Version 3 Last updated 19 December 2018

ab217602 Fatty Acid Oxidation Assay

For the convenient measurement of Fatty Acid Oxidation (FAO) in live cells when used in combination with Extracellular Oxygen Consumption Assay (ab197243).

View kit datasheet: www.abcam.com/ab217602
(use www.abcam.cn/ab217602 for China, or www.abcam.co.jp/ab217602 for Japan)

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Fatty Acid Oxidation Assay (ab217602) allows the detection of Fatty Acid Oxidation (FAO) in live cells. This product is designed to be used in combination with our Extracellular Oxygen Consumption Assay (ab197243).

The assay uses the 18C unsaturated fatty acid Oleate as substrate, and includes two FAO modulators, etomoxir and FCCP. Etomoxir, an inhibitor of the carnitine transporter CPT1, prevents Oleate import and thereby limits the supply of reducing equivalents to the ETC, reducing oxygen consumption in turn. The remaining ETC (electron transport chain) activity is driven by non-long chain FAO. FCCP treatment induces maximal ETC activity by dissipating the mitochondrial membrane potential, while the increased demand for reducing equivalents causes a concomitant increase in the FAO activity. If exogenous long-chain fatty acid is unavailable or import is inhibited, FAO activity will be limited.

Fatty acid oxidation (FAO) is the primary metabolic pathway for degradation of fatty acids. Figure 1 gives an overview of long-chain fatty acid activation, import and oxidation. FAO is an important process in many tissues during periods of glucose deprivation. In organs, such as liver and skeletal muscle, FAO can provide over 75% of cellular ATP while in cardiac tissue it can be responsible for up to 90% of cellular energy requirements. FAO is also now acknowledged as a key factor in cancer metabolism and is also implicated in drug-induced microsteatosis.

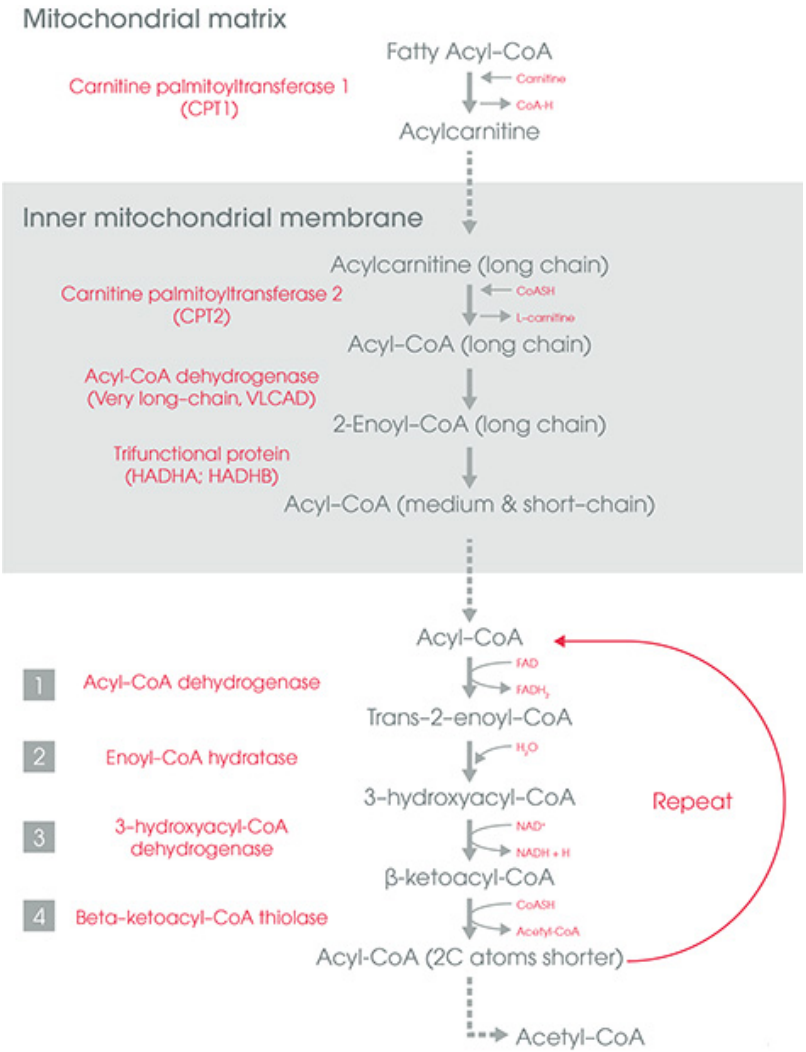

Figure 1. Overview of long-chain fatty acid activation, import and oxidation.

2. Protocol Summary

Day 1

Plate cells and return to culture overnight

Prepare Base Measurement Media

⇩ Incubate Over Night

Day 2 (OPTIONAL)

Prepare Glucose-Deprivation Media

Replace culture media with Glucose-Deprivation Media and return cells to culture

⇩ Incubate Over Night

Day 3

Prepare FA-Free & FA Measurement Media

Prepare FAO kit controls (Etoxomir, FCCP and BSA)

Prepare O_2 Consumption reagent (ab197243)

⇩

Wash cells twice with FA-Free Measurement Media

Add FA/FA-Free Measurement Media and O_2 consumption reagent

Add controls (Etoxomir, FCCP, BSA)

Overlay plate with High Sensitivity mineral oil (ab197243)

⇩

Measure on fluorescence plate reader

Analyze kinetic data output to determine FAO-driven ETC activity

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All assay kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Kit has a storage time of 1 year from receipt. Please observe storage conditions of each individual component described in the Materials Supplied section for correct storage upon receipt.

Δ Note: Reconstituted reagents are stable for 3 months.

5. Limitations

- Kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
FAO Conjugate (3 mM)	1 mL	4°C	4°C
FAO Control (1.5 mM)	500 µL	4°C	4°C
FAO Tablet (base media)	1 tablet	RT	4°C
L-Carnitine	1 vial	4°C	-20°C
FCCP	1 vial	-20°C	-20°C
Etomoxir	1 vial	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Extracellular Oxygen Consumption Assay (ab197243)
- Microplate reader capable of measuring fluorescence, with suitable filter and plate temperature control – see Instrument and Measurement Settings section on the Extracellular Oxygen Consumption Assay (ab197243) protocol for suitable plate readers
- MilliQ water or other type of double distilled water (ddH_2O)
- DMSO
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Sterile 96-well plate (black wall with clear flat bottom), or standard clear plates for cell culture
- Cell culture media
- Base glucose deprivation media: glucose-free DMEM media, 1 mM glucose, 1 mM L-glutamine, 1% FBS, Penicillin/streptomycin solution (100 U/mL /0.1 mg/mL)
- Glucose: to prepare FA-Free Measurement Media
- (Optional) Plate block heater for plate preparation.

To prepare Base measurement media:

- HCl and NaOH: to bring media to pH7.4
- Appropriate 0.2 µm filter to sterilize media

8. Technical Hints

- **This kit is sold based on number of tests. Number of tests based on described procedure, stock dilutions, concentrations and wash steps. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.
- Refer to Instrument and Measurement Settings Table (Table 1) in the user manual for Extracellular O_2 Consumption Assay (ab197243) for recommended settings for your plate reader.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 FAO Conjugate (Oleate-BSA conjugate, 3 mM) (1mL):

Ready to use as supplied. Equilibrate to room temperature prior to use. Store at 4°C.

9.2 FAO Control (BSA, 1.5 mM) (500 µL):

Ready to use as supplied. Equilibrate to room temperature prior to use. Store at 4°C.

9.3 FAO Tablet (1 Base media tablet):

To prepare Base Measurement Media, dissolve tablet provided in 100 mL ddH_2O and warm solution to 37°C to ensure table is completely dissolved. Adjust pH to 7.4 using HCl and NaOH. Filter sterilize base media. Filtered Base Measurement Media can be stored at 4°C for 3 weeks.

9.4 L-Carnitine (L-carnitine hydrochloride, 4 mg):

Prepare a 50 mM L-Carnitine stock solution (100X) by dissolving vial contents in 400 µL ddH_2O. Aliquot L-Carnitine stock solution so that you have enough volume to perform the desired number of assays (recommendation: 100 µL to cover 100 tests). Store at -20°C. Once reconstituted, use component within 3 months.

9.5 FCCP (0.004 mg):

Prepare a 250 µM FCCP stock solution (100X) by dissolving vial contents in 60 µL of DMSO. Aliquot FCCP stock solution so that you have enough volume to perform the desired number of assays (recommendation: 20 µL to cover 20 tests). Store at -20°C. Once reconstituted, use component within 3 months.

9.6 Etomoxir (0.074 mg):

Prepare a 400 µM Etomoxir stock solution (10X) by dissolving vial contents in 550 µL ddH_2O. Aliquot Etomoxir stock solution so that you have enough volume to perform the desired number of assays (recommendation: 50 µL or 100 µL to cover 10 or 20 tests respectively). Store at -20°C. Once reconstituted, use component within 3 months.

10. Sample Preparation

General Sample Information:

- Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.
- Cells are seeded at a density to achieve full confluence on the day of measurement. Plating density, cell type and basal metabolic rate will determine oxygen consumption rate.
- If performing an overnight glucose deprivation step using non-terminally differentiated cells, seeding densities should be adjusted downwards to facilitate doubling.
- We recommend following the plate map suggested in figure 2 to facilitate assay procedure.

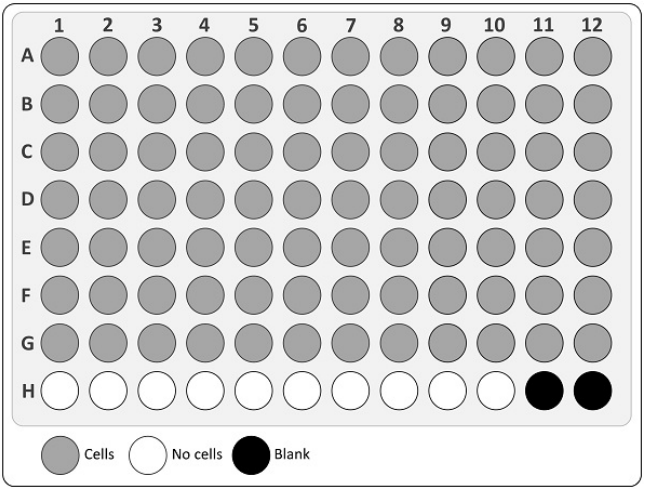

Figure 2. Plate Map.

– If using extended culture periods (> 2 days), we recommend following plate map (figure 3), adding 200 µL of culture medium to all outer wells. This minimizes plate effects related to inconsistent cell growth across the microplate.

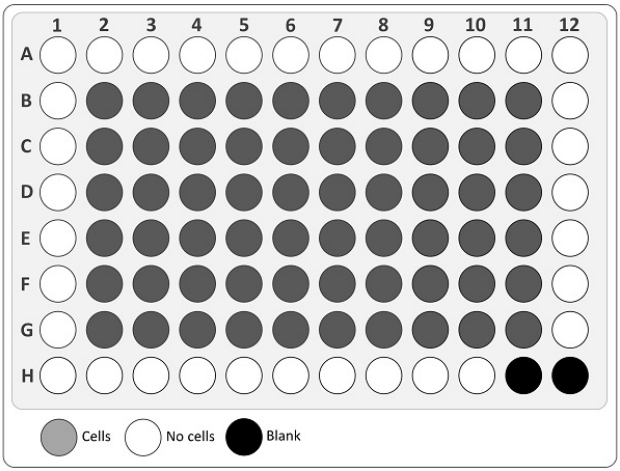

Figure 3. Recommended plate map for extended culture times (> 2 days)

– Inconsistent growth in some cell types can be additionally reduced by allowing the plate to stand at RT for 30 minutes after plating before returning plate to cell culture.

10.1 Adherent cells (2D cell culture):

10.1.1 Seed cells in a 96-well plate a density of 6 x 10^4 cells/well in 200 µL culture medium.

10.1.2 Incubate overnight in a CO_2 incubator at 37°C (typical incubation time > 14 hours).

10.1.3 OPTIONAL: Glucose deprivation step.

Δ Note: Including a glucose deprivation step before performing the assay increases cellular dependence on FAO. For maximum FAO dependence, concentrations of L-Carnitine, glucose and FAO Conjugate should be optimized for each cell type.

10.1.3.1 Prepare Base glucose deprivation media as described in Section 7 (media can be stored for 2 weeks at 4°C).

10.1.3.2 Prepare Glucose deprivation media by adding 0.5 mM L-Carnitine (1:100 of stock) to Base glucose deprivation media.

10.1.3.3 Wash cells twice with Base glucose deprivation media.

10.1.3.4 Add 200 µL of Glucose deprivation media.

10.1.3.5 Incubate overnight in a CO_2 incubator at 37°C (typical incubation time > 14 hours).

10.2 3D cell culture:

10.2.1 Plate cells for 3D cultures at a higher density than optimized for 2D cultures. When plating 3D cultures, prepare the plate or matrix solution in advance as per manufacturer's instructions.

Δ Note: test compounds can take longer to penetrate and affect 3D cultures and longer treatment times may be necessary.

10.2.2 OPTIONAL: Glucose deprivation step.

Δ Note: Including a glucose deprivation step before performing the assay increases cellular dependence on FAO. For maximum FAO dependence, concentrations of L-Carnitine, glucose and FAO Conjugate should be optimized for each cell type.

10.2.2.1 Prepare Base glucose deprivation media as described in Section 7 (media can be stored for 2 weeks at 4°C).

10.2.2.2 Prepare Glucose deprivation media by adding 0.5 mM L-Carnitine (1:100 of stock) to Base glucose deprivation media.

10.2.2.3 Wash cells twice with Base glucose deprivation media.

10.2.2.4 Add 200 µL of Glucose deprivation media.

10.2.2.5 Incubate overnight in a CO_2 incubator at 37°C (typical incubation time > 14 hours).

11. Assay Controls set up

General guidelines for optimal concentrations and treatment times for assay controls:

11.1 FAO modulators:

- FCCP exhibits a bell-shaped dose-response which can vary between cell types. The concentration which delivers maximum respiratory activity should be titrated for each cell type: run a FCCP serial dilution (15-0.5 µM) in the presence of FAO Conjugate. Higher FCCP concentrations may be required when using FAO Conjugates as compared with glucose-based measurement due to the ability of BSA to bind FCCP (FAO Conjugate is a 2:1 Oleate-BSA conjugate).
- FAO Conjugate is typically used at 150 µM. However, the concentration at which maximum respiratory activity is observed can be cell type dependent. Optimum concentration can be determined by measuring oxygen consumption at varying FAO Conjugate concentrations (typically 50-200 µM) in the presence of FCCP. Users may also wish to add FAO Conjugate to Glucose Deprivation Media (typically 100 µM).
- L-Carnitine is typically used at 0.5 mM. However, the optimum concentration to facilitate LCFA transport is cell type-dependent. Optimum concentration can be determined by measuring oxygen consumption at varying L-Carnitine concentrations in the presence of FCCP.
- Etomoxir can exhibit 'off-target' effects if used at > 40 µM. Etomoxir efficacy can be reduced in presence of high serum and BSA concentrations. In these situations, use higher Etomoxir concentrations to ensure CPT-1 inhibition. A minimum of 10 minutes should elapse between Etomoxir treatment and the commencement of measurement to ensure CPT-1 inhibition has impacted oxygen consumption prior to measurement. To maximize inhibition, Etomoxir can be pre-incubated in FA-Free Media prior to the addition of FAO Conjugate or BSA control.

11.2 Optional additional controls:

- Antimycin A can be used as optional negative biological control. Antimycin blocks the ETC thereby inhibiting ETC-related oxygen consumption. To use in the assay, add 1 µL of Antimycin A 100 µM stock solution (in DMSO) to wells.
- Oligomycin can be used as optional coupling control. Oligomycin blocks the F1/Fo ATPase highlighting the portion of O_2 consumption driving aerobic ATP production. Remaining O_2 consumption is typically due to uncoupled mitochondria. To use in this assay, add 1 µL of Oligomycin 100 µM stock solution (in DMSO) to wells.

Control	Typical concentration
FCCP	Titration recommended to establish best concentration
FAO-Conjugate	150 µM
L-Carnitine	0.5 mM
Etomoxir	< 40 µM
Antimycin A	1 µM
Oligomycin	1 µM

12. Assay Procedure

- This assay is designed to be used as companion kit together in combination with Extracellular Oxygen Consumption Assay (ab197243) The Extracellular Oxygen Consumption Assay User Manual describes instrument set-up, assay optimization, data analysis and troubleshooting. The described instrument set-up and signal optimization steps should be performed prior running an FAO assay.
- We recommend that you assay all controls and samples in triplicate.
- Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature (typically 37°C).
- Compounds are typically added immediately pre-treatment to determine their effect on FAO and related mitochondrial functional. For some 3D models, a pre-incubation step can be incorporated to ensure compounds access cells within the 3D construct. Longer treatment times can be used as required: in these instances, compound should be present in both culture media (during incubation) and measurement media.
- Long-term measurement with CO_2 control: additional media buffering capacity is required when conducting long term measurements (> 2 h) outside 5% CO_2. This is achieved by supplementing Measurement Media with 5 mM HEPES. Supplementation is not required if plate reader has environmental gas control where 5% CO_2 can be maintained within the measurement chamber.

12.1 Prepare additional reagents:

12.1.1 FA-Free Measurement Media: To Base Measurement Media (Step 9.3), add 0.5 mM L-Carnitine (1:100 final dilution) and 2.5 mM glucose.

Δ Note: Optimal L-Carnitine and glucose concentration may be cell-type specific and maybe require additional optimization.

Δ Note: If long term measurements are being performed outside 5% CO_2 a HEPES supplement is recommended.

12.1.2 FA Measurement Media: FA-free Measurement Media + 150 µM FAO Conjugate (1:20 dilution from 3 mM stock – Step 9.1).

12.1.3 Extracellular O_2 Consumption Reagent: prepare reagent as described in Extracellular O_2 Consumption Assay (ab197243) user manual.

12.2 Wash cells:

12.2.1 Place the plate on a plate block heater set to assay temperature (typically 37°C) and remove spent culture media with an aspirator (be careful not to dislodge cells from the base of the wells).

12.2.2 With a multichannel or repeater pipette, add 100 µL of the pre-warmed FA-Free Media to each well.

12.2.3 Repeat wash step one more time.

12.3 Add assay media to wells:

- Signal control wells (wells with no cells; row H) = 90 µL of pre-warmed FA Measurement Media.
- Blank control wells (H11 and H12) = 90 µL of pre-warmed FA Measurement Media.
- Sample wells = 90 µL of pre-warmed FA Measurement Media.
- FA-Free control wells =85 µL of FA-Free Measurement Media + 5 µL of BSA control.

Δ Note: FA-Free Measurement Media is used as a control to measure O_2 consumption without FAO Conjugate. BSA control is added to ensure that the free concentrations of test compounds are consistent between FA and FA-Free conditions. BSA concentration in FA-free control wells should be consistent with the BSA concentration in samples containing FAO Conjugate.

12.3.1 Add 10 µL of Extracellular O_2 Consumption Reagent (Step 12.1.3) to each sample, FA-Free control and signal control wells. Do not add to blank control wells.

12.3.2 Add 10 µL of FA Measurement Media to Blank Control wells.

ΔNote: If measuring a full 96-well plate, we recommend diluting reconstituted ab197243 stock 1 in 10 in the relevant measurement media and, using a multichannel pipette, to add 100 µL to each well. Add 100 µL of FA Measurement Media (no ab197243 reagent) to the Blank Control wells.

12.4 Treat cells:

Add relevant treatment or compounds to cells (see Section 13 for more detailed information on how to perform assays). The procedure below describes how to perform drug screening:

12.4.1 Add test compound or vehicle (typically 1-5 µL) to test wells (Step 12.3): we recommend using 6-8 compound dilutions.

Δ Note: We recommend keeping volume of added compound as low as possible to minimize any potential vehicle effects.

Δ Note: Additional BSA control stock is added to wells without Oleate (Step 12.3.1-12.3.2). Cells should be co-treated with FCCP if impact on maximal FAO is being determined. Etomoxir is used as a control.

12.5 Measurement:

12.5.1 Seal each well with 100 µL of pre-warmed High Sensitivity Mineral Oil (component from ab197243), taking care to avoid bubbles.

12.5.2 Read plate immediately in a fluorescence microplate reader as described in the protocol booklet for Extracellular Oxygen Consumption Assay (ab197243).

12.6 Calculations:

12.6.1 Process data as described in Section 15 of the protocol booklet for Extracellular Oxygen Consumption Assay (ab197243).

13. Typical Assays/Data

13.1 Sample FAO-Driven Oxygen Consumption

Cellular dependence on, or preference for FAO can be determined using Etomoxir and FCCP in the presence or absence of FAO substrate (Oleate). Etomoxir blocks long chain fatty-acid (LCFA) uptake, while FCCP increases cellular energy demand, thereby increasing FAO dependence.

For compound addition, add 10 µL of Etomoxir and 1 µL FCCP stock to relevant samples on Step 12.3.

Δ Note: Ensure a minimum of 10 minutes between Etomoxir addition and assay measurement.

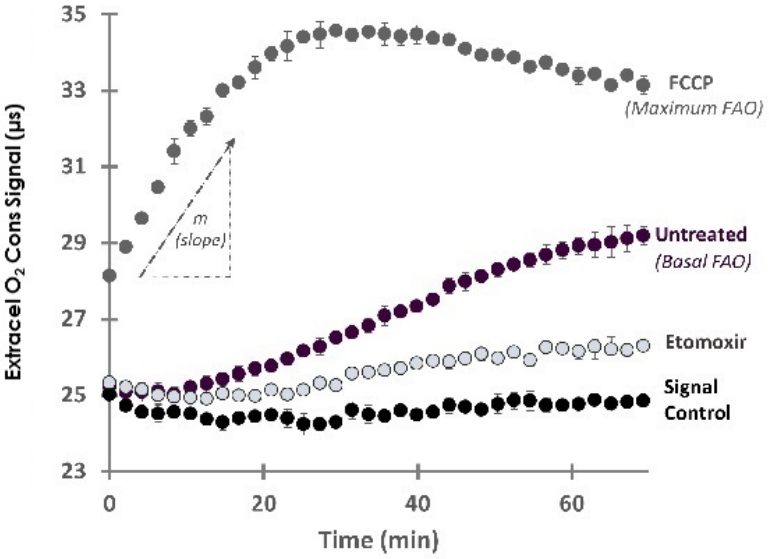

Figure 4. FAO-driven oxygen respiration in HepG2 cells treated with the CPT-1 inhibitor Etomoxir (white) and uncoupler FCCP (gray).

- **Untreated cells** curve shows a steady increase of the Extracellular O_2 Consumption Reagent signal reflecting ETC (electron transfer chain)-driven oxygen consumption.
- **Signal Control** shows probe signal in the absence of cell respiration.

- **Etomoxir treatment** prevents oleate import, resulting in reduced availability of reducing equivalents and a resultant decrease in ETC activity. The remaining ETC activity (difference between Etoxomir treatment and Signal Control) is driven by metabolic activity other than long chain FAO.
- **FCCP treatment** induces maximal ETC activity by dissipating the mitochondrial membrane potential. Increased demand for reducing equivalents causes a concomitant increase in FAO as indicated by the rapid increase in Extracellular O_2 Consumption Reagent signal. This strong increase in ETC activity is not observed where exogenous LCFA is unavailable or where import is inhibited.

13.2 Evaluating Exogenous and Endogenous FAO

FAO-driven respiratory activity can be investigated further by calculating the rate of signal change for each FAO assay profile, facilitating assessment of exogenous FAO (Oleate supplied), endogenous FAO (Oleate-free) and non-LC FAO (Etomoxir treated). This can be determined using slopes (*m*) calculated from the linear portion of each profile:

Exogenous FAO = $m_{Oleate} - m_{Etomoxir}$

Endogenous FAO = $m_{Oleate\text{-}free} - m_{Etomoxir}$

Non-LC FAO = $m_{Etomoxir} - m_{Signal\ Control}$

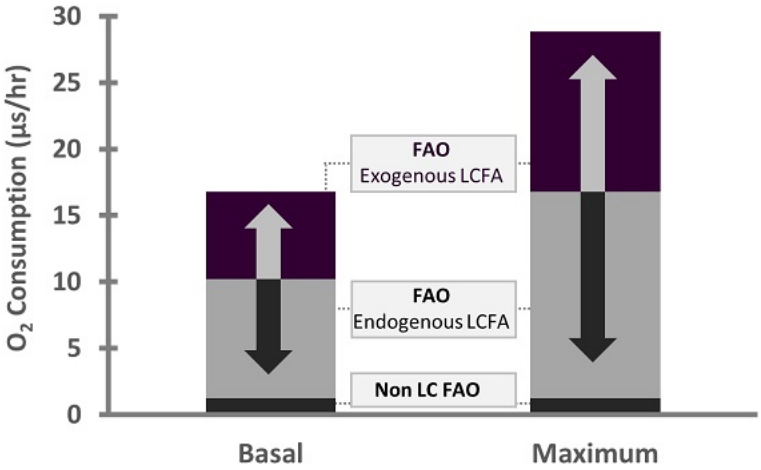

Figure 5: FAO-driven respiration of HepG2 cells treated with the CPT-1 inhibitor Etomoxir and uncoupler FCCP. The figure summarizes the balance between these parameters under "Basal" and "Maximum" (FCCP treated) conditions. The increased energy demand imposed by FCCP treatment is met by increased FAO.

14. Notes

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