 1988; Weir et al., 2018).

TROUBLESHOOTING

Problem 1
Glycerol and fatty acid levels too low to detect.

Potential solution
Fluorescence-based assays for fatty acid detection or highly sensitive absorbance-based assays for glycerol detection can be used to measure low glycerol or fatty acid levels. In addition, this protocol can be performed under shaking conditions to maximize the release of glycerol and fatty acids (Sveidahl Johansen et al., 2021). As BSA is known to bind to fatty acids, it is crucial to use fatty acid-free BSA when running the protocol. The use of conventional BSA or the absence of BSA in the incubation buffer will reduce the yield of fatty acids released from fat depots. For long-term analysis (e.g., >2 h), we recommend adding Triacsin C (5 μM) to the incubation buffer. Triacsin C inhibits acyl-CoA-synthetase and blocks fatty acid re-esterification, which may lower fatty acid levels.

Problem 2
Variability in glycerol and fatty acid levels.

Potential solution
Assay reagents may have been freeze-thawed multiple times. To minimize variability, we strongly suggest to aliquot and freeze assay reagents and use fresh aliquots every time. When ready, aliquots are thawed at 20°C–25°C. The lipolytic potential may also vary based on the time of the day, as reported by others (Shostak et al., 2013). Expression levels of HSL and ATGL, the primary lipolytic enzymes, fluctuate during the day. Therefore, we recommend to keep the time (ideally between 10 am and 4 pm) of the day during which the experiments are conducted consistent. Under normal feeding conditions, the lipolytic potential in rodents (i.e., mice and rats) is high during the day (inactive phase). Therefore, careful planning and tissue dissection at a specific time of the day can greatly minimize variability when measuring the lipolytic potential in adipose tissue.

Problem 3
Lipids in the adipose tissue lysate.

Potential solution

Eliminating lipids in adipose tissue protein lysate can be challenging. We recommend maximizing the volume of the lysis buffer as described in the protocol (i.e., 600 μL for 100 mg of adipose tissue). This will enable repeated centrifugation steps and careful pipetting (using a long pipette tip) to maximize lipid removal. Users can also use a 23-gauge needle to remove the aqueous layer without disturbing the fat layer. Delipidation using chloroform/methanol represents another valuable option to consider (Harney et al., 2021).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Andrea Tedeschi (andrea.tedeschi@osumc.edu).

Materials availability

This study did not generate any new reagents.

Data and code availability

The original data for Figures 3 and 4 will be available from the lead contact on request.

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AUTHOR CONTRIBUTIONS

D.R. designed and performed the experiments, optimized procedures, and wrote the protocol. J.M.M. performed the experiments and wrote the protocol. A.T. conceived and supervised the study and wrote the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

An, Y.A., Sun, K., Joffin, N., Zhang, F., Deng, Y., Donze, O., Kusminski, C.M., and Scherer, P.E. (2017). Angiopoietin-2 in white adipose tissue improves metabolic homeostasis through enhanced angiogenesis. Elife 6, e24071. https://doi.org/10.7554/eLife.24071.

Arner, P., Bolinder, J., Eliasson, A., Lundin, A., and Ungerstedt, U. (1988). Microdialysis of adipose tissue and blood for in vivo lipolysis studies. Am. J. Physiol. 255, E737–E742. https://doi.org/10.1152/ajpendo.1988.255.5.E737.

Girousse, A., Tavernier, G., Valle, C., Moro, C., Mejhert, N., Dinel, A.L., Houssier, M., Roussel, B., Besse-Patin, A., Combes, M., et al. (2013). Partial inhibition of adipose tissue lipolysis improves glucose metabolism and insulin sensitivity without alteration of fat mass. PLoS Biol. 11, e1001485. https://doi.org/10.1371/journal.pbio.1001485.

Greenberg, A.S., Shen, W.J., Muliro, K., Patel, S., Souza, S.C., Roth, R.A., and Kraemer, F.B. (2001).

Stimulation of lipolysis and hormone-sensitive lipase via the extracellular signal-regulated kinase pathway. J. Biol. Chem. 276, 45456–45461. https://doi.org/10.1074/jbc.M104436200.

Lee, M.J., Wu, Y., and Fried, S.K. (2013). Adipose tissue heterogeneity: implication of depot differences in adipose tissue for obesity complications. Mol. Aspect. Med. 34, 1–11. https://doi.org/10.1016/j.mam.2012.10.001.

Roy, D., and Tedeschi, A. (2021). The role of lipids, lipid metabolism and ectopic lipid accumulation in axon growth, regeneration and repair after CNS Injury and disease. Cells 10, 1078. https://doi.org/10.3390/cells10051078.

Shostak, A., Meyer-Kovac, J., and Oster, H. (2013). Circadian regulation of lipid mobilization in white adipose tissues. Diabetes 62, 2195–2203. https://doi.org/10.2337/db12-1449.

Sveidahl Johansen, O., Ma, T., Hansen, J.B., Markussen, L.K., Schreiber, R., Reverte-Salisa,

L., Dong, H., Christensen, D.P., Sun, W., Gnad, T., et al. (2021). Lipolysis drives expression of the constitutively active receptor GPR3 to induce adipose thermogenesis. Cell 184, 3502–3518.e33. https://doi.org/10.1016/j.cell.2021.04.037.

Weir, G., Ramage, L.E., Akyol, M., Rhodes, J.K., Kyle, C.J., Fletcher, A.M., Craven, T.H., Wakelin, S.J., Drake, A.J., Gregoriades, M.L., et al. (2018). Substantial metabolic activity of human Brown adipose tissue during warm conditions and cold-induced lipolysis of local triglycerides. Cell Metab. 27, 1348–1355.e4. https://doi.org/10.1016/j.cmet.2018.04.020.

Harney, D.J., Cielesh, M., Chu, R., Cooke, K.C., James, D.E., Stöckli, J., and Larance, M. (2021). Proteomics analysis of adipose depots after intermittent fasting reveals visceral fat preservation mechanisms. Cell Rep. 34, 108804. https://doi.org/10.1016/j.celrep.2021.108804.