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Measurement of long-chain fatty acid uptake into adipocytes

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Abstract

The ability of white and brown adipose tissue to efficiently take up long-chain fatty acids is key to their physiological functions in energy storage and thermogenesis respectively. Several approaches have been taken to determine uptake rates by cultured cells as well as primary adipocytes including radio- and fluorescently labeled fatty acids. In addition, the recent description of activatable bioluminescent fatty acids has opened the possibility for expanding these in vitro approaches to real-time monitoring of fatty acid uptake kinetics by adipose depots in vivo. Here we will describe some of the most useful experimental paradigms to quantitatively determine long-chain fatty acid uptake by adipocytes in vitro and provide the reader with detailed instruction on how bioluminescent probes for in vivo imaging can be synthesized and used in living mice.

Keywords

Fatty acid uptake; adipocytes; white adipose tissue; brown adipose tissue; long-chain fatty acids; fatty acid transport proteins; in vivo imaging

1. Introduction

1.1 Fatty acid uptake by adipocytes

Long-chain (LCFA) fatty acid uptake by adipocytes plays an important role in maintaining lipid homeostasis. Adipose tissue produces lipoprotein lipase (Wang & Eckel 2009), which can generate LCFAs in the local vasculature through its action on triacylglycerol (TAG) rich lipoprotein particles. Following transition across the endothelium (Hagberg *et al.* 2010), interstitial albumin bound LCFAs can interact with adipocytes. This interaction is thought to involve dissociation from albumin, fatty acid translocation across the plasma membrane, and interaction with cytosolic fatty acid binding proteins and/or activation to acyl-CoA (Bernlohr, Coe & LiCata 1999), which subsequently can participate in a variety of metabolic process such as mitochondrial β -oxidation, particularly in brown adipose tissue (BAT), and

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TAG synthesis, particularly in white adipose tissue (WAT). Depending on the assay system and physiological condition several, if not all, of these steps can become rate limiting for the net flux of exogenous LCFAs into adipocytes. However particular attention has been devoted to identifying proteins involved in the binding and transfer of LCFAs on the plasma membrane leading to the identification of the solute carrier family 27 (Fatty Acid Transport Proteins, FATPs) (Anderson & Stahl 2013), the scavenger receptor CD36 (Su & Abumrad 2009), and the mitochondrial aspartate amino transferase (FABPpm) (Isola *et al.* 1995). Of these potential membrane LCFA transporters WAT has been shown to express robust levels of FATP1, FATP4, CD36, and FABPpm (Hui & Bernlohr 1997). Importantly, physiological stimuli such as insulin stimulation of white adipocyte (Wu *et al.* 2006b) and adrenergic stimulation of brown adipocyte (Wu *et al.* 2006a) cell lines can drastically change cellular LCFA uptake rates. Also, genetic loss- and gain-of-function models for membrane transporters such as FATPs (Doerge & Stahl 2006) and CD36 (Coburn *et al.* 2000) have shown the expected alterations in uptake rates in a variety of tissues including liver, heart, WAT, and BAT. Specifically, loss of FATP1 function in vitro and in vivo results in the loss of insulin stimulatory, but not basal, LCFA uptake by WAT (Wu *et al.* 2006b) and 3T3-L1 adipocytes (Lobo *et al.* 2007), as well as in diminished LCFA uptake by BAT resulting in severe cold sensitivity (Wu *et al.* 2006a). Conversely, overexpression of the same transporter in heart leads to TAG accumulation and symptoms of diabetic cardiomyopathy (Chiu *et al.* 2001). Homozygote CD36 null mutations have been created in *Mus musculus* (Febbraio *et al.* 1999) and also occur spontaneously in humans (Hirano *et al.* 2003) with resulting profound alterations in fatty acid uptake rates by various tissues including adipose. Given the clear evidence that cellular fatty acid uptake rates can be dynamically regulated and the importance of free fatty acid uptake for cellular energetics as well as insulin sensitivity (Samuel, Petersen & Shulman), several experimental routes have been taken to determine LCFA uptake kinetics by WAT and other tissues both in vitro and in vivo.

1.2 In vitro fatty acid uptake assays

A variety of approaches have been developed to determine LCFA uptake that fall into the general categories of either tracing labeled fatty acids or indirect detection of transition of fatty acids across the plasma membrane. The most common indirect approach to determine fatty acid uptake is the measurement of cellular TAG stores, which is technically straight forward and can be easily done in vivo as well as in vitro but has the obvious short coming of not only being driven by cellular LCFA uptake but also by rates of lipolysis, fatty acid catabolism, LCFA efflux, and de novo synthesis from glucose and other substrates.

Additional indirect approaches taken include the use of fluorescent intracellular fatty acid binding proteins (Kampf & Kleinfeld 2004), pH indicators (Berk & Stump 1999), and growth assays of yeast cells plated on oleate media following the expression of murine fatty acid transporter proteins on oleate media (Dirusso *et al.* 2000). Use of intracellular pH indicators for LCFA uptake assays assumes that fatty acids translocate across the plasma membrane as a protonated species and that fatty acid-induced proton fluxes across the plasma membrane relate to actual LCFA fluxes. However, recent studies of the function of the mitochondrial uncoupling protein 1 (UCP1), found in BAT mitochondria, has shown that unprotonated fatty acids can also be moved across membranes (Fedorenko, Lishko &

Kirichok 2012). Forcing yeast to grow on oleate as their primary carbon source following the ablation of the endogenous yeast FATP has demonstrated that certain mammalian FATPs, including FATP1 and -4 which are highly expressed by adipocytes, can functionally rescue growth and thus opened the door to rapid screening of point mutations (DiRusso *et al.* 2005). However, yeast is not able to functionally express all human FATPs and other transporters such as CD36 have not been tested. As is the case for all reductionist approaches, focusing on one adipocyte protein expressed ectopically in a model cell system also carries the risk of missing important complex interactions that could be present at the adipocyte surface to mediate efficient LCFA uptake. An alternative strategy is the detection of intracellular fatty acid concentrations using a fluorescent fatty acid binding protein (ADIFAB) (Kampf, Parmley & Kleinfeld 2007). As with TAG levels, fluorescent binding proteins are unable to distinguish exogenous from endogenous LCFAs, an important point particularly in adipocytes, which are able to mobilize large numbers of fatty acids during induction of lipolysis. In addition, this approach requires the microinjection of cells and cannot be translated to *in vivo* measurements.

Uptake assays with labeled fatty acids have either relied on radiolabeled fatty acids or used fatty acid analogs conjugated to fluorescent or bioluminescent probes. A variety of ^{14}C and ^3H radiolabeled fatty acids are commercially available and have the additional advantage of faithfully mimicking the biochemical properties of natural fatty acids. Thus, radiolabeled LCFAs have been extensively used for LCFA uptake assays in many *in vitro* cell systems including brown (Wu *et al.* 2006a) and white adipocytes (Stahl *et al.* 2002). They also have found use *in vivo* after delivery by gavage or injection (Doerge *et al.* 2008) and have even been used for high-throughput screens (HTS) aimed at identifying FATP4 inhibitors (Blackburn *et al.* 2006). Disadvantages of this approach include the use of radioactive materials, the lack of dynamic monitoring of cellular uptake, and high costs that frequently prevent their use in HTS applications. Fluorescently labeled fatty acid analogs, particularly C1-Bodipy-C12, have proven to be suitable alternatives to radiolabeled LCFAs. Uptake kinetics of C1-Bodipy-C12 have been determined for several cell types including adipocytes (Fig. 1) and the fluorescent fatty acids are capable of participating in downstream metabolic reactions following uptake (Kasurinen 1992). While C1-Bodipy-C12 has been the most widely used fluorescent fatty acid, bodipy-conjugated fatty acids are available with acyl chain lengths ranging from C5 to C15. In side-by-side comparisons we found that all bodipy-LCFAs but not the medium chain bodipy-C5 are readily taken up by 3T3-L1 adipocytes (Fig. 2), which is congruent with studies demonstrated that FATPs have a substrate preference for C 8 fatty acids (Stahl *et al.* 1999). Fluorescent LCFA uptake can be quantitated using either plate readers or fluorescence-activated cell scanners (FACS). FACS has the advantage of being able to assess additional parameters such as cell size, surface markers, or cell viability and thus lends itself to applications for heterogeneous cell populations such as isolations of primary BAT or WAT cells. In contrast, plate reader based assays offer a higher throughput. Traditionally, both approaches have relied on end-point assays with each time point requiring the removal of the uptake solution, washing of cells, and subsequent analysis. A convenient alternative to this approach is the combination of water soluble, cell impermeable, quenchers with C1-bodipy-C12 fatty acids, which provides

a homogeneous assay system that allows for the continuous real-time assessment of fatty acid uptake by adherent cells (Liao *et al.* 2005).

1.3 In vivo fatty acid uptake assays

Given the recent surge in interest in imaging BAT and its activation, there is also a need to develop suitable clinical as well as pre-clinical assays to determine LCFA uptake rate by BAT in vivo. Current approaches have mainly relied on PET using ^{18}F -2deoxyglucose (Cypess & Kahn 2010), however LCFAs are the predominant substrates for BAT uncoupled respiration (Ma & Foster 1986). PET imaging approaches are costly and, due to the short half-life of ^{18}F (~110 min), do not lend themselves for imaging of slow metabolic changes nor longitudinal studies. As a suitable alternative, we recently developed a bioluminescent approach based on an activatable LCFA probe that can generate luciferin following cellular uptake. Thus, in the presence of luciferase, light generation is proportional to cellular fatty acid uptake (Henkin *et al.* 2012). Cellular uptake of this probe was shown to occur via physiological pathways and could be monitored in live animals in real-time using bioluminescent imagers (Henkin *et al.* 2012). Importantly, using whole-body luciferase expressing mice, uptake of fatty acids by classical BAT pads could be quantitatively determined. The generation of tissue-specific luciferase expressing animals, e.g. utilizing adipose-specific promoters, should further expand the utility of this approach.

2. Adipocyte Sources

There are several white, e.g. OP9, and brown, e.g. HIB1B, adipocyte lines available but 3T3-L1 preadipocytes remain the most widely used in vitro system. Primary adipocytes can also be isolated from a variety of adipose depots and are particularly useful for the characterization of fatty acid uptake alterations in transgenic animals.

2.1 3T3-L1 Adipocyte differentiation

A. Materials Required—Cell culture dishes (Corning Inc., Corning, NY; Cat. # 430167)

Medium 1 (DMS)

Materials:

DMEM (Life Technologies, Carlsbad, CA; Cat. # 11995-065)

500 ml

Fetal Bovine Serum (Life Technologies, Carlsbad, CA; Cat. # 10437-028)

50 ml (or 10%)

Pen/Strep/Glutamine (Life Technologies, Carlsbad, CA; Cat. # 10378-016) 5 ml (or 1%)

Combine and sterile filter in a tissue culture (TC) hood

Medium 2 (DM1)

Materials:

DMS (Medium 1)	500 ml
Dexamethasone (Sigma-Aldrich, St Lois, MO; Cat. # D4902)	500 µl of 0.25 mM
IBMX (Sigma-Aldrich, St Lois, MO; Cat. # I5879)	5 ml of 50 mM
Insulin (Sigma-Aldrich, St Lois, MO; Cat. # I2643)	100 µl of 5 mg/ml

Combine and sterile filter in TC hood

Medium 3 (DM2)**Materials:**

DMS (Medium 1)	500 ml
Insulin	100 µl of 5 mg/ml

Combine and sterile filter in TC hood

B. Protocol—All cell culture work should be done in a TC hood under sterile conditions.

Cell maintenance:

1. For propagation, cells must be kept at a low confluency without cells touching each other. For splitting cells, remove DMS from dish and add 0.5 ml of Trypsin (Life Technologies, Carlsbad, CA; Cat. # 15400-054).
2. Swirl Trypsin onto the entire dish and put the dish into a 37 °C 5% CO₂ incubator for 1–3 minutes.
3. Remove cells from incubator and add 10 ml of DMS to dish. Pipette up and down to detach cells from the dish.
4. Pipette the contents of the dish into a 15ml conical vial and centrifuge the vial at 600 × g for 2 mins at room temperature. A cell pellet should be visible at the bottom of the vial after the spin is complete.
5. Carefully aspirate the media as close to the pellet as possible without sucking up the pellet. Resuspend the pellet in DMS. Cells used for maintenance should be split every other day at a ratio of 1:10.

Cell Differentiation:

1. Seed 200,000 cells evenly onto a 10 cm cell culture dish and add 10 ml of DMS to the dish.
2. Exchange the DMS every other day until cells reach 100% confluency.

3. Upon confluency, replace DMS with 10 ml DM1 to initiate differentiation. Once DM1 is added, cells are in Day 0 of differentiation.
4. On day 2, replace DM1 with 10 ml of DM2.
5. On day 4, replace DM2 with DMS and continue replacing DMS every other day until day 8.
6. By day 8 adipocytes should have visible lipid droplets and are ready for uptake assay.

2.2 Primary adipocytes

A. Materials Required—Sterile cheesecloth

Shaking water bath

Solutions:

Transfer solution: 5% BSA in Ringer – 50 ml

Wash Solution: 1% BSA in Ringer – 100 ml

0.1% fatty acid free BSA_{in} 1× HBS – 50 ml

Collagenase Solution: Dissolve 1 g of type I collagenase (Life Technologies, Carlsbad, CA; Cat. # 17100-017) in 1l of DMEM with 50 g of fatty acid free BSA.

Sterile filter, aliquot, and store at –20 °C.

B. Procedure

1. Prepare small Petri dish (60 × 15 mm) containing 2.25 ml of Transfer Solution on ice.
2. Euthanize mice using CO₂ or isoflurane + cervical dislocation.
3. Remove desired adipose depots (using sterile tools and alcohol) do not allow hair to mix with sample.
4. Place fat pads into Petri dish on ice and transfer into sterile tissue culture cabinet.
5. Chop gently with scissors and add 0.25 ml Collagenase Solution.
6. Transfer cells using 25 ml pipet into a 15 ml conical
7. Incubate at 37 °C while gently shaking for 55 min
8. Place cheesecloth atop a 50 ml conical, decant cells in collagenase into 50 ml conical and rinse cheesecloth twice with 10 ml of Wash Solution.
9. Remove cheesecloth and pull up the entire volume of collagenase/Wash Solution into a 25 ml pipet
10. Let the pipet stand upright for 10 min, cells will slowly rise to the top.

11. Slowly release cell free solution until cell layer approaches end of pipet
12. Wash cells by pulling up 10 ml of fresh Wash Solution and resuspend cells by bubbling air through the pipet.
13. Repeat washing procedure twice by going back to step 9.
14. Wash once in 5–10 ml of 0.1% fatty acid free BSA in 1× HBS and collect cells into a 1.5 ml centrifuge tube after discarding the cell free solution.

3. Uptake assay with radioactive fatty acids

This assay can be performed with a variety of ^3H - and ^{14}C -labeled fatty acids and is useful for both cultured and primary adipocytes.

A. Materials Required

Scintillation counter (Beckman LS6500)

Scintillation cocktail (EcoLume™; MP Biomedicals, Solon, OH, Cat. #0188247001)

10× Dulbecco's Phosphate Buffered Saline (Life Technologies, Carlsbad, CA; Cat. # 14200-075)

Fatty acid free BSA (Sigma-Aldrich, St Louis, MO; Cat. # A7511)

EDTA (Sigma-Aldrich, St Louis, MO; Cat. # E5134)

Sodium-deoxycholate (Sigma-Aldrich, St Louis, MO; Cat. # D6750)

NP-40 (Calbiochem; Cat. #492016)

Sodium Dodecyl Sulfate (Sigma-Aldrich, St Louis, MO; Cat. # L3771)

Oleic acid [1- ^{14}C] (American Radiolabeled Chemicals; Cat. #ARC0297)

Octanoic acid [1- ^{14}C] (American Radiolabeled Chemicals; Cat. #ARC0149)

Arachidonic acid [1- ^{14}C] (American Radiolabeled Chemicals; Cat. #ARC0290)

Palmitic acid [1- ^{14}C] (American Radiolabeled Chemicals; Cat. #ARC0172A)

24 well cell culture dish (BD Falcon, Franklin Lakes, NJ; Cat. # 353043)

Solutions:

Radiolabeled fatty acid solution:

Fatty acid stock solution (55 mCi/mmol, 1 mCi/ml)	5 μL
0.1% fatty acid free BSA PBS	445 μL

Serum free DMEM	450 μ L
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Final fatty acid concentration will be 0.1 mM

Control solution:

100% ethanol	5 μ L
0.1% fatty acid free BSA PBS	450 μ L
Serum free DMEM	450 μ L

RIPA buffer:

50 mM Tris-HCL, pH7.4	25 ml of 1M
1% NP-40	5 mL
0.5% Na-deoxycholate	2.5 g
0.1% SDS	0.5 g
150 mM NaCl	15 ml of 5 M
2 mM EDTA	2 ml of 0.5 M

in 500 mL distilled water

B. Protocol for attached cells

1. Fully differentiate 3T3-L1 fibroblasts into adipocytes (see differentiation protocol above) in a 24 well cell culture dish. Perform experiment on day 8 of differentiation. Alternatively, use primary cells.
2. If treating the cells, e.g. with insulin, serum starve the cells for 3–8 h to establish basal conditions. Rinse cells with serum free medium 3 times treat with desired hormone/growth factor etc.
3. Add 20 μ L of radiolabeled fatty acid solution or control solutions to cells. (The final concentration of each fatty acid should be 1 μ M)
4. After 1–60 mins remove media and carefully wash cells twice in 2 ml ice cold 0.1% fatty acid free BSA in PBS.
5. Add 200 μ L of ice cold RIPA buffer and incubate plates on ice for 5 min.
6. Scrape the cells into RIPA buffer, transfer cell lysates into microcentrifuge tubes and centrifuge at 16,000 g in a tabletop centrifuge for 10 min.
7. Use 50 μ L of supernatant for protein assay of your choice and add 100 μ L of lysate to 4 ml of scintillation fluid.
8. Using a beta scintillation counter determine activity and calculate the uptake rate taking into account protein amount and incubation time (pmol/min/mg).

3. Uptake Assay with Flow Cytometer

This assay relies on determining the uptake of a fluorescently labeled fatty acid analog and is particularly useful in combination with primary cells as non-adipocytes and dead cells can easily be excluded. However, the assay can also be performed as an endpoint assay for adherent cells.

A. Materials Required

Flow Cytometer (BD FACSCalibur™, BD Franklin Lakes, NJ, or equivalent)

FACS Tubes (USA Scientific, Ocala, FL; Cat. # 1450 2000)

BODIPY Fatty Acids (Invitrogen – Life Technologies, Carlsbad, CA; Cat. #'s D-3821 D-3822 D-3823 D-3834 D-3862)

Propidium Iodide (Invitrogen – Life Technologies, Carlsbad, CA; Cat. # P3566)

10× Hank's Balanced Salt Solution (HBSS, Gibco – Life Technologies, Carlsbad, CA; Cat. # 14065-056)

Fatty acid free BSA (Sigma-Aldrich, St Louis, MO; Cat. # A7511)

EDTA (Sigma-Aldrich, St Louis, MO; Cat. # E5134)

Solutions:

Cell Buffer: Fatty acid free 0.1% BSA in 1× HBSS

BODIPY Solution: 2.0 μM final concentration of fatty acid-BODIPY in Cell Buffer
Vortex, sonicate, and keep at 37°C in a water bath

FACS Buffer: 10% Fetal Bovine Serum
10 mM EDTA
50 μg/ml propidium iodine
in 1× HBSS
keep on ice

Stop Solution: 0.2% BSA in 1× PBS
keep on ice

B. Protocol for detached cells

1. Fully differentiate 3T3-L1 fibroblasts into adipocytes in a 10 cm cell culture dish (see differentiation protocol above). Perform experiment with Day 8 differentiated cells. Alternatively, use primary adipocytes and go to step 7.
2. If treating the cells, e.g. with insulin, serum starve the cells for 3–8 h to establish basal conditions. Rinse cells with serum free medium 3 times treat with desired hormone/growth factor etc.
3. Aspirate and remove media from the 10 cm dish. Add 0.5 ml of trypsin to the dish. Swirl around for a few seconds so that trypsin covers the dish.
4. Carefully aspirate trypsin without detaching cells.
5. Add 1 ml trypsin and place the cells in a 37 °C 5% CO₂ incubator for 3 minutes.
6. Carefully aspirate the trypsin. Tap on edge of dish to dislodge adipocytes. Cells should all detach and slide off.
7. Add 9.5 ml of Cell Buffer to dish and gently pipette up and down so that cells are evenly mixed.
8. Transfer the 9.5 ml of Cell Buffer with the cells to a 15 ml conical tube and put the tube in a 37 °C water bath.
9. Prepare a 2 μM fatty acid BODIPY Solution in 0.5ml of Cell Buffer and warm to 37 °C.
10. Prepare and label FACS tubes and place them on ice.
11. Pipette 4 ml of Stop Solution into each FACS tube on ice.
12. Add 0.5 ml of fatty acid BODIPY solution to 9.5 ml of cells in a 37°C water bath and invert gently. This is the beginning of your time course.
13. At appropriate time point (there is enough solution for 20 FACS assays, for example quadruplicates at 30 seconds, 1 minute, 2 minute, 5 minute, 10 minute) gently invert solution with cells and remove 500 μL of cells and add them to the appropriate FACS tube.
14. After completing the time course, spin down FACS tubes at 600 × g for 10 mins in a tissue culture centrifuge with appropriate adaptors.
15. Cell pellet should be visible at the end of the spin. Carefully decant or aspirate supernatant and discard.
16. Add 500 μL FACS Buffer with Propidium Iodide to each FACS tube with cells.
17. Resuspend the cells by gently pipetting up and down several times and briefly vortex each FACS tube immediately before collecting data.
18. Run FACS by setting one gate in a forward/side scatter plot to include single cell mature adipocytes (see Fig. 1A) and set a second gate in the FL3 channel to exclude propidium iodine positive cells (see Fig. 1B).

19. Determine fatty acid uptake as fluorescence (in arbitrary units) in the FL1 channel (see Fig. C).

C. Protocol for attached cells

1. Fully differentiate 3T3-L1 fibroblasts into adipocytes (see differentiation protocol above) in a 12 well cell culture dish (BD Falcon, Franklin Lakes, NJ; Cat. # 353043). Perform experiment on Day 8 differentiated cells.
2. Prepare 12 ml of Cell Buffer with a final concentration of 2.0 μ M BODIPY.
3. Warm BODIPY solution to 37°C.
4. Prepare and label FACS tubes and place them on ice (duplicates).
5. Prepare 2 ml of Stop Solution per well (24 ml total) and place on ice.
6. Prepare FACS buffer with propidium iodide and keep on ice.
7. Aspirate and remove media from the 12 well dish.
8. Add 1 ml BODIPY solution to each of the wells in the 12 well dish.
9. At each of the time points (e.g. 30 sec, and 1, 2, 5, or 10 mins) add 2 ml of cold stop solution to the appropriate wells.
10. After completing the time course, aspirate and discard the stop solution.
11. Pipette 100 μ L of Trypsin per well into each of the 12 wells. Swirl gently and aspirate and discard the trypsin.
12. Pipette 200 μ L of Trypsin into each of the 12 wells. Place the dish of cells in a 37 °C 5% CO₂ incubator for 3 minutes.
13. Carefully aspirate trypsin. Tap on dish gently to shake cells loose. Cells should all detach and slide off.
14. Add 500 μ L FACS Buffer per well.
15. Pipette up and down to detach the rest of the cells. Pipette the 500 μ Ls of FACS buffer and cells into the respective FACS tube.
16. Resuspend the cells by gently pipetting up and down several times with a 100 μ L pipette.
17. Vortex each FACS tube with cells right before collecting data.
18. Perform FACS run as described above.

E. Variation of Bodipy-FFA chain length

Alternatively, experiments B and C above can be performed with Bodipy-fatty acids of different chain length, such as C5, C11, C12, and C15 to determine chain length specificity of uptake. A representative experiment using 3T3-L1 adipocytes is shown in Fig. 2.

5. Quencher based LCFA uptake assay

Plate reader based assays have the advantage of allowing for high throughput and convenience but rely on homogeneous cell populations and high well-to-well consistency in viability and cell number. Importantly, through the addition of a water-soluble cell impermeable quencher to the uptake solution, this assay can be used to ascertain live uptake kinetics for fatty acids into adipocytes.

A. Materials Required

Bottom read capable spectrophotometer (Molecular Devices, Spectra Max Gemini EM)

QBT™ Fatty Acid Uptake Assay Kit (Molecular Devices; Cat. # R8132)

10× Hank's Balanced Salt Solution (Gibco; Cat. # 14065-056)

Fatty acid free BSA (Sigma-Aldrich, St Louis, MO; Cat. # A7511)

1 M Hepes buffer solution

Insulin (Sigma-Aldrich, St Louis, MO; Cat. # I2643)

Solutions:

Modified HBSS Buffer

1 g BSA

10 ml of 1M Hepes buffer solution

50 ml 10× HBSS

add 440 ml of sterile TC quality water to make up 500 ml

B. Protocol

1. Fully differentiate 3T3-L1 fibroblasts into adipocytes (see differentiation protocol above) in a 12 well cell culture dish. Perform experiment on day 8 of differentiation.

If treating the cells, e.g. with insulin, serum starve the cells for 3–8 h to establish basal conditions. Rinse cells with serum free medium 3 times treat with desired hormone/growth factor etc.

2. Prepare a 1× Loading Buffer by adding 10 ml of HBSS Buffer to 1 vial from the QBT Fatty Acid Uptake Kit.
3. Keep the 1× Loading Buffer at 37 °C until ready to add to cells. Vortex before adding.
4. Aspirate the media from the wells and put 1 ml of serum free media (warmed to 37°C) into each of the 12 wells. Incubate for 1 hr in the 37°C 5% CO₂ incubator.

5. Aspirate and discard the serum free medium.
6. Add 800 μ L of the 1 \times Loading Buffer per well and immediately transfer the dish to the fluorescent spectrophotometer (set to 37°C).
7. Start kinetic assay
8. Kinetic assay settings:
 - a. Measure fluorescence with bottom-read mode.
 - b. Excitation= 485 nm. Emission= 515 nm. Cutoff= 495 nm.
 - c. Read every 20 sec for 60 min

Representative results for short- and long-term uptake assays with undifferentiated and differentiated 3T3-L1 adipocytes are shown in Fig. 3.

4. Bioluminescent fatty acid uptake assays

Assays with bioluminescent fatty acids can be performed in vitro using cells or tissues as well as in vivo. In both cases as sensitive optical detection system, e.g. an Ivis Spectrum, is required. Since the bioluminescent LCFA analogs are, at the time of this writing, not commercially available in the US, we will also provide the reader with instruction for probe synthesis.

1. Probes synthesis

- 1.1. **Required materials**—Aldrithiol-2 (Sigma-Aldrich, St Lois, MO; Cat. # 143049)
3-Mercapto-1-propanol (Sigma-Aldrich, St Lois, MO; Cat. # 405736)
Triphosgene (Fluorochem West Columbia, SC; Cat. # M03560)
Pyridine (Sigma-Aldrich, St Lois, MO; Cat. # 270970)
2-Cyano-6-hydroxybenzothiazole (Shanghai Chemical Pharm-Intermediate Tech. Co., Ltd)
16-Mercaptohexadecanoic acid (Sigma-Aldrich, St Lois, MO; Cat. # 674435)
Methyl-16-mercaptohexadecanoate (Asemblon INC)
D-cysteine hydrochloride (Anaspec INC, Fremont, CA; Cat. # 61814-5)
Potassium carbonate (Sigma-Aldrich, St Lois, MO; Cat. # 209619)
Triethylamine (Acros Organics – Thermo Fisher, Waltham, MA; Cat. # 219510500)
Dichloromethane (Sigma-Aldrich, St Lois, MO; Cat. # 270997)
Methanol (Sigma-Aldrich, St Lois, MO; Cat. # 322415)
Hexane (Sigma-Aldrich, St Lois, MO; Cat. # 32293)

- Ethyl acetate (Sigma-Aldrich, St Lois, MO; Cat. # 45760)
- Tetrahydrofuran (Sigma-Aldrich, St Lois, MO; Cat. # 401757)
- Dimethylformamide (Sigma-Aldrich, St Lois, MO; Cat. # 227056)
- Acetic acid (Sigma-Aldrich, St Lois, MO; Cat. # 320099)
- Chloroform-d (Sigma-Aldrich, St Lois, MO; Cat. # 431915)
- Methan(ol-d) (Sigma-Aldrich, St Lois, MO; Cat. # 151939)
- Acetone-d⁶ (Sigma-Aldrich, St Lois, MO; Cat. # 175862)
- Deionized water (obtained from Milli-Q purification system)
- Hydrochloric acid 1 M solution in water (Sigma-Aldrich, St Lois, MO; Cat. # 71763)
- Silica gel (Silicycle SiliaFlash P60 230–400 mesh; Cat. # R12030B)
- Flash chromatograph (Biotage FLASH + column Biotage Si 12+M or equivalent)
- Glass-backed silica gel 60 Å F254 plates for analytical thin layer chromatography (Silicycle; Cat. # TLG-R10011B-333)
- Reversed phase analytical LC-MS system (Agilent Technologies 6120 Quadrupole LC-MS with 1260 Series HPLC system, Agilent Technologies, Santa Clara, CA, or equivalent)
- Reversed phase preparative high performance liquid chromatography system (RP-HPLC Varian Pro Star + UV-Vis detector model 330 + preparative column Microsorb C-18 (21.4 × 250mm) or equivalent)
- NMR spectrometer (AVQ-400 MHz or equivalent)

1.2. Synthesis of FFA-SS-luc probe—The synthesis of FFA-SS-luc probe is outlined in Scheme 1.

3-mercaptoopropanol **1** is converted into the activated disulfide derivative **3** by reaction with aldrithiol-2 **2**. In a separate route, 2-cyano-6-hydroxybenzothiazole **4** is transformed *in situ* into chloroformate **5** which gives carbonate **6** after coupling with the compound **3**. Subsequent reaction of **6** with 16-mercaptohexadecanoic acid leads to the displacement of the thiopyridyl moiety to produce the conjugate **7**. In the last step, condensation of **7** with D-cysteine formed *in situ* from D-cysteine hydrochloride and K₂CO₃ results in the FFA-SS-luc probe **8**.

1.2.1. Synthesis of 3-(pyridin-2-yl)disulfanylpropan-1-ol (**3**)

1. Place 3.84 g (17.44 mmol, 3 equivalents) of aldrithiol-2 under nitrogen in an oven-dried 25 mL flask equipped with a stir bar. Add 12 ml of methanol previously purged with nitrogen for 20 min.

2. Add to the reaction mixture 0.50 mL (5.81 mmol, 1 equivalent) of 3-mercapto-1-propanol dropwise.
3. Allow the solution to stir for 3 hrs at room temperature.
4. Evaporate the solvent *in vacuo*.
5. Purify the residue by flash silica gel chromatography using hexane/ethyl acetate 1:1 (v/v) as an eluent. Determine which fractions contain the product by TLC and evaporate the solvent *in vacuo*.
6. Prove the structure and check the purity of the product after purification by ¹H and ¹³C NMR using CD₃OD as a solvent for NMR sample preparation and compare the spectra with those previously reported (Henkin *et al.* 2012).

1.2.3. Synthesis of 16-((3-((2-cyanobenzo[d]thiazol-6-yloxy)carbonyloxy)propyl)disulfanyl)hexadecanoic acid (7)

1. Place 376 mg (0.93 mmol, 1 equivalent) of the compound **6** and 269 mg (0.93 mmol, 1 equivalent) of 16-mercaptohexadecanoic acid under nitrogen in an oven dried 100 mL flask equipped with a stir bar. Add 40 mL of dry DMF and 207 μL (1.49 mmol, 1.6 equivalents) of dry triethylamine to the reaction flask.
2. Allow the reaction mixture to stir at room temperature for 2 hours.
3. Evaporate the solvent *in vacuo*.
4. Purify the residue by flash silica gel chromatography using hexane/ethyl acetate 4:1 (v/v) as an eluent. Determine which fractions contain the product by TLC and evaporate the solvent *in vacuo*.
5. Prove the structure and check the purity of the product after purification by ¹H and ¹³C NMR using CDCl₃ as a solvent for NMR sample preparation and compare the spectra with those previously reported (Henkin *et al.* 2012).

1.2.4. Synthesis of (S)-2-(6-((3-((15-carboxypentadecyl)disulfanyl)propoxy)carbonyloxy)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (FFA-SS-luc) (8)

1. Place 49.9 mg (0.09 mmol, 1 equivalent) of the compound **7** and 13.5 mg (0.09 mmol, 1 equivalent) of D-cysteine hydrochloride under nitrogen in a 25 mL flask equipped with a stir bar.
2. Add to the reaction flask 5 ml of methanol and 5 ml of dichloromethane. These solvents should be purged with nitrogen for 20 min prior to use.
3. Prepare in a separate flask a solution of potassium carbonate (11.8 mg, 0.09 mmol, 1 equivalent) in a mixture of water (2 mL) and methanol (5 mL). Purge this solution with nitrogen for 20 min.
4. Add the prepared solution of potassium carbonate to the reaction flask and allow the reaction mixture to stir for 30 min at room temperature in darkness.

- 5 Quench the reaction mixture by addition of 1 M HCl in water to pH 3–4.
- 6 Evaporate the solvents *in vacuo*.
- 7 Purify the residue by flash silica gel chromatography using a mixture of DCM/ethyl acetate 4:1 (v/v) containing 2% (v/v) of acetic acid as an eluent. Determine which fractions contain the product by TLC and evaporate the solvent *in vacuo*.
- 6 Prove the structure and check the purity of the product after purification by ^1H and ^{13}C NMR using acetone- d^6 as a solvent for NMR sample preparation and compare the spectra with those previously reported (Henkin *et al.* 2012).

1.2.4. Synthesis of (S)-2-(6-((3-((15-carboxypentadecyl)disulfanyl)propoxy)carbonyloxy)-benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (FFA-SS-luc) (8)

- 8 Place 49.9 mg (0.09 mmol, 1 equivalent) of the compound **7** and 13.5 mg (0.09 mmol, 1 equivalent) of D-cysteine hydrochloride under nitrogen into a 25 mL flask equipped with a stir bar.
- 9 Add to the reaction flask 5 ml of methanol and 5 ml of dichloromethane. These solvents should be purged with nitrogen for 20 min prior to use.
- 10 Prepare in a separate flask a solution of potassium carbonate (11.8 mg, 0.09 mmol, 1 equivalent) in a mixture of water (2 mL) and methanol (5 mL). Purge this solution with nitrogen for 20 min.
- 11 Add the prepared solution of potassium carbonate to the reaction flask and allow the reaction mixture to stir for 30 min at room temperature in darkness.
- 12 Quench the reaction mixture by addition of 1 M HCl in water to pH 3–4.
- 13 Evaporate the solvents *in vacuo*.
- 14 Purify the residue by flash silica gel chromatography using a mixture of DCM/ethyl acetate 4:1 (v/v) containing 2% (v/v) of acetic acid as an eluent. Determine which fractions contain the product by TLC and evaporate the solvent *in vacuo*.
- 15 Prove the structure and check the purity of the product after purification by ^1H and ^{13}C NMR using acetone- d^6 as a solvent for NMR sample preparation and compare the spectra with those previously reported (Henkin *et al.* 2012).

1.3. Synthesis of Me-FFA-SS-luc control compound—The synthesis of Me-FFA-SS-luc probe is outlined in Scheme 2.

Carbonate **6** which contains thiopyridyl moiety is converted to the conjugate **10** by reaction with methyl-16-mercaptohexadecanoate **9** in DMF in the presence of triethylamine. In the last step, condensation of **10** with D-cysteine formed *in situ* from D-cysteine hydrochloride and K_2CO_3 results in the desired Me-FFA-SS-luc probe **11**.

1.3.1. Synthesis of methyl 16-((3-((2-cyanobenzo[d]thiazol-6-yl)oxy)carbonyloxy)propyl)disulfanyl)hexadecanoate (10)

1. Place 56 mg (0.14 mmol, 1.4 equivalents) of the compound **6** and 30.6 mg (0.1 mmol, 1 equivalent) of methyl-16-mercaptohexadecanoate **9** under nitrogen in an oven dried 25 mL flask equipped with a stir bar. Add 10 mL of dry DMF and 29 μ L (0.21 mmol, 2.1 equivalents) of dry triethylamine to the reaction flask.
2. Allow the reaction mixture to stir at room temperature for 2 hrs.
3. Evaporate the solvent *in vacuo*.
4. Purify the residue by RP-HPLC changing the eluent from 40% methanol/60% water to 100% methanol for 45 min then 100% methanol for 30 min at a flow rate 10 ml/min using a preparative column Microsorb C-18 (21.4 \times 250mm) or equivalent. Determine which fractions contain the product by LC-MS.
5. Evaporate the solvent from fractions containing the product and purify the product again by flash silica gel chromatography using gradient elution with hexane/ethyl acetate 9:1 \rightarrow 7:1 (v/v). Determine the fractions that contain the product by TLC and evaporate the solvent *in vacuo*.
6. Prove the structure and check the purity of the product after purification by ^1H and ^{13}C NMR using CDCl_3 as a solvent for NMR sample preparation and compare the spectra with those previously reported (Henkin *et al.* 2012).

1.3.2. Synthesis of (S)-2-(6-((3-((16-methoxy-16-oxohexadecyl)disulfanyl)propoxy)carbonyloxy)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (Me-FFA-SS-luc) (11)

1. Place 37.2 mg (0.06 mmol, 1 equivalent) of the compound **10** and 10.7 mg (0.07 mmol, 1.17 equivalents) of D-cysteine hydrochloride under nitrogen in a 25 mL flask equipped with a stir bar.
2. Add to the reaction flask 3.4 ml of methanol and 3.4 ml of dichloromethane. These solvents should be purged with nitrogen for 20 min prior to use.
3. Prepare in a separate flask a solution of potassium carbonate (8.7 mg, 0.06 mmol, 1 equivalent) in a mixture of water (1.4 mL) and methanol (3.4 mL). Purge this solution with nitrogen for 20 min.
4. Add the prepared solution of potassium carbonate to the reaction flask and allow the reaction mixture to stir for 30 min at room temperature in darkness.
5. Quench the reaction mixture by addition of 1 M HCl in water to pH 3–4.
6. Evaporate the solvents *in vacuo*.
7. Purify the residue by RP-HPLC changing the eluent from 40% methanol/60% water to 100% methanol for 45 min then 100% methanol for 30 min at a flow rate 10 ml/min using a preparative column Microsorb C-18 (21.4 \times 250mm) or equivalent. Determine the fractions that contain the product by LC-MS.

8. Evaporate the solvent from fractions containing the product and purify the product again by RP-HPLC changing the eluent from 80% methanol/20% water to 100% methanol for 45 min then 100% methanol for 30 min at a flow rate 10 ml/min using a preparative column Microsorb C-18 (21.4 × 250mm) or equivalent. Determine which fractions contain the product by LC-MS.
9. Evaporate the solvent from fractions containing the product.
10. Prove the structure and check the purity of the product after purification by ¹H and ¹³C NMR using acetone-d₆ as a solvent for NMR sample preparation and compare the spectra with those previously reported (Henkin *et al.* 2012).

2. In vitro bioluminescent uptake assays with adipocytes

Required material:

3T3-L1 adipocytes stably transfected with the pGL4.51[luc2/CMV/Neo] vector (Promega, Madison, WI) (3T3-L1-luc) and differentiated as described above Black-wall/clear-bottom-96-well plates (Costar, Corning Inc., Corning, NY) Bioluminescent Imager (Ivis Spectrum, Caliper Life Sciences – PerkinElmer, Waltham, MA; or equivalent)

Solutions:

Uptake buffer:

Dissolve bioluminescent fatty acid in DMSO to yield a 1 mM stock solution.

Prepare a 0.1% (w/v) solution of fatty acid free BSA in HBSS and warm to 37 °C. Slowly add bioluminescent fatty acid stock solution to BSA/HBSS to a final concentration of 2–100 μM and use immediately.

1. Install the plate adapter in the imager (see Fig. 4) and pre-warm the imaging stage to 37 °C.
2. Seed 5000 cells/well of differentiated 3T3-L1-luc cells into a blackwall/clear-bottom 96 well plate and let adhere for 6–8 h.
3. Rinse once with pre-warmed 0.1% (w/v) solution of fatty acid free BSA in HBSS.
4. Add 100 μl of pre-warmed uptake buffer containing 2–100 μM of either FFA-SS-luc or control compounds.
5. Immediately place plate on heated (37 °C) imaging stage and commence image acquisition using either auto exposure or predetermined exposure times, i.e. 5 min. exposures.
6. Continuously image plates for 60 min.
7. Analyze images using IVIS Living Image software (or equivalent) by aligning a 96 well grid region of interest with the plate. Fatty acid uptake rates can be expressed as photon flux (photons/sec) or as radiance (photons/sec/cm²/sr).

5. In vivo imaging of BAT fatty acid uptake rates

Material

Transgenic mice expressing luciferase in BAT such as whole body luciferase expressing animals (L2G85 mice, The Jackson Laboratory)

Bioluminescent Imager (Ivis Spectrum, Caliper Life Sciences – PerkinElmer, Waltham, MA; or equivalent) with anesthesia set up.

Solutions:

Injection solution:

Dissolve bioluminescent fatty acid in DMSO to yield a 1 mM stock solution. Add bioluminescent fatty acid to a pre-warmed solution of 0.1% (w/v) solution of fatty acid free BSA in PBS to yield a final concentration of 200 μ M. Prepare sufficient injection solution to allow for 100 μ l per animal. Sterile filter using a syringe filter and use immediately.

Imaging protocol

- 1) Anesthetize mice with isoflurane/oxygen and inject intraperitoneally with 100 μ l of injection solution.
- 2) Place animals on heated imaging stage with dorsal site facing the camera. Securely place nose cones over animals and maintain anesthesia with isoflurane/oxygen.
- 3) Adjust imaging stage height to achieve optimal magnification of the interscapula area and commence imaging using either auto exposure or a predetermined fixed exposure time, e.g. 3 min.
8. Continue imaging for 30 min. Analyze images using IVIS Living Image software (or equivalent) after identifying a region of interest such as the interscapular BAT of each mouse (see Fig. 4B). Fatty acid uptake rates can be expressed as photon flux (photons/sec) or as radiance (photons/sec/cm²/sr).

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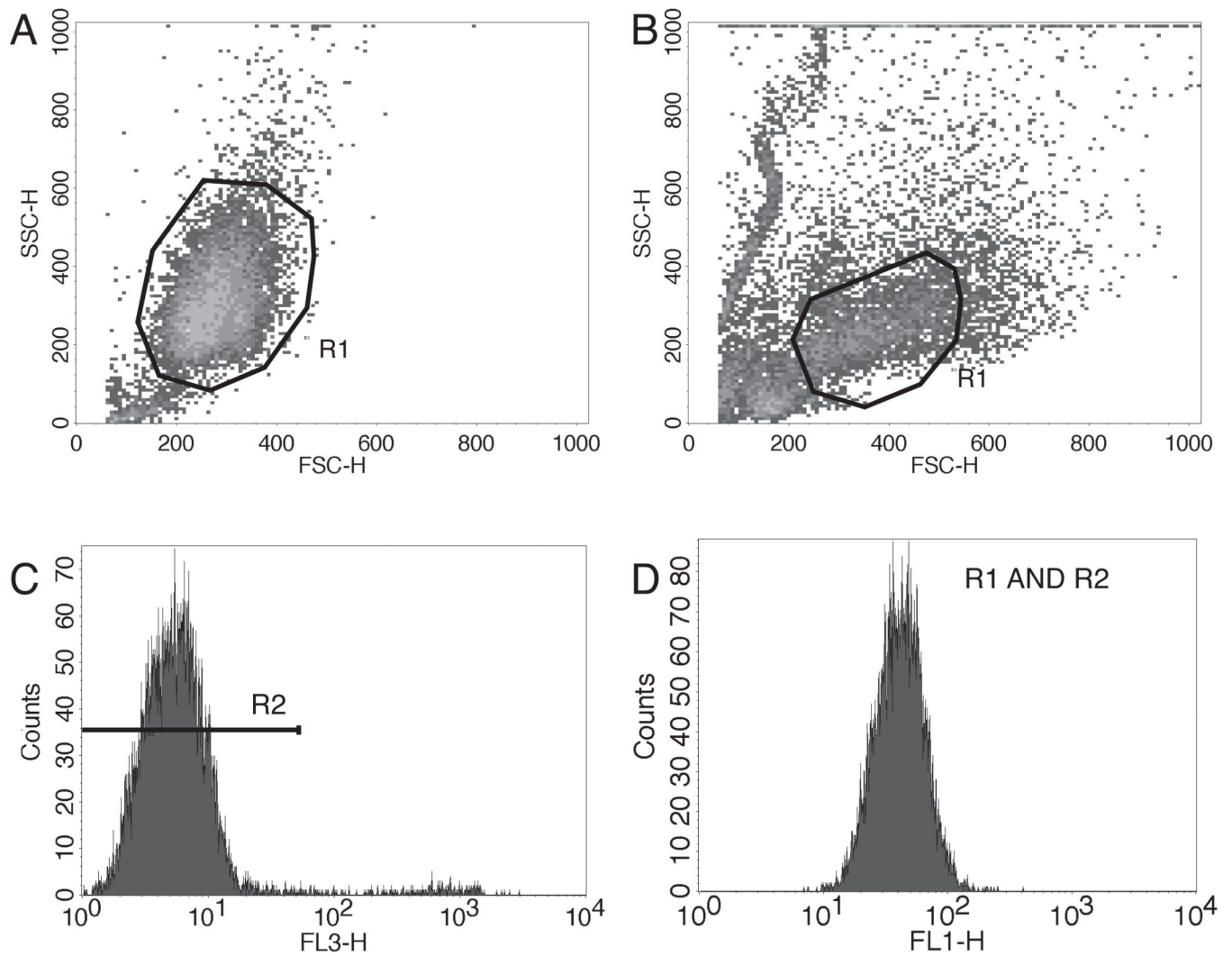


Figure 1. FACS based fatty acid uptake assays

A) Forward and side scatter dot blot of mature 3T3-L1 adipocytes. B) Forward and side scatter dot blot of primary WAT derived adipocytes. Gate R1 identifies intact adipocytes. C) PI staining of 3T3 L1 adipocytes as recorded in FL3. Gate R2 identifies viable cells. D) C1-Bodipy-C12 uptake of 3T3 L1 adipocytes with the logical gate R3=R1 AND R2.

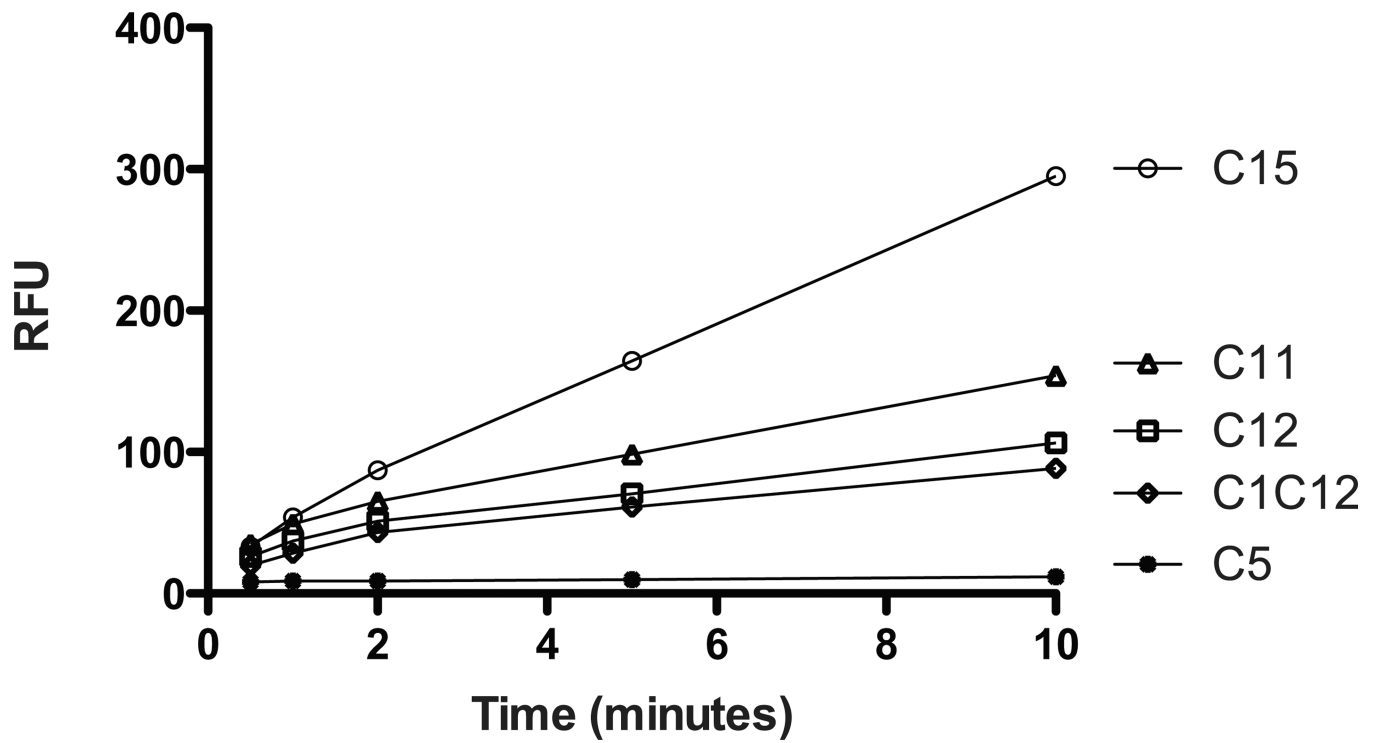


Figure 2. FACS based fatty acid uptake assays with different bodipy-LCFAs

Mature 3T3-L1 adipocytes were incubated with 2 μ M of either BODIPY C15 (Cat. # 3821), BODIPY C11 (Cat. # 3862), BODIPY C12 (Cat. # 3822), BODIPY C1C12 (Cat. # 3823), or BODIPY C5 (Cat. # 3834) for the indicated amount of time. Each time point was performed in duplicate.

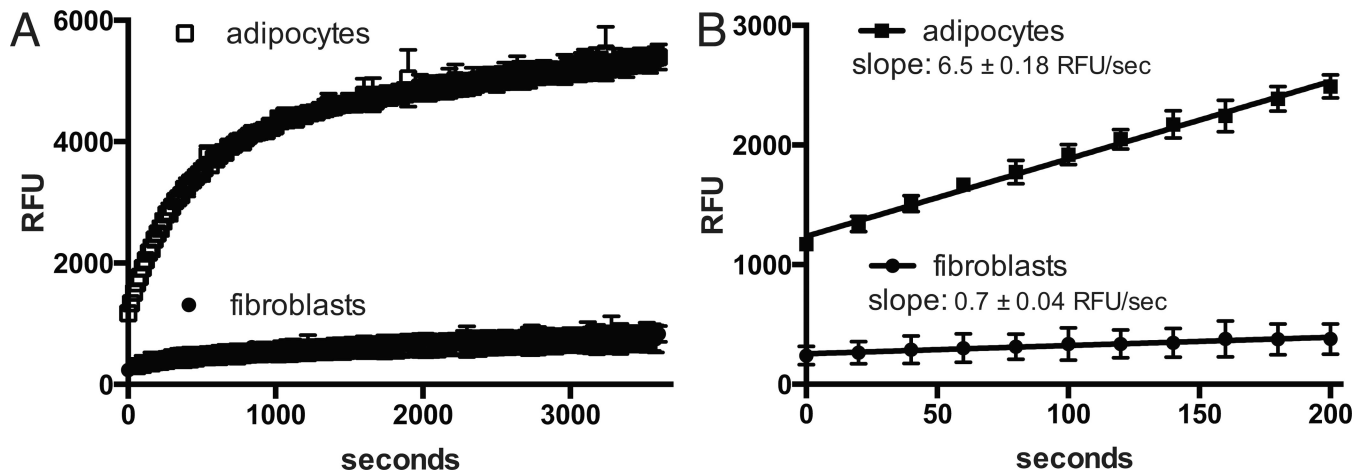


Figure 3. Quencher based real-time fatty acid uptake assay

Mature 3T3-L1 adipocytes or undifferentiated fibroblasts were treated with bodipy-fatty acid quencher mix as described under protocol 5. A) Real-time uptake over 1 h. B) Linear regression through initial time points to determine uptake velocities.

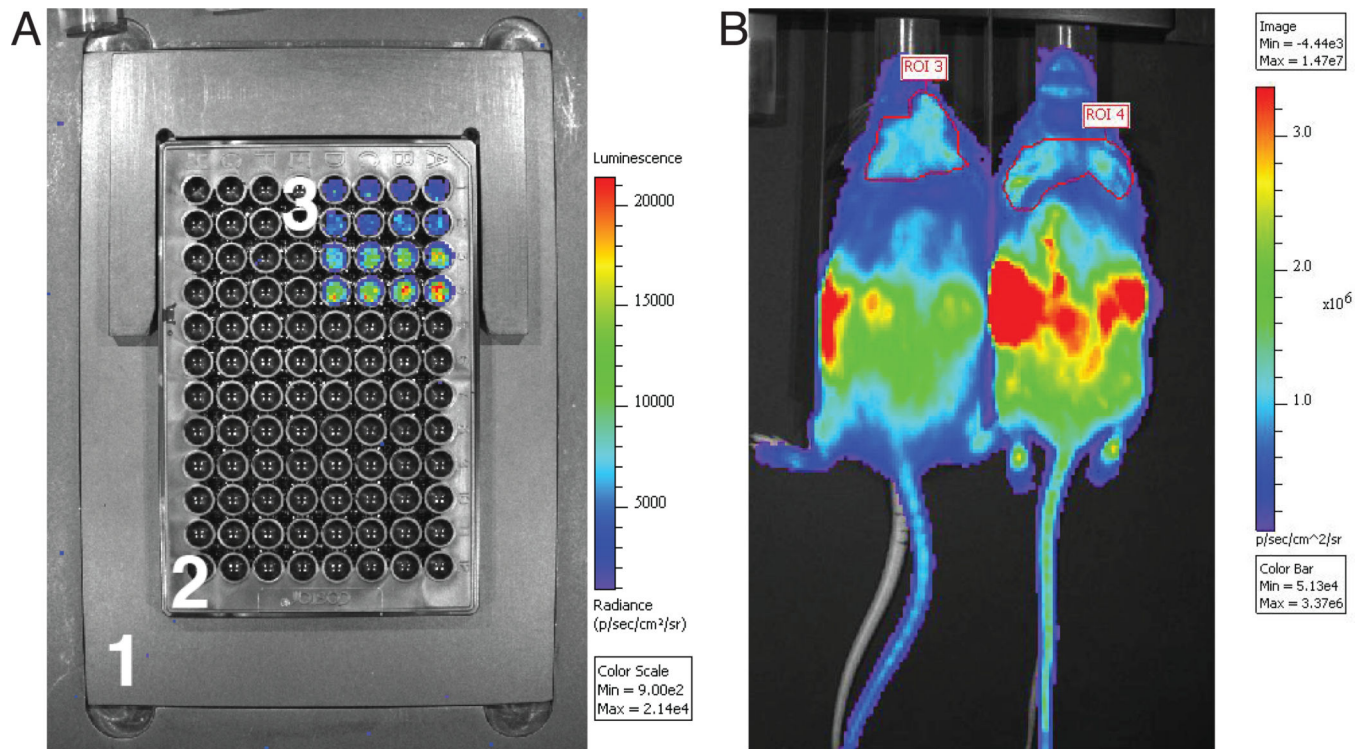
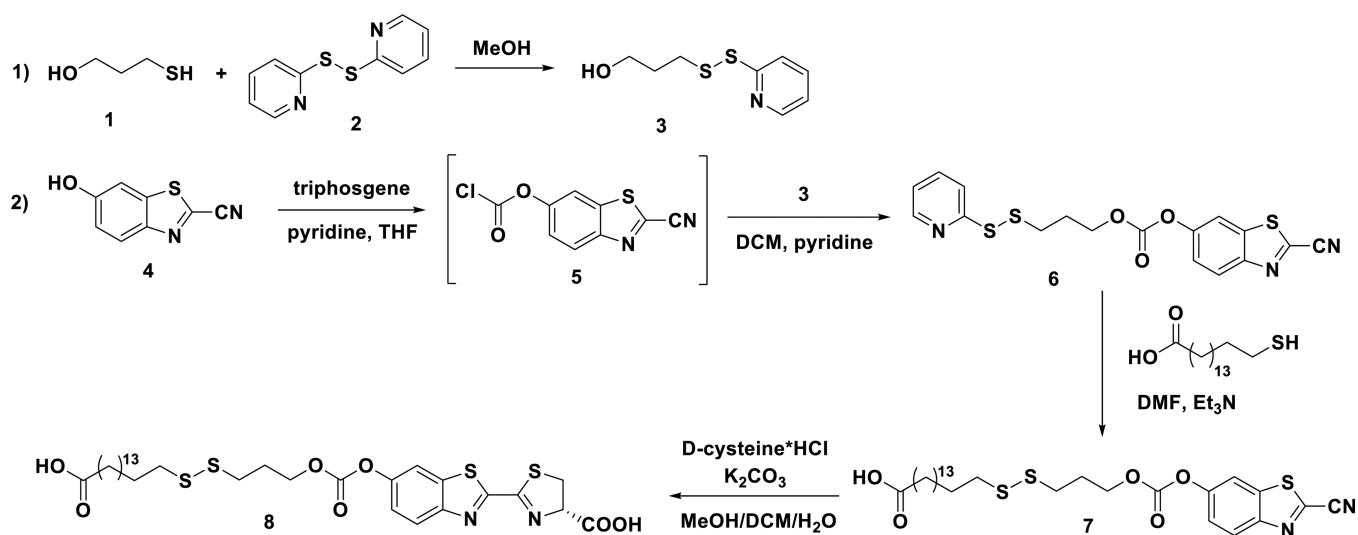
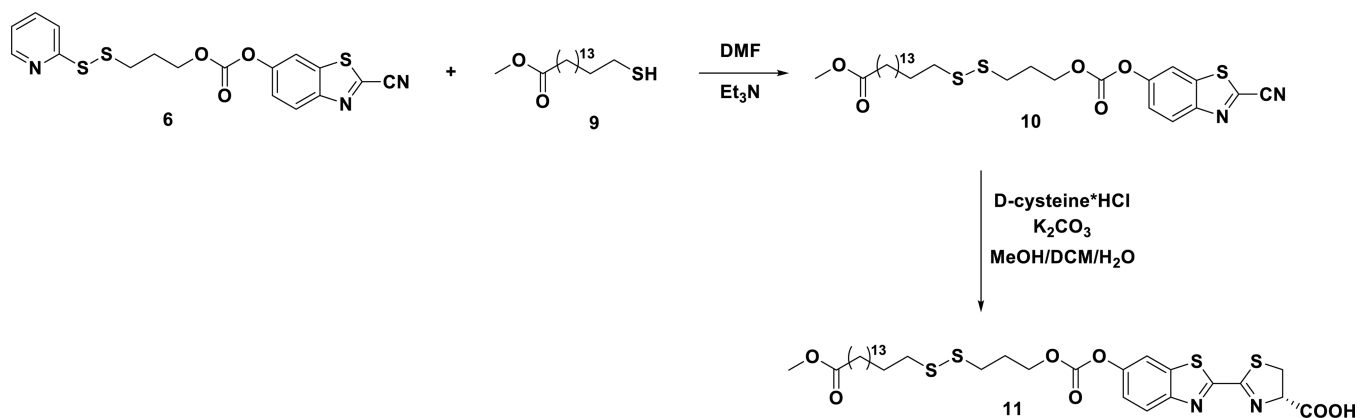


Figure 4. Bioluminescent fatty acid uptake assays

A) Using a plate adapter (1) a 96 well plate (2) containing mature 3T3-L1 adipocytes and undifferentiated fibroblasts (3) were placed into an Ivis Spectrum. Fatty acid uptake rates were monitored following the addition of 100 μ l of 100 μ M BSA bound 100 μ M FFA-SS-luc. B) L2G85 mice injected with 100 μ l of a 200 μ M solution intraperitoneally were imaged using auto exposure settings. Red regions of interest denote the BAT area in both animals.



scheme 1.
Synthesis of FFA-SS-luc probe.



scheme 2.
Synthesis of Me-FFA-SS-luc probe.