Using PMP to Measure Substrate Specific ETC/OxPhos Activity in Permeabilized Cells

Technical Overview

Introduction

Mitochondrial respiration measurements using intact cells can help define metabolism and its dysregulation in fields such as cancer, metabolic disease, immunology, and neurodegeneration. Using intact cells in combination with the Agilent Seahorse XF Cell Mito Stress Test provides a general mitochondrial bioenergetic profile. Changes in respiration may correlate to altered mitochondrial substrate oxidation. Therefore, examining the effect of specific substrates is one approach to determine the mechanism underlying the observed differences.

Many oxidizable substrates are unable to cross the plasma membrane freely, preventing control over which substrates the mitochondria are oxidizing. Moreover, while the assay medium can be supplemented with various substrates, many cell types can store and oxidize endogenous pools of substrates (for example, glycogen and triglyceride), making it difficult to determine precisely which metabolic pathways are fueling respiration.

Consequently, pinpointing the precise mechanism underlying metabolic changes has typically involved isolating mitochondria. While mitochondrial respiration measurements in isolated mitochondria enable control over which substrates are oxidized, the process is complex, and the yield and quality of the end product is often poor and prone to subselection during isolation.

XF Plasma Membrane Permeabilizer (XF PMP) provides a solution to this challenge, allowing experimental controlover the specific substrates offered to *in situ* mitochondria. A combination of XF PMP, substrates, and inhibitors yields a powerful approach to understanding mitochondrial function.

XF PMP forms pores in the plasma membranes of adherent cell monolayers with demonstrably less mitochondrial outer membrane damage and cell lifting than detergent-based options such as digitonin or saponin[1,2]. Moreover, a fixed concentration of XF PMP sufficiently permeabilizes a broad range of cell types[1], reducing the time spent optimizing assay conditions.

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By exploiting the fact that substrates feed differentially into mitochondrial pathways[1,3], it is possible to isolate which metabolic pathways are responsible for the altered oxygen consumption rate (OCR) originally observed in intact cells (Figure 1).

This Technical Overview describes using XF PMP-treated cells and the Agilent Seahorse XF^{e}96/24 or XF96/24 Analyzer to measure substrate oxidation in relation to specific respiratory complexes. This Technical Overview also provides a table of common substrates, inhibitors, and assay designs that measure respiratory complex activity.

Materials and Methods

The assay workflow (Figure 2) describes the procedure used to prepare the cells and the XF PMP reagent. For detailed materials and methods, refer to *Assessing Mitochondrial Respiratory Complexes Using Cells Permeabilized with XF Plasma Membrane Permeabilizer (PMP)*[7].

The experiments described in this Technical Overview used an Agilent Seahorse XF96 Extracellular Flux Analyzer. These methods can be adapted for all Seahorse XF^{e}96/24 and XF96/24 Analyzers.

Note: When planning an XF PMP assay, Agilent recommends characterizing the cell seeding density and optimal reagent concentrations prior to starting the assay. Please contact Agilent Technical Support with any questions.

Cell culture

All indicated cell lines were cultured as specified by the manufacturer. Twenty-four hours prior to the assay, the cells were counted and seeded at a predetermined optimal density in an XF96 Cell Culture Microplate (p/n 101085-004).

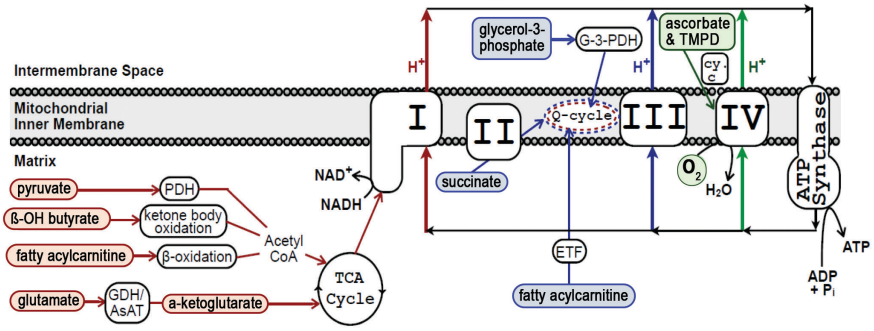

Figure 1. Oxidizable substrates feed into different parts of the respiratory chain. OCR is a measure of respiratory chain activity involving subsets of electron transport chain complexes. Complex I-linked substrates are labeled in red, and those linked directly to the ubiquinone pool are labeled in blue (note that fatty acid oxidation involves both). Electrons are delivered to cytochrome oxidase (Complex IV) with ascorbate and TMPD, as labeled in green. PDH = pyruvate dehydrogenase; GDH = glutamate dehydrogenase; AsAT = aspartate aminotransferase; ETF = electron transfer flavoprotein-related enzymes; G-3-PDH = glycerol-3-phosphate dehydrogenase; TMPD = N,N,N′,N′ Tetramethyl-p-phenylenediamine; cy. c = cytochrome c; Pi = inorganic phosphate. Adapted from Divakaruni; *et al.* 2013[1].

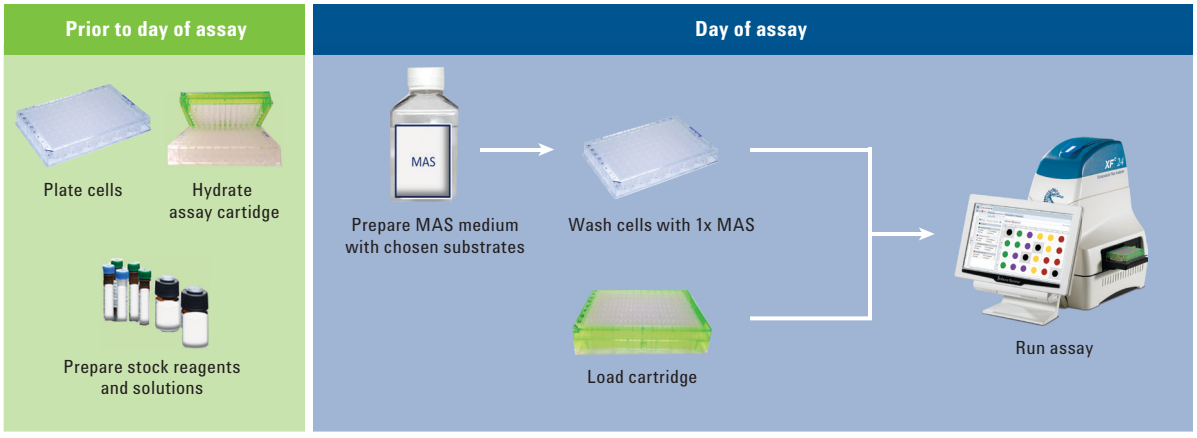

Figure 2. XF PMP assay workflow.

Reagent preparation

All substrates and inhibitors used in this XF assay (Table1) were freshly prepared, as indicated in *Assessing Mitochondrial Respiratory Complexes Using Cells Permeabilized with XF Plasma Membrane Permeabilizer (PMP)*[7]. XF PMP reagent (p/n 102504-100) was used according to the manufacturer's instructions.

Data analysis

All data analysis employed Wave. Data shown are mean ±SEM.

Interpretation of Results

Cells pretreated with XF PMP examine mitochondrial substrate oxidation by measuring OCR changes.

Complex I

Measuring the oxidation of NADH-linked substrates (such as pyruvate or glutamate) in permeabilized cells alongside succinate characterizes defects in complex I activity (Figure 3 and Figure 4). For example, permeabilized cells treated with fenofibrate, a complex I inhibitor at supraphysiological concentrations, exhibited a depressed respiration rate for NADH-linked substrates but no effect on succinate-driven respiration (Figure 3 and Figure 4).

Table 1. Substrates and inhibitors.

Substrate	Final conc.	Add with...	Relevant inhibitors
Pyruvate	10 mM	1 mM malate 2 mM DCA*	2 µm rotenone 2 µm UK5099
Glutamate	10nM	10nM malate	2 µm rotenone titrated aminooxyacetate
β-hydroxybutyrate	10 mM	1 mM malate	2 µm rotenone
Palmitoylcarnitine/ Octanoylcarnitine	40 µm	1 mM malate	2 µm antimycin A
α-ketoglutarate	10 mM	No additions necessary	2 µm rotenone
Succinate	10 mM	2 µm rotenone	2 µm antimycin A 2 µm myxothiazol 20 mM malonate
Glycerol-3-phosphate	5–10 mM	2 µm rotenone	2 µm antimycin A 2 µm myxothiazol
Ascorbate	10 mM	100 µm TMPD 2 µm antimycin A	

NADH-linked (Complex I) substrates

Q-linked (Complex II or III) substrates

Cytochrome oxidase-linked (Complex IV) substrates

* Optional: DCA will relieve potential kinase inhibition of pyruvate dehydrogenase. Adapted from Divakaruni; *et al*. 2014[4].

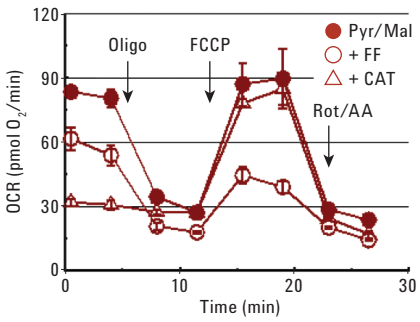

Figure 3. Changes in OCR due to substrate utilization. Primary skeletal muscle myocytes, were offered either pyruvate (pyruvate, malate, and dichloroacetate (DCA)), fenofibrate (FF: open circles), or carboxyatractyloside (CAT: open triangles) prior to the experiment. Data are mean ±SEM.

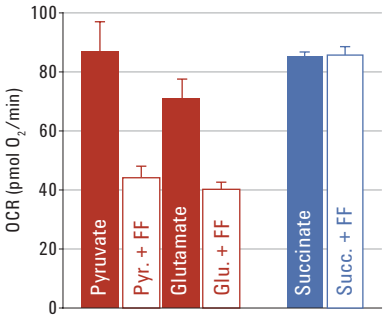

Figure 4. Complex I-linked respiration. Human skeletal muscle myocytes were offered pyruvate, succinate (succinate with rotenone), or glutamate (glutamate and malate) and fenofibrate, as indicated in the presence of oligomycin and FCCP. Data are mean ±SEM.

Complexes II and III

Using FADH2-linked substrates (such as succinate or glycerol-3-phosphate) in addition to measurements of ascorbate-driven respiration reveals defects in respiratory complexes II and III (Figure 5). The complex III inhibitor myxothiazol blocks succinate-driven respiration, but does not affect complex IV activity, as demonstrated by respiration driven by TMPD with ascorbate.

Careful selection of substrates and inhibitors can distinguish between effects at either complex II or III. For example, the complex II inhibitor malonate depresses the rate of succinate-driven respiration, but has no effect on complex IV activity, and a minimal effect on glutamate-driven respiration.

Since glutamate oxidation requires a functional Q-cycle, a *bona fide* complex III inhibitor such as myxothiazol collapses the rate of glutamate-driven respiration, giving a different profile than malonate. Measurement of another Q-cycle-linked substrate, such as glycerol-3-phosphate, provides a definitive measurement[6] and a distinction between complex II and III.

Complex IV

Measuring respiration in the presence of TMPD with ascorbate assesses cytochrome oxidase activity (Figure 6). The results are verified by measuring sensitivity to the complex IV inhibitor azide.

Complex V (ATP synthase)

By measuring phosphorylating and uncoupler-stimulated respiration, the Seahorse XF Analyzer probes the activity of the enzymes involved in ATP synthesis (Figure 7). Decreased activity of ATP synthase appears as a decrease in phosphorylating respiration without a concomitant drop in maximal, uncoupler-stimulated respiration.

Note: This profile is independent of the substrate offered. Patient fibroblasts with defects in the adenine nucleotide translocase (as illustrated in Figure 3), the phosphate carrier, or the ATP synthase (See Havlícková; *et al.*[5] as an example) would exhibit qualitatively similar results.

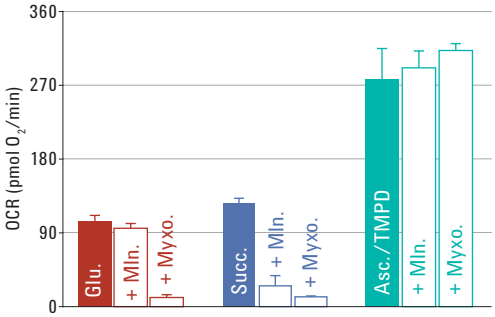

Figure 5. Complex II and III-linked respiration. Human skeletal muscle myocytes were initially offered glutamate and malate (Glu.), succinate (Succ.), or TMPD with ascorbate (Asc./TMPD). Open bars indicate assay the addition of malonate (Mln.) or myxothiazol (Myxo.) to the medium immediately prior to the experiment. Data are mean ±SEM.

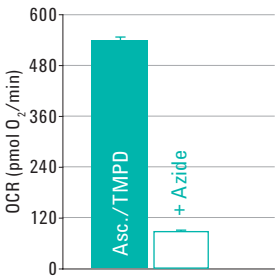

Figure 6. Complex IV activity. FCCP-stimulated respiration was measured in permeabilized fibroblasts oxidizing TMPD with ascorbate. The open bar indicates the addition of azide to the assay medium prior to measurement. Data are mean ±SEM.

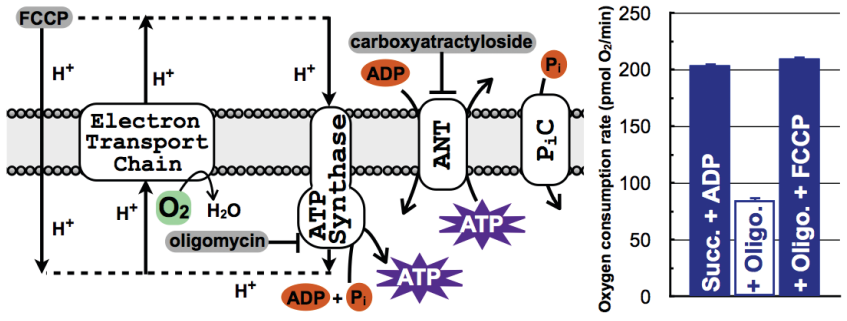

Figure 7. Complex V activity.(Left) Inhibition of the ATP synthase, adenine nucleotide translocase (ANT), or phosphate carrier (PiC) will cause a decrease in phosphorylating respiration. (Right) Permeabilized fibroblasts were offered succinate with rotenone. Inhibition of the ATP synthase by oligomycin decreases phosphorylating respiration, but has no effect on FCCP-stimulated respiration. Data are mean ±SEM.

Summary

The ability to identify, as well as quantify, changes in specific components of mitochondrial respiration provides mechanistic insight into the complex relationship between mitochondrial function and cellular phenotype. Studying these changes is critical to understanding disease etiology, pathology, and potential therapeutic mechanism of action.

By forming pores in the cellular plasma membrane and allowing the user to study the oxidation of specific substrates, XF PMP provides a powerful and precise approach to studying mitochondrial function. This type of controlled experimental design can isolate the mechanism associated with an observed metabolic change.

This Technical Overview describes methods that use XF PMP-treated cells to examine *in situ* mitochondrial substrate oxidation. Using a combination of specific substrates, inhibitors, and mitochondrial effectors, the function of each respiratory complex can be studied (Figure 2).

Conducting substrate oxidation analysis using permeabilized cells overcomes the disadvantages associated with isolated mitochondria from cells, including poor quality and yield as well as subselection during the isolation procedure. Moreover, unlike detergent-based permeabilization methods, such as digitonin or saponin, XF PMP is considerably less prone to cell lifting or mitochondrial outer membrane damage[1,2].

While the method outlined in this Technical Overview focuses on specific respiratory chain complexes, a different approach can use XF PMP to determine a general overview of respiratory chain activity when sample size is limited. Refer to *Using PMP to Measure Mitochondrial ETC Complex Activity in Limited Biomaterials*[8] for an alternative method to investigate mitochondrial substrate oxidation. Agilent Seahorse XF technology combined with XF PMP is a powerful tool that enables further mechanistic analysis of cellular metabolism.

Assay Optimization Hints

- Table 1 lists the starting concentration for oxidizable substrates and inhibitors used in the assay. Cells may have specific substrate preferences based on the tissue origin and culture conditions.
- Assay duration should be as short as possible to avoid cell lifting from the microplate.
- Perform the wash steps as quickly (but gently) as possible to minimize the cell-exposure time to the MAS buffer.
- When pipetting BSA-supplemented assay medium, bubbles can form in the assay plate. Therefore, do not push past the stopper when either washing or loading the plate.

References

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