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Measurement of Fatty Acid β-Oxidation in a Suspension of Freshly Isolated Mouse Hepatocytes

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TITLE:

Measurement of Fatty Acid β-Oxidation in a Suspension of Freshly Isolated Mouse Hepatocytes

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SUMMARY:

Fatty acid β-oxidation is an essential metabolic pathway responsible for generating energy in many different cell types, including hepatocytes. Here, we describe a method to measure fatty acid β-oxidation in freshly isolated primary hepatocytes using ^{14}C-labeled palmitic acid.

ABSTRACT:

Fatty acid β-oxidation is a key metabolic pathway to meet the energy demands of the liver and provide substrates and cofactors for additional processes, such as ketogenesis and gluconeogenesis, which are essential to maintain whole-body glucose homeostasis and support extra-hepatic organ function in the fasted state. Fatty acid β-oxidation occurs within the mitochondria and peroxisomes and is regulated through multiple mechanisms, including the uptake and activation of fatty acids, enzyme expression levels, and availability of cofactors such as coenzyme A and NAD^+. In assays that measure fatty acid β-oxidation in liver homogenates, cell lysis and the common addition of supraphysiological levels of cofactors mask the effects of these regulatory mechanisms. Furthermore, the integrity of the organelles in the homogenates is hard to control and can vary significantly between preparations. The measurement of fatty acid β-oxidation in intact primary hepatocytes overcomes the above pitfalls. This protocol describes a method for the measurement of fatty acid β-oxidation in a suspension of freshly isolated primary mouse hepatocytes incubated with ^{14}C-labeled palmitic acid. By avoiding hours to days of culture, this method has the advantage of better preserving the protein expression levels and metabolic pathway activity of the original liver, including the activation of fatty acid β-oxidation observed in hepatocytes isolated from fasted mice compared to fed mice.

INTRODUCTION:

Fatty acid β-oxidation is an essential process in lipid metabolism, providing a catabolic pathway

to balance fatty acid synthesis and intake from the diet. This process generates energy for multiple organs, including the cardiac muscle, kidney cortex, and fasted liver, and utilizes fatty acids obtained from the diet, adipose tissue lipolysis, and internal triglyceride stores[1,2].

Oxidation of fatty acid through the β-oxidation pathway results in the sequential shortening of the fatty acyl chain by two carbons at a time, released as acetyl-CoA, and this process occurs both in the mitochondria and the peroxisomes. While most fatty acids undergo only β-oxidation, some are oxidized at different carbons before entering this pathway. For example, 3-methyl-substituted fatty acids, such as phytanic acid, undergo removal of one carbon by α-oxidation in the peroxisomes before entering the β-oxidation pathway. Similarly, some fatty acids are first converted to dicarboxylic fatty acids by oxidation of the terminal methyl group (ω-oxidation) in the endoplasmic reticulum before being preferentially oxidized in the peroxisomes by β-oxidation[3].

Regardless of the specific organelle, a fatty acid must first be converted to a coenzyme A (CoA) thioester, or acyl-CoA, to be oxidized through the β-oxidation pathway. β-Oxidation of long-chain acyl-CoAs in the mitochondrial matrix requires the carnitine shuttle for their translocation, where carnitine palmitoyltransferase 1 (CPT1) catalyzes the conversion of acyl-CoAs to acylcarnitines and is the rate-limiting enzyme in this process[4]. Once translocated to the mitochondrial matrix, the acyl-CoAs are re-formed and serve as substrates for the mitochondrial β-oxidation machinery. In the fasted state, the acetyl-CoA produced through β-oxidation in hepatic mitochondria is primarily channeled to ketogenesis. Peroxisomes serve as the primary site for the β-oxidation of very-long-chain, branched-chain, and dicarboxylic fatty acids. Peroxisomes do not require the carnitine shuttle to import fatty acid substrates, instead of importing the correspondent acyl-CoAs through the activity of the ATP-binding cassette (ABC) transporters ABCD1-3[5]. Within the peroxisomes, acyl-CoAs are then oxidized by a dedicated set of enzymes, distinct from the mitochondrial fatty acid β-oxidation machinery. Both mitochondria and peroxisomes also require a supply of NAD^+ and free CoA to oxidize fatty acyl chains. CoA levels in the liver have been shown to increase in response to fasting, supporting the increased rate of fatty acid oxidation which occurs in this state[6]. Furthermore, increased CoA degradation in the peroxisomes results in a selective decrease in peroxisomal fatty acid oxidation[7]. Therefore, the process of fatty acid oxidation within the cell is regulated by the expression levels and activities of enzymes involved in the activation, transport, and oxidation of fatty acids, as well as the concentrations of cofactors and other metabolites throughout multiple subcellular compartments.

Procedures using tissue homogenates to measure fatty acid oxidation destroy the cellular architecture regulating and supporting this process, leading to a collection of data that does not accurately reflect the *in vivo* metabolism. While techniques using plated primary hepatocytes preserve this system, culturing isolated cells for extended periods of time results in a loss of the *in vivo* gene expression profile that was present in the cells when they were still living within the animal[8,9]. The following protocol describes a method to isolate primary hepatocytes and assay their capacity for fatty acid β-oxidation immediately after isolation and in suspension, using [1-^{14}C]palmitic acid. The assay is based on the measurement of the radioactivity associated with the

acid-soluble metabolites (ASM) or products, like acetyl-CoA, produced by the β-oxidation of [1-^{14}C]palmitic acid[10,11].

PROTOCOL:

All experimental procedures on mice (C57BL/6J, males, 9–11 weeks of age) were approved by the Institutional Animal Care and Use Committees (IACUC) of West Virginia University.

1. Hepatocyte isolation

1.1. Preparation

1.1.1 In the days before the hepatocyte isolation, prepare the buffers and cell culture media listed in **Table 1.** Set up a water bath with the temperature set to 37 °C close to where the surgery will be performed.

1.1.2 On the day of the hepatocyte isolation, under a laminar flow hood, transfer 35 mL of Buffer 1 to a sterile 50 mL centrifuge tube and 70 mL of Buffer 2 to a 100 mL sterile beaker or bottle.

1.1.3 Add antibiotics gentamicin (50 µg/mL) and penicillin/streptomycin (1x) to both buffers.

1.1.4 Transfer 20 mL of Buffer 2 as prepared in step 1.1.3 to a 100 mm cell culture dish and place on ice.

1.1.5 Transfer the remaining 50 mL of Buffer 2 to a sterile 50 mL centrifuge tube. Place the 50 mL tubes containing the antibiotic-supplemented Buffers 1 and 2 in a water bath set at 37 °C and let them warm up for at least 15 min before starting the perfusion.

NOTE: If conducting multiple hepatocytes isolations in a session, scale up the number of antibiotic-supplemented aliquots of Buffers 1 and 2 to prepare accordingly.

1.1.6 Thaw an aliquot of collagenase solution and keep on ice.

NOTE**:** If properly stored, there is no significant loss of enzyme activity in collagenase solutions frozen and thawed up to 3 times and used within 3 weeks from preparation.

1.1.7 Prepare the surgical instruments and peristaltic pump. Sterilize the lines of the peristaltic pump by circulating 15 mL of 70% ethanol, followed by 15 mL of sterile water.

1.1.8 Connect a 22 G needle to the exit line (**Figure 1A**). The hollowed filter of a catheter works well as a connector. Fill the lines with Buffer 1 and inspect the lines, connector, and needle to ensure no air bubbles are trapped.

[Insert **Figure 1** here]

1.2. Liver perfusion and dissociation

1.2.1. Anesthetize a mouse via isoflurane inhalation, using 4% isoflurane for induction and 1.5% isoflurane to maintain anesthesia. Verify the depth of anesthesia by assessing the loss of pedal reflex.

1.2.2. If there is no response to toe pinching, place the mouse in a supine position on a surgery board, outstretch the limbs, and secure them to the board with pins.

1.2.3. Liberally spray the abdomen and chest of the mouse with 70% ethanol.

1.2.4. Using forceps, pull up the skin and abdominal wall near the base of the abdomen and cut laterally, on either side of the midline and up to the diaphragm, to expose the organs.

1.2.5. Expose the inferior vena cava (IVC) by moving the intestines to the right side and gently flipping the lobes of the liver up. Insert a small cylindrical object, such as a needle cap, under the back of the mouse to slightly tilt the IVC and facilitate its cannulation (**Figure 1B**).

1.2.6. Start the pump at the lowest speed and, with Buffer 1 flowing, insert the needle into the IVC.

1.2.7. Cut the portal vein to relieve the pressure and allow for drainage of blood and perfusion buffers, then immediately increase the flow rate to 7 mL/min. If done correctly, the liver will uniformly blanch within a few seconds (**Figure 1B**).

1.2.8. For more consistent results, hold the needle in position by hand for the entire duration of the perfusion.

1.2.9. Perfuse the liver with warm Buffer 1. To avoid introducing air bubbles, ensure that the line inserted in the tube containing Buffer 1 remains continuously submerged.

1.2.10. While perfusion occurs, add 130 µL of collagenase solution to Buffer 2 and mix by pipetting up and down or stirring with a 5 mL or 10 mL serological pipette.

1.2.11. As the volume in the tube containing Buffer 1 decreases to about 5 mL, slowly add 5 mL of Buffer 2 to Buffer 1 by pipetting on the side of the tube. The goal is to avoid introducing air bubbles in the line while changing from Buffer 1 to Buffer 2.

1.2.12. Wait until the volume decreases again to 5 mL and slowly add another 5 mL of Buffer 2. Repeat one more time. As Buffer 2 replaces Buffer 1 and the dissociation starts, the liver will swell.

1.2.13. Add the remaining Buffer 2 to the tube originally containing Buffer 1. Stop the perfusion when there is about 5–10 mL of Buffer 2 left in the tube.

NOTE**:** While Buffer 2 is perfusing the liver, the portal vein can be intermittently clamped with forceps for 5 s. This step is optional, but the resultant increase in pressure throughout the liver can improve its dissociation and, thus, the final hepatocyte yield.

1.2.14. Carefully excise the liver and transfer it to the 100 mm culture dish containing the 20 mL of ice-cold Buffer 2 set aside at step 1.1.4.

1.2.15. Under the laminar flow hood, gently break the liver apart using surgical scissors and tweezers.

1.2.16. Add about 20 mL of ice-cold M199 to the hepatocyte suspension and filter it through a 100 µm cell strainer using the plunger of a syringe to gently promote the release of additional hepatocytes from larger liver pieces.

1.2.17. Wash the 100 mm culture dish and the cell strainer with additional M199 until the collection tube is full.

1.2.18. Centrifuge the suspension at 50 x *g* for 2 min at 4 °C. Carefully aspirate the supernatant and gently resuspend the hepatocyte pellet in 30 mL of cold M199 by swirling.

1.2.19. Pellet the hepatocytes as mentioned in step 1.2.18. Repeat the wash one more time.

1.2.20. Resuspend the hepatocytes in 10 mL of warm M199 and determine the viability and yield using the trypan blue exclusion method and a hemocytometer[12].

1.2.21. Dilute the cells in M199 to a final concentration of 1.0 x 10^6 viable cells/mL and immediately start the assay.

2. Fatty acid β-oxidation assay

NOTE: The assay is conducted in triplicate, and each reaction mixture contains 750,000 cells, 1.35 mg/mL bovine serum albumin (BSA), 100 µM palmitic acid, and 0.4 µCi [1-^{14}C]palmitic acid in a final volume of 2 mL.

CAUTION: Radioactive compounds are hazardous. Purchase, handle, store, and dispose of radioactive material in accordance with Institutional, State, and Federal regulations.

2.1. Preparation

2.1.1 In the days before the assay, prepare the palmitic acid and BSA solutions (**Table 1**) and store them at -20 °C.

2.1.2 On the day of the assay, complete steps 2.1.3–2.1.9 before starting the liver perfusion.

2.1.3 Thaw the palmitic acid and BSA solutions. Prepare the substrate mixture for multiple reactions plus a 20%–30% excess, with a typical assay setup shown in **Table 2**.

2.1.4 Aliquot 13.5 µL of BSA solution per reaction in a microcentrifuge tube and warm to 41 °C, then add 1 µL of the 200 mM palmitic acid solution (BSA: palmitic acid molar ratio = 1:5) per reaction.

2.1.5 Vortex vigorously and incubate at 41 °C to facilitate the formation of the soluble palmitic acid: BSA complex. Vortex occasionally during the incubation period.

2.1.6 The mixture will initially appear cloudy but will clarify completely after 20–30 min of incubation at 41 °C. Keep it at 41 °C until ready to start the reactions.

NOTE: It is preferable to dispense solutions prepared using organic solvents, such the radioactive and non-radioactive palmitic acid solutions, with a positive displacement pipette and appropriate tips.

2.1.7 Aliquot 133 µL of 1 M perchloric acid in 1.5 mL microcentrifuge tubes to stop the reactions.

CAUTION: Perchloric acid is a strong acid and a strong oxidant. Appropriate protective gear is required for handling this compound.

2.1.8 Aliquot 485.5 µL of M199 per reaction into a tube and keep it at 37 °C to dilute the radioactive BSA: palmitic acid complex prepared at steps 2.1.3–2.1.4 before starting the reactions.

2.1.9 Dispense 750 µL of M199 in as many 14 mL round-bottom tubes as the samples. If desired, add inhibitors of fatty acid β-oxidation, such as etomoxir, rotenone, and antimycin, including a vehicle control (**Table 2**).

2.1.10 During the hepatocyte wash steps, 10–15 min before starting the reactions, transfer the tubes to a shaking water bath set to 37 °C and shaking at 180–200 rpm.

2.2. Starting, stopping, and analyzing the fatty acid β-oxidation reactions

2.2.1 If the viability of the hepatocytes is acceptable (typically ≥75%, **Figure 2**), for each reaction, transfer 0.8 µL of [1-^{14}C]palmitic acid (0.5 mCi/mL) to the microcentrifuge tube containing the clarified BSA: palmitic acid solution (steps 2.1.3–2.1.4). Vortex and return to the water bath at 41 °C.

2.2.2 To equilibrate the hepatocytes to 37 °C and to pre-incubate them with inhibitors (if present) immediately after the final hepatocyte resuspension (step 1.2.21), transfer 750 µL of the hepatocyte suspension with a 1 mL pipette to each of the 14 mL round-bottom tubes in the shaking water bath (step 2.1.9–2.1.10) and vortex briefly at low speed to mix.

2.2.3 Stagger each addition by 30 s and incubate for 15 min. To save a sample for protein determination, transfer another aliquot of hepatocytes to a 1.5 mL microcentrifuge tube and spin at 3,000 x g for 5 min.

2.2.4 Remove the supernatant and store the pellet at -80 °C until ready to measure the total amount of protein in the sample to normalize the results (**Figure 3**).

NOTE: While dispensing, the hepatocyte suspension needs to be continuously swirled or gently stirred with the dispensing 1 mL pipette to prevent settling and large variability in cell number across samples.

2.2.5 While the hepatocytes are under pre-incubation at 37 °C, add the radioactive BSA: palmitic acid complex to the warm medium in 2.1.8, and keep at 37 °C until ready to start the reactions. This is the final substrate mix.

2.2.6 To start the reactions, remove the hepatocytes from the water bath and add 500 µL of substrate mix.

2.2.7 Vortex at low speed for 5 s to completely resuspend the cells and return to the water bath. Repeat with all the samples, staggering by 30 s.

2.2.8 Incubate for 15 min. Start a set of reactions and immediately stop (see steps 2.2.10–2.2.11) to determine the background radioactivity (**Table 2**).

2.2.9 Transfer duplicate aliquots (200–250 µL) of the leftover substrate mix to 6 mL scintillation vials and set aside to count. Use these counts to calculate the radioactivity corresponding to the total nmoles of palmitic acid available for oxidation in 500 µL of substrate mix.

2.2.10 To stop the reactions, remove the hepatocytes from the water bath, resuspend the hepatocytes by vortexing at moderate speed, and then transfer 400 µL of the hepatocyte suspension to the microcentrifuge tubes containing perchloric acid.

2.2.11 Immediately cap the tubes and vortex. Repeat this sequence for all the samples, staggering by 30 s.

2.2.12 Spin down the 1.5 mL microcentrifuge tubes at 13,000 x *g* for 10 min.

2.2.13 Transfer 300 µL of the supernatant to a 6 mL scintillation vial, add 4 mL of scintillation fluid, and count the radioactivity in the samples and the substrate mix aliquots (step 2.2.9) in a

scintillation counter.

CAUTION: After centrifuging, open the tubes under a fume hood to avoid breathing the ^{14}C-CO_2 produced by the complete oxidation of ^{14}C-acetyl-CoA generated by fatty acid β-oxidation and released by the acidic conditions.

REPRESENTATIVE RESULTS:

The liver perfusion described here typically yields 30–40 million cells/liver with average viability of 80%, as estimated by trypan blue exclusion (**Figure 2**). The typical concentration of glucose in the Krebs-Henseleit buffer (KHB), which is used to prepare the perfusion Buffers 1 and 2, is 11 mM. When measuring fatty acid β-oxidation in hepatocytes isolated from fasted mice, the concentration of glucose in the KHB can be lowered to better represent the fasted state. As shown in **Figure 2**, lowering the glucose concentration to 5.6 mM has no negative effect on the yield or viability of the hepatocytes.

Table 2 shows a typical experimental setup for a hepatocyte suspension assayed in triplicate in the presence and absence of etomoxir, a potent inhibitor of CPT1 and thus, mitochondrial fatty acid oxidation[10,13]. In the presence of this or other inhibitors of mitochondrial fatty acid oxidation, any residual ^{14}C-labeled products generated by [1-14C]palmitic acid oxidation can be ascribed to the first cycle of β-oxidation in the peroxisomes. Thus, the contribution of mitochondrial fatty acid oxidation to total fatty acid β-oxidation can be calculated as the difference between total (-etomoxir) and peroxisomal (+ etomoxir) fatty acid oxidation[7,14,15] (**Figure 3**).

For hepatocytes, more than 95% of the radioactivity associated with the products of the β-oxidation of [1-^{14}C]palmitic acid is found in the ASM, and the rest is released as ^{14}C-CO_2[10]. The counts per minute (CPM) associated with the background radioactivity vary with the batch of [1-^{14}C]palmitic acid. However, they are still significantly lower than the CPM obtained in samples allowed to incubate with the substrate mix for 15 min (**Figure 3A**). As expected, hepatocytes isolated from fasted mice show a robust increase in the rates of both mitochondrial and peroxisomal fatty acid β-oxidation, consistent with the known activation of these pathways[16–19].

FIGURE AND TABLE LEGENDS:

Table 1: Buffers, media, and other solutions required for the hepatocyte isolation and the fatty acid β-oxidation assay

Table 2: Example of the experimental setup for a hepatocyte suspension assayed in triplicate in the presence and absence of etomoxir.

Figure 1: Perfusion apparatus and perfused liver. (**A**) Peristaltic pump with outlet line connected to the needle used to cannulate and perfuse the liver. (**B**) Successful cannulation is indicated by immediate and homogeneous blanching of the liver.

Figure 2: Viability and yield of hepatocytes isolated using the procedure described herein. Hepatocytes were isolated from male mice fed *ad libitum* or fasted overnight for 16–18 h. (**A**)

Hepatocyte viability and (**B**) yield per liver. Data are reported as the mean (bars) of measurements on individual hepatocyte preparations (circles) ± SEM. Hepatocytes isolated from fed and fasted mice were compared using an unpaired two-tailed Student's t-test. * $p < 0.05$.

Figure 3: Fatty acid β-oxidation capacity in hepatocytes isolated from fed and fasted male mice and assayed in suspension. Freshly isolated hepatocytes were pre-incubated with etomoxir (45 µM, +Eto) or DMSO (vehicle, -Eto) before the addition of the substrate mix. (**A**) Total CPM introduced in each assay and recovered in the ASM fraction of reactions set up to estimate the background radioactivity, total (-Eto), peroxisomal (+Eto), and mitochondrial fatty acid β-oxidation. These data are shown before any correction (for background, cell number, or protein levels) or any other calculations were applied. (**B**) Data in (**A**) corrected for the background, the total volume of the assay, normalized to 1 million viable cells and expressed as the rate at which palmitic acid is oxidized in hepatocytes isolated from fed and fasted mice. (**C**) Total protein corresponding to the estimated 750,000 hepatocytes/assay used. (**D**) Data in (**A**) corrected as in (**B**) but normalized to mg of protein. Data are reported as the mean (bars) of measurements on individual hepatocyte preparations (circles) ± SEM. Hepatocytes isolated from fed and fasted mice were compared using an unpaired two-tailed Student's t-test. * $p < 0.05$; ** $p < 0.01$.

DISCUSSION:

During the liver perfusion, it is critical to avoid the introduction of air bubbles, as they block the microcapillaries in the liver, preventing or restricting the buffer circulation and overall decreasing the hepatocyte yield and viability[20,21]. Precautions, such as closely inspecting the buffer-filled inlet line before cannulation of the IVC and avoiding lifting the inlet line off the tube containing Buffer 1 to switch to Buffer 2, as described herein, can successfully decrease the number of failed perfusions (viability <70%). The use of bubble traps in the perfusion system can also significantly reduce this risk[20,21].

The collagenase activity is another critical parameter for the isolation of hepatocytes, historically requiring testing and optimization of each new batch acquired[12,20]. The use of a highly purified and defined blend of collagenases dramatically reduces the batch-to-batch variability, eliminating the need to test each new batch. Furthermore, when these blends are used, small adjustments in the volume used (±10–20 µL) are usually sufficient to restore high yields or viability of the hepatocyte preparations.

Hepatocytes are delicate cells. All resuspension and dispensing steps should be done gently by swirling or pipetting slowly to reduce shear damage and lysis. The use of wide-bore tips can also further minimize hepatocyte damage. Vortexing at steps 2.2.2 and 2.2.7 of the assay should be done at the lowest setting possible that still ensures good mixing of the components.

Compared to traditional protocols[20–22], one of the major changes introduced in the hepatocyte isolation procedure described here is the replacement of the intravenous catheter insertion and ligation with the insertion of a hypodermic needle held in position by the operator. This modification provides two main advantages. First, it decreases the risk of introducing air bubbles when connecting the end of the intravenous catheter to the line, as the buffer is already flowing

when the hypodermic needle is inserted in the IVC. Second, it decreases the risk of perforating the IVC during manipulations of the catheter, such as the retraction of the needle or its securing with sutures. One of the drawbacks of this modification is that it is usually necessary to hold the needle in position by hand for the duration of the perfusion to ensure the consistent success of the procedure. This can be tiring for the person performing the surgery and could limit the number of consecutive perfusions that can be done in a session. To limit the movements of the person holding the needle, which could cause inadvertent perforation of the IVC, it is advisable to work in pairs, with one person conducting the surgery and another person changing the perfusion buffers without interruptions in the perfusion. Multiple back-to-back perfusions would require a third person to start the fatty acid β-oxidation assay within 1–2 min of each completed hepatocyte isolation.

Similar to other hepatocyte isolation methods, the procedure described here yields hepatocytes that can be used in suspension or in culture to assess a variety of other liver processes, including additional metabolic pathways and changes in gene expression due to various treatments[23–25]. To culture the hepatocytes, steps 1.2.20–1.2.21 can be easily modified by resuspending the cells in the appropriate medium, followed by plating in cell culture dishes and incubation[20,22,26]. Furthermore, while not required for the β-oxidation assay, if needed by other applications, the percentage of viable hepatocytes can be increased by removing the dead cells through a Percoll layer[22,26].

In conclusion, this protocol describes a robust assay to measure the rate of fatty acid β-oxidation in intact hepatocytes and without the addition of exogenous cofactors, thus preserving the endogenous regulatory mechanisms of this pathway.

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DISCLOSURES

The authors have no conflicts of interest to disclose.

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Figure 1

Click here to access/download;Figure;Fig1_perfusion apparatus and mouse_combined_flat.tif

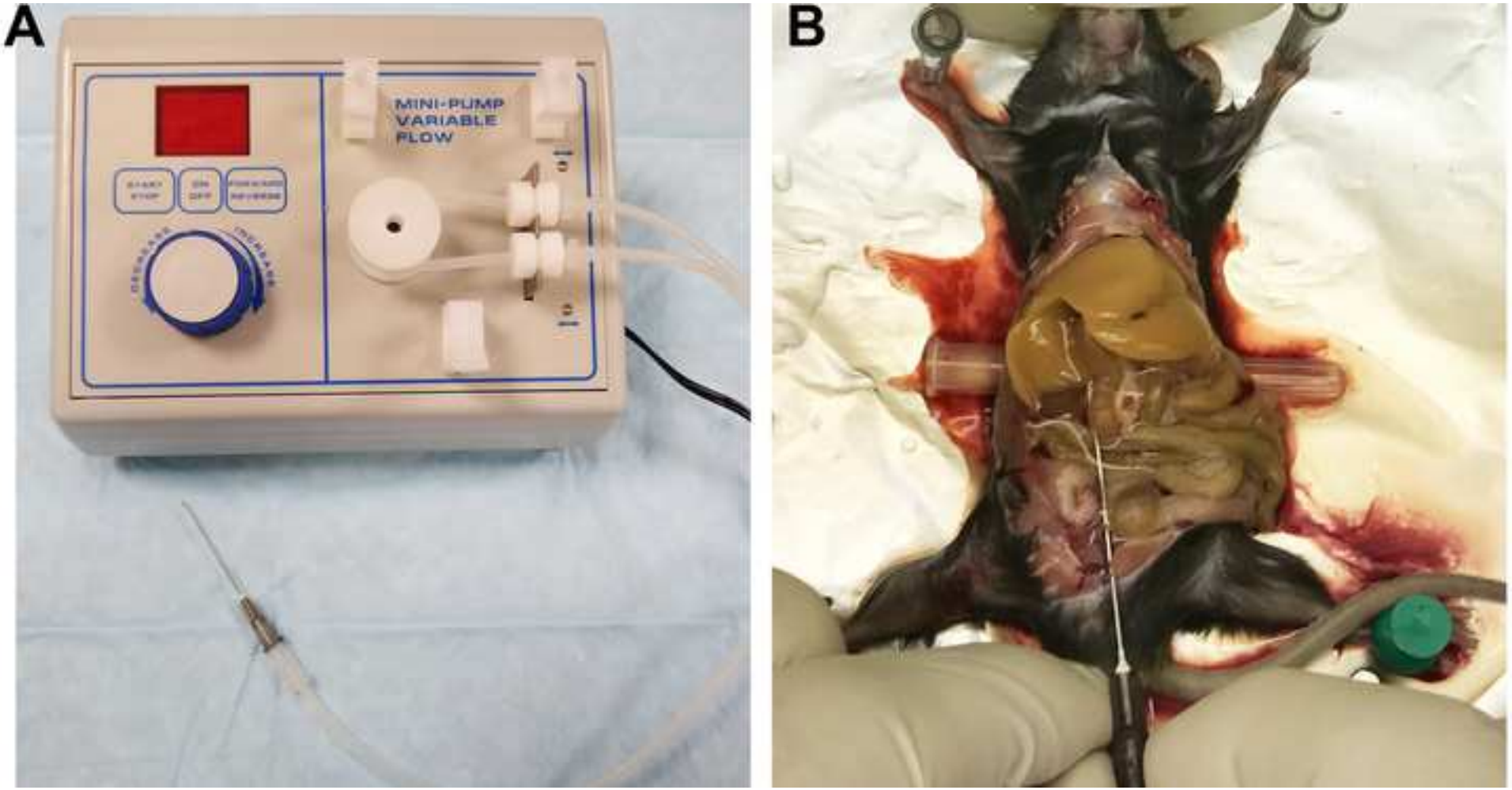

Figure 2

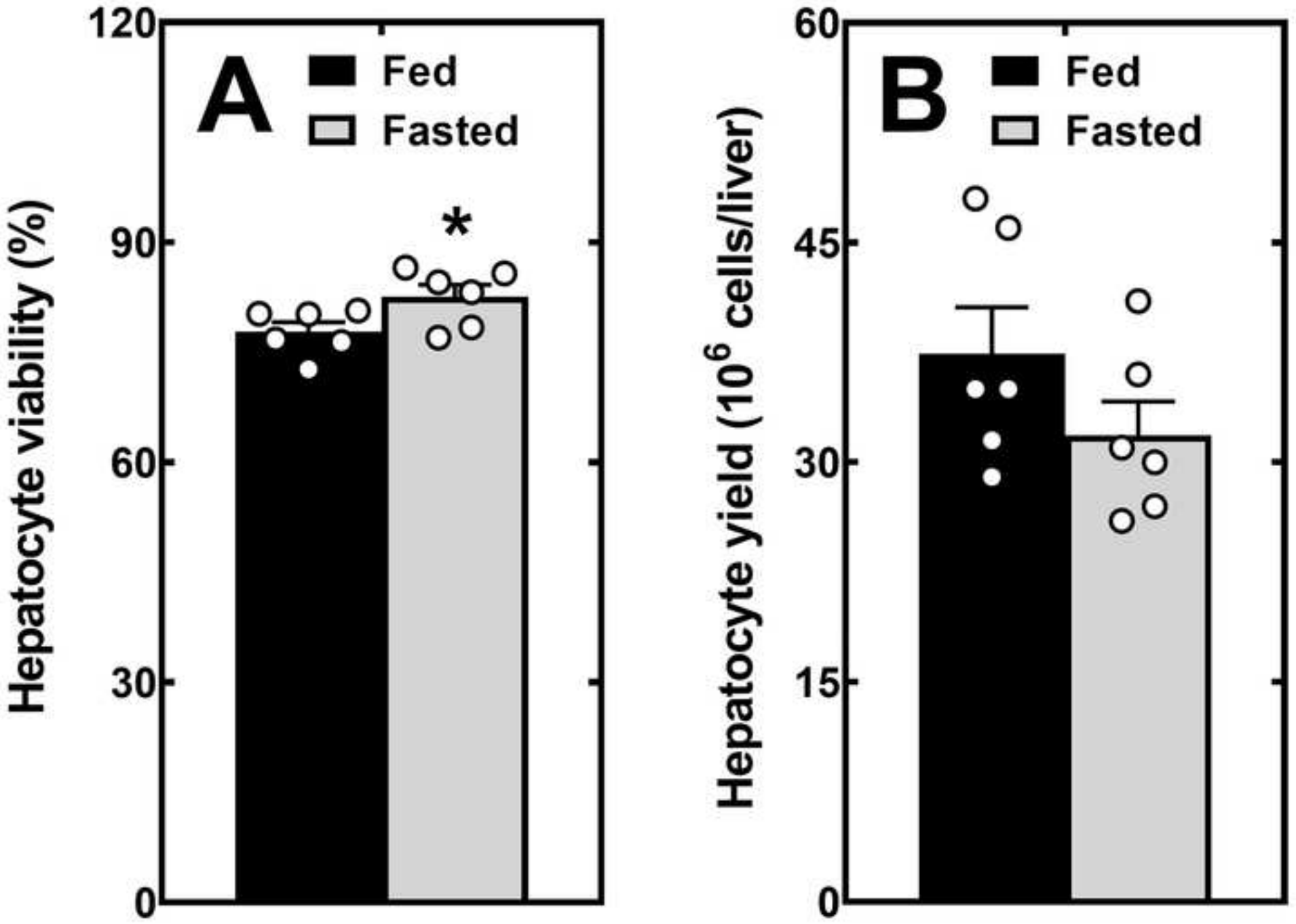

Figure 3

Click here to access/download;Figure;Fig 3_b-oxidation results.tif

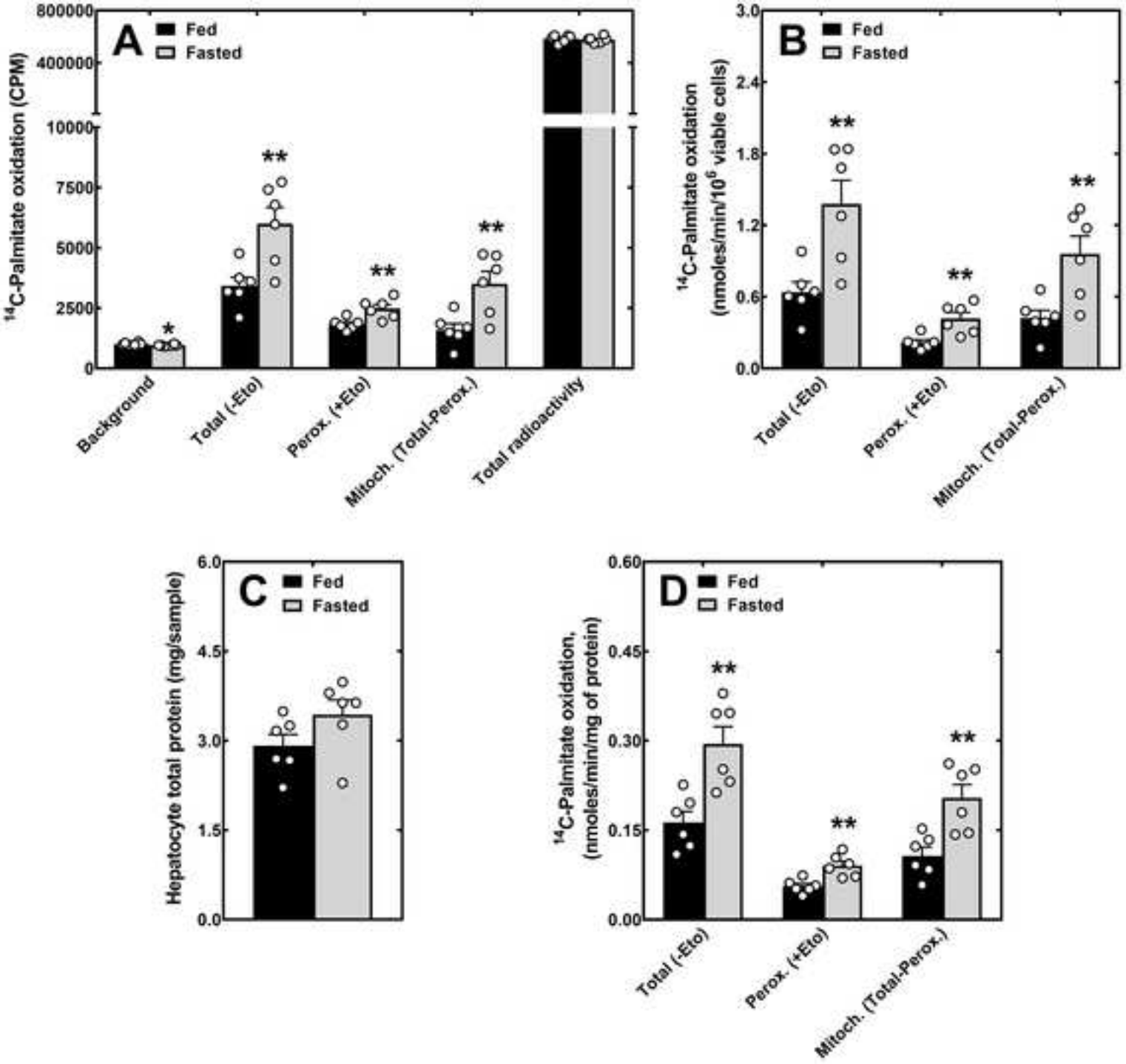

Table 1

Buffers/Media	Amount	Final Concentration
Solution C		
KCl	1.79 g	480 mM
$MgSO_4$ heptahydrate	1.48 g	120 mM
KH_2PO_4	0.81 g	119 mM
Krebs-Henseleit Buffer (KHB), calcium-free		
NaCl	7.0 g	120 mM
$NaHCO_3$	2.0 g	24 mM
1 M HEPES pH 7.45	5 mL	5 mM
Glucose	1 or 2 g	5.6 or 11 mM
Solution C	10 mL	
Buffer 1		
KHB	500 mL	
50 mM EGTA	1.0 mL	0.1 mM
Buffer 2		
KHB	500 mL	
1 M $CaCl_2$ dihydrate	686 µL	1.4 mM
Gentamicin solution		
Gentamicin sulphate	0.5 g	50 mg/mL
Collagenase solution		
Collagenase I and II blend	10 mg	7 mg/mL
M199		
M199	1 pouch	
$NaHCO_3$	2.2 g	26 mM
1 M HEPES (cell culture grade)	25 mL	25 mM
Extra glucose (only for fed mice)	1 g	11 mM
BSA solution		
Fatty acid-free BSA	400 mg	20% (w/v)
Non-radioactive palmitic acid solution		
Palmitic acid	103 mg	200 mM
1 M Perchloric acid		
70% Perchloric acid	3.5 mL	1 M

Instructions
Add water to 50 mL. Store at 4 °C
Add water to 900 mL, adjust the pH to 7.4, and bring the final volume to 1 L. Store at 4 °C
Mix components and filter sterilize. Store at 4 °C
Mix components and filter sterilize. Store at 4 °C
Add water to 10 mL and filter sterilize. Aliquot and store at -20 °C
Dissolve the entire content of the vial in 1.43 mL of water. Aliquot and store at -20 °C
Add water to 900 mL and adjust the pH to 7.2-7.4. Bring the final volume to 1 L and filter sterilize
Dissolve in 2 mL of water. Aliquot and store at -20 °C
Dissolve in 2 mL of ethanol, store at -20 °C
Dilute to 40 mL with water. Store at room temperature

Table 2

Reaction number	M199 ± Inhibitors			Hepatocyte suspension (µL)	
	Volume (µL)	Etomoxir			
1	750	-	Pre-warm at 37 °C	750	Pre-incubate at 37 °C for 15 min
2					
3					
4		+			
5					
6					
7		+			
8					
9					

Substrate mix (µL)	
500	Incubate at 37 °C for 15 min
	Stop immediately

Response to the reviewers

Manuscript ID: JoVE62904

Title: Measurement of Fatty Acid β-Oxidation in a Suspension of Freshly Isolated Mouse Hepatocytes

Authors: Schuyler D. Vickers, Dominique C. Saporito, and Roberta Leonardi

We are thankful to the reviewers for their insightful comments and we have addressed the below.
Please refer to the separately submitted revised manuscript to see all the changes (underscored) made to the original version.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
All the authors have proofread the manuscript and edits have been tracked in the revised document.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
These changes have been made as requested.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Exel Safelet, etc.
We have now removed the commercial names Liberase (replaced by 'collagenase solution') and Exel Safelet in the revised version of the manuscript.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
We have changed the action description throughout the manuscript by using the imperative tense. Safety procedure were already included in the original version of the manuscript.

5. For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2
We have corrected the abbreviation for liters to L throughout the manuscript.

6. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks
We have complied with this requirement.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software

actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
We have added more details throughout the protocol, as requested by both the editorial staff and the reviewers.

8. Line 92: Please specify the antibiotics used in this protocol.
We have clarified that gentamicin, penicillin and streptomycin mentioned at line 99 are the antibiotics used in this protocol by slightly modifying sections 1.1.2 and 1.1.3.

9. Line 112: Please specify the dose of isoflurane used for induction and maintenance of anesthesia.
We have added the isoflurane doses in section 1.2.1.

10. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
We have highlighted what we think are the critical steps of the protocol, which roughly correspond to 3 pages.

11. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
The discussion has been re-organized and expanded so that points a) and b) are now covered, together, in the first paragraph; points c) and d) are covered in the second paragraph, and e) is covered in the third paragraph.

12. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.
The References have been modified as requested.

13. Figures: Please revise the Y axis of the figures to include the units within parenthesis.
The Y axis of the figures have been modified as requested.

14. Table 1/2: Please use standard abbreviation for units (e.g., 7 mg/ mL, 3.5 mL, 20%, µL etc.)
Tables 1 and 2 have been updated as requested.

15. Please sort the Table of Materials in alphabetical order.
The Table of Materials has been sorted as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
A method for measuring the oxidation of fatty acids in hepatocytes.

Major Concerns:
none

Minor Concerns:
1. The introduction should describe the major end product of hepatic beta oxidation - Ketone body synthesis.
We have added this detail at lines 59-60.

2. Section 2.2.1 has the investigator pipetting 0.8 uls of solution. It is not possible to accurately measure this small amount. I would suggest changing this to a larger volume.
We have now clarified, in section 2.1.3, that the substrate mixture is prepared for multiple reactions (typical assay set up, which includes 9 reactions, is shown in Table 2) and the 0.8 µl of 14C-palmitic acid is the volume required per reaction. To further stress this point, we have also explicitly mentioned that the assay is conducted in triplicate in section 2, line 176.

3. The authors say that the elimination of beta oxidation by etomoxir shows peroxisomal oxidation. What about omega oxidation? why is there no mention of this pathway.
ω-Oxidation converts a monocarboxylic fatty acid to a dicarboxylic fatty acid without fatty acyl chain shortening. Therefore, ω-oxidation, by itself, does not produce any acid soluble metabolites (e.g. acetyl-CoA). Instead, the resultant dicarboxylic fatty acids are broken down by β-oxidation in the peroxisomes and, to a lesser extent, in the mitochondria. So, even if ω-oxidation converts some 14C-palmitic acid to the correspondent hexadecanedioic acid, in the presence of etomoxir, any acid soluble metabolite derived from the oxidation of this acid would still be generated by the peroxisomes. We have added a short description of ω- and also α-oxidation processes in the introduction, lines 46-52.

4. The introduction should make clear that this is for long chain fatty acids. Of course medium and short chain fatty acids can be measured this way but etomoxir would not differentiate the organeller oxidation.
To clarify that only long-chain acyl-CoAs require the activity of CptI, which is the target of etomoxir, we have now added the word 'long-chain' in line 54 of the Introduction.

Reviewer #2:

Major Concerns:
On page 6, line 208, 'be used to normalize the results'. Will it be better to use the numbers of viable cells for normalization because the damaged or non-viable cells still contribute to the protein amount, but these cells may not be functional anymore for the assay. The cell pellets in this protocol include both viable and non-viable cells.
We have now added a new panel B to Fig. 3 showing the data as normalized to 1 million viable cells (we use 750,000 viable cells in each sample). As it can be seen comparing the new panels B and D, normalizing the counts to total protein or viable cells yields the same results, arguing that the proteins that are still potentially associated with the cell debris and pelleted together with the viable cells do not interfere with the conclusions in any significant manner. The main advantage of normalizing the data to total protein is that assays that measure protein concentration do not rely on the sensitivity and color

perception of the human eye, which is what the trypan blue exclusion method is based on, to determine whether an hepatocyte should be counted as viable or not.

Minor Concerns:

* On page 5, line 133. Is 'the line' the perfusion tube line?

Yes. We have now specified what line we are talking about by adding the words 'inserted in the tube containing Buffer 1' in section 1.2.5

* On page 5, line 162. Can authors please provide information regarding how to keep the freshly isolated hepatocytes before the assay? Are the hepatocytes kept on ice, or room temperature or at 37 °C?

The assay is started immediately after the final re-suspension (step 1.2.11). We had previously mentioned this detail in the discussion but we have now better clarified the timing of the assay with respect to the hepatocyte isolation by adding more details in throughout section 2.1.

* On page 6, line 184. Can authors please explain why 41°C is used? (is it because of the solubility?)

Correct. We have clarified this point in section 2.1.3 by modifying the text as: 'Vortex vigorously and incubate at 41 °C to facilitate the formation of the soluble palmitic acid:BSA complex'.

* On page 6, line 203, 'transfer 750 ul'. Can authors please provide details regarding how to transfer (presumably by pipetting)?

Yes, we transfer the hepatocyte suspension to tubes with pipetting. This detail has been added to section 2.2.2.

If it needs to pipette 750 ul of hepatocytes, wide-bore tip is recommended because it is less forceful and reduces the cell damage of hepatocytes. * On page 6, line 210, 'dispensing pipette'. Please use wide-bore tips for pipetting hepatocytes to avoid cell damage.

While we use standard 1 ml tips, we do pipette slowly when transferring hepatocytes from one vessel to another. We have now explicitly indicated that hepatocytes are delicate and need to be transferred and mixed gently in the revised Discussion (lines 327-330), also including a mention to the use of wide bore tips to minimize damage.

* On page 6, line 205, 'shaking at a moderate speed'. Can authors please specify the range of the speed?

Between 180 and 200 rpm. We have added this detail at step 2.1.6. In general, while working with primary hepatocytes requires a gentle touch, efficient exposure to inhibitors and substrates, including oxygen (the final acceptor of the electrons generated by fatty acid β-oxidation), still requires some mixing to break the clumps and shaking (see also other points below). Importantly, the way we do the assay described in this protocol consistently produces high quality data that reveal not only major differences in the rate of fatty acid oxidation between the fed and fasted states (Fig 3) but also more subtle differences in mitochondrial vs peroxisomal fatty acid oxidation due to genetic manipulations (Shuman SA, J. Lipid Res 2019, 60:1005).

* On page 6, line 218, 'vortex gently for 5 seconds'. Need to avoid vortex because it causes hepatocyte damage.

See comment above. However, to be more precise, in section 2.2.4, we replaced the expression 'vortex gently' with 'vortex at low speed for 5 s to completely re-suspend the cells'. Indeed, the hepatocytes settle even if shaken.

* On page 7, line 225, 'vortex at moderate'. Will pipetting with wide-bore tip be better?

This step is done to thoroughly resuspend the hepatocytes (again, they settle even with shaking) before stopping the reaction. At this point of the assay, it does not matter if the cells break, as the

suspension is transferred to a tube with perchloric acid to precipitate the palmitic acid, lyse the hepatocytes, and release the acid soluble metabolites.

Response to the requested revisions

Manuscript ID: JoVE62904

Title: Measurement of Fatty Acid β-Oxidation in a Suspension of Freshly Isolated Mouse Hepatocytes

Authors: Schuyler D. Vickers, Dominique C. Saporito, and Roberta Leonardi

Editorial comments:

1. Line 94-95: Please include the strain, age, and sex of the mice used in this study.

This information (underlined) has been added, as requested

To make corrections or further clarify a few steps, additional minor changes (underlined) were made at lines:

113
185
222
225
252
263
289-291
303

Additionally, we have un-highlighted lines 180-182, as we don't routinely clamp the portal vein and we were not going to show it in the video.