Measuring Fatty Acid Oxidation using the MitoXpress® FAO Kit

Companion Kit for MitoXpress® Xtra - Oxygen Consumption Assay

- Convenient high-throughput, 96/384 well plate assay, compatible with conventional fluorescence plate readers
- Allows convenient delineation of endogenous and exogenous FAO
- Control compounds and media tablet included in kit to simplify assay workflow
- Oleate-BSA provided, removing the time consuming and often inconsistent process of fatty acid conjugation
- Compatible with 2D & 3D cell cultures and can be multiplexed with other fluorescence-based metabolic readouts

Introduction

Fatty acid oxidation (FAO) is the primary metabolic pathway in a variety of tissues, becoming particularly important 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 [1, 2]. FAO is also now acknowledged as a key factor in cancer metabolism [3] and is also implicated in drug-induced microsteatosis [4].

The primary pathway for the degradation of fatty acids is mitochondrial fatty acid β-oxidation. Long-chain fatty acids (LCFAs) are imported into the mitochondria as acyl carnitine. Once inside, acyl-CoAs are released to undergo an iterative four-step oxidation until the entire chain is oxidized to acetyl-CoA, while carnitine returns to facilitate further LCFA transport. The Acetyl CoA produced typically enters the TCA, although in liver it can also fuel the production of ketone bodies, an important energy source for other tissues. Both TCA & β-oxidation contribute to the pool of reducing equivalents (NADH and $FADH_2$) which, in turn, drive the activity of the electron transport chain (ETC) and subsequent ATP generation. These processes are summarised in Figure 1.

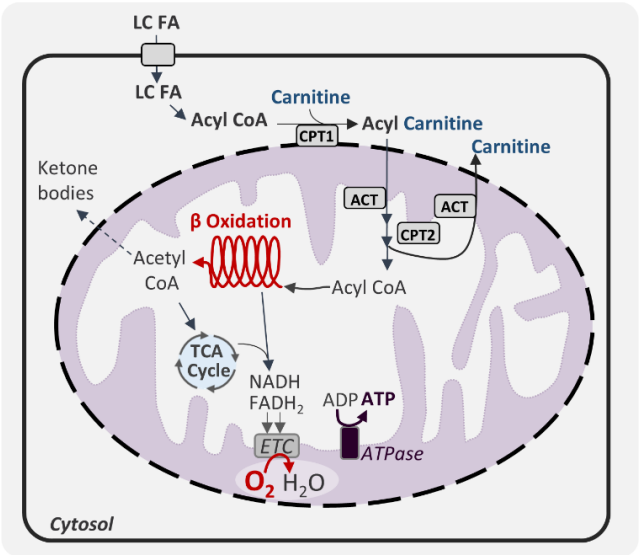


Figure 1: *Overview of long-chain fatty acid activation, import and oxidation.*

The MitoXpress® FAO Kit

The MitoXpress® FAO Kit (MXC-500) enables the convenient high-throughput measurement of FAO and is designed for use in combination with Luxcel's MitoXpress® Xtra - Oxygen Consumption Assay (MX-200). The companion kit contains the unsaturated fatty acid Oleate as a substrate (supplied as 2:1 BSA conjugate), as well as control compounds and a buffer tablet to simplify measurement workflow. Kit contents are summarised in Table 1.

Table 1: *Summary of MitoXpress FAO Kit components.*

Oleate-BSA	unsaturated C18 fatty acid conjugated to BSA
BSA control	BSA control for use with compound treatments
Base Measurement Media Tablet	KHB-based Base Measurement Media provided in convenient tablet form
L-Carnitine	facilitates LCFA cellular uptake
FCCP	uncouples ETC and F_o/F_1 ATPase activity
Etomoxir	CPT1 inhibitor preventing of LCFA import

By using Oleate as the primary energy source, oxygen consumption, as measured using MitoXpress® Xtra, becomes a convenient high-throughput indicator of FAO activity. In addition, using a common dietary unsaturated FA such as Oleate means that, as well as monitoring the core machinery of β-oxidation, the assay also assesses the function of critical cellular isomerases necessary for the metabolism of dietary fatty acids.

The provision of Oleate-BSA conjugate as a kit component also removes the time consuming process of FA conjugation and delivers a conjugate consistency often difficult to achieve with in-house conjugations.

Data Analysis and Interpretation

Sample FAO-Driven Oxygen Consumption

The following example (Figure 2), demonstrates the typical MitoXpress® FAO assay data output. HepG2 cells are measured in the presence of 150 µM Oleate and treated with the CPT-1 inhibitor Etomoxir and the uncoupler FCCP.

Untreated cells show a steady MitoXpress® Xtra signal increase reflecting ETC-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 LC FAO.

FCCP treatment induces maximal ETC activity by dissipating the mitochondrial membrane potential. Increased demand for reducing equivalents causes a concomitant increases in FAO as indicated by the rapid increase in MitoXpress® Xtra signal. This strong increase in ETC activity is not observed where exogenous LCFA is unavailable or where import is inhibited.

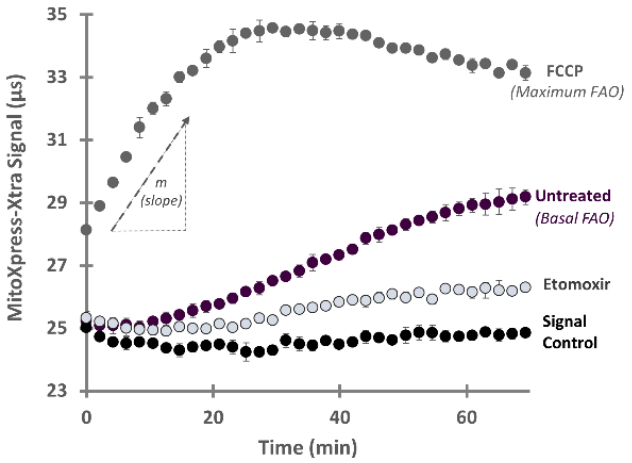


***Figure 2**: FAO-driven respiration of HepG2 Cells.*

Evaluating Exogenous and Endogenous FAO

FAO-driven respiratory activity can be interrogated further by calculating the rate of signal change for each MitoXpress® Xtra 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 3 summarises the balance between these parameters under 'Basal' and 'Maximum' (FCCP treated) conditions and clearly illustrates that the increased energy demand imposed by FCCP treatment is met by increased FAO.

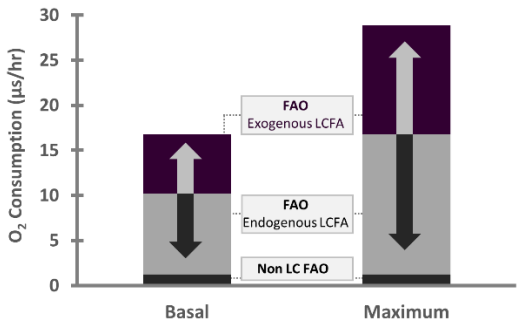


***Figure 3:** FAO-driven respiration of HepG2 Cells.*

Metabolic Characterisation

MitoXpress® FAO kit facilitates interrogation of oxygen consumption due to exogenous FA substrates such as Oleate, endogenous FA stores, and non long chain FA substrates. Figure 4 illustrates this approach to determine the impact of differentiation on C2C12 cell metabolism, measuring maximal respiratory capacity of fully confluent C2C12 myoblasts (undifferentiated) and multinucleated myotubes (differentiated), and the proportion of this capacity driven by long chain FAO. Differentiation from myoblasts to multinucleated myotubes significantly increases maximal respiratory capacity, measured after a 1h glucose deprivation step. This is driven by both endogenous substrates and an increased capacity to metabolise exogenous FA substrates (Oleate-BSA) to meet an increased demand for reducing equivalents imposed by FCCP treatment.

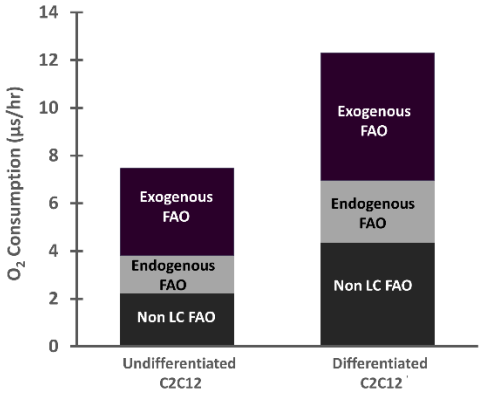


***Figure 4:** Maximal FAO-driven respiration of differentiated and undifferentiated C2C12 cells (FCCP treated). Data courtesy of Dr. Ben Buehrer, Zen-Bio Inc.*

The MitoXpress® FAO kit is a powerful tool when used in conjunction with the MitoXpress® Xtra - Oxygen Consumption Assay to interrogate FAO-driven respiration, allowing convenient high-throughput metabolic characterisation and FAO modulator screening.

Materials & Methods

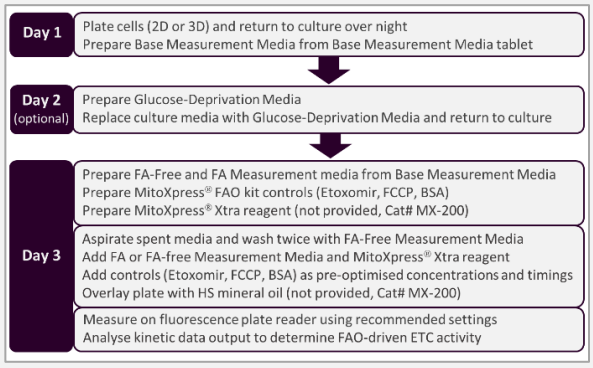
Materials

MitoXpress FAO Kit (MXC-500), MitoXpress®Xtra - Oxygen Consumption Assay (MX-200), Glucose free DMEM (Sigma Cat# D5030).

Reagents

Glucose Deprivation Media (optional): *DMEM, 1 mM glucose, 0.5 mM L-carnitine*
Base Measurement Media: *kit buffer tablet dissolved in 100 mL of sterile water*
FA-Free Measurement Media: *Base media, 0.5 mM L-Carnitine, 2.5 mM glucose*
FA Measurement Media: *FA-free Measurement Media + 150 µM Oleate*

Typical Workflow



References

[1] Eaton *et al., Biochem. J.* (320) 345-357, 1996. **[2]** Schulz *et al., J. Nutr*. (124) 165-171 1994. **[3]** Carracedo *et al* , *Nat Rev Cancer.(4)*:227-32, 2013, **[4]** Begriche *et al., J Hepatol.*, (54): 773-794, 2011