Using PMP to Measure Mitochondrial ETC Complex Activity in Limited Biomaterials

Technical Overview

Introduction

The study of mitochondrial function is central to both clinical and basic scientific research. The Agilent Seahorse XF Cell Mito Stress Test provides a general mitochondrial bioenergetic profile. However, examining the precise enzyme or pathway driving observed changes can provide additional insight and further link-specific alterations in metabolic enzymes with disease states, such as the correlation between decreased complex I activity and Parkinson's disease[1].

Using the Agilent Seahorse XF Analyzer to obtain a highly analytical, mechanistic understanding of mitochondrial respiratory complexes and metabolic pathways requires controlling the specific substrates offered to the mitochondria. This is difficult in cells with an intact plasma membrane. Although the assay medium can be supplemented with various substrates, many cell types will continue to utilize endogenous substrates and prevent the identification of a pathway-specific effect.

The traditional method of studying substrate oxidation involves isolating mitochondria, but there are several disadvantages when isolating mitochondria, including limited quantity and a bias stemming from the subselection of mitochondria during isolation.

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Because substrates enter the mitochondria using different pathways (Figure 1), controlling the specific substrate or group of substrates offered to permeabilized cells can correlate molecular mechanisms of action changes with changes in the oxygen consumption rate (OCR)[2,3].

XF Plasma Membrane Permeabilizer (PMP) forms pores in the plasma membrane of adherent cells without causing any concomitant damage to the mitochondrial membrane. This reagent overcomes the challenges associated with using isolated mitochondria or substrate-supplemented media with intact cells. Furthermore, a fixed concentration of XF PMP sufficiently permeabilizes a broad range of cell types[2], reducing the time spent optimizing assay conditions.

One method of using XF PMP-treated cells involves testing specific respiratory chain complexes by employing a variety of substrates and inhibitors (refer to *Using PMP to Measure Substrate Specific ETC/OxPhos Activity in Permeabilized Cells*[4]). The assay described in this document is very similar to the aforementioned assay; however, this assay uses relevant substrates and inhibitors to gain information as to the function of multiple mitochondrial complexes from a single well. As such, the assay described in this document is customized for limited sample availability.

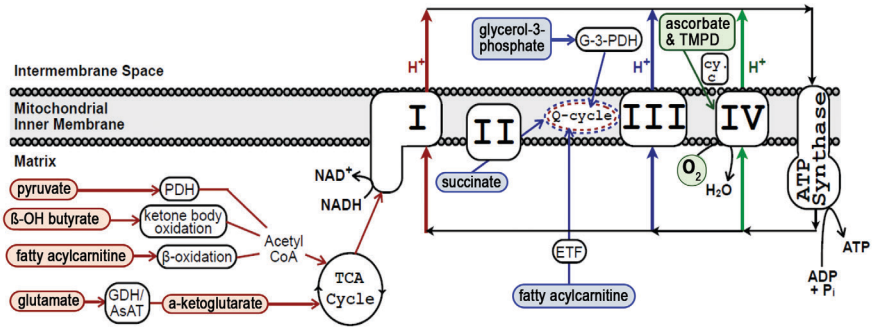

Figure 1. Oxidizable substrates feed into different parts of the respiratory chain. OCR is a measure of respiratory chain activity involving subsets of electron transport chain complexes. Complex I-linked substrates are labeled in red, and those linked directly to the ubiquinone pool are labeled in blue (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[2].

This Technical Overview describes a method using XF PMP-treated cells and the Seahorse XF Analyzer to interrogate respiratory chain complexes within a single group of wells. It measures uncoupler-stimulated respiration fueled by various substrates in a single assay. This type of assay is most appropriate when there is either limited cell sample availability or when only a general overview of the respiratory chain complexes is required.

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)*[5].

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

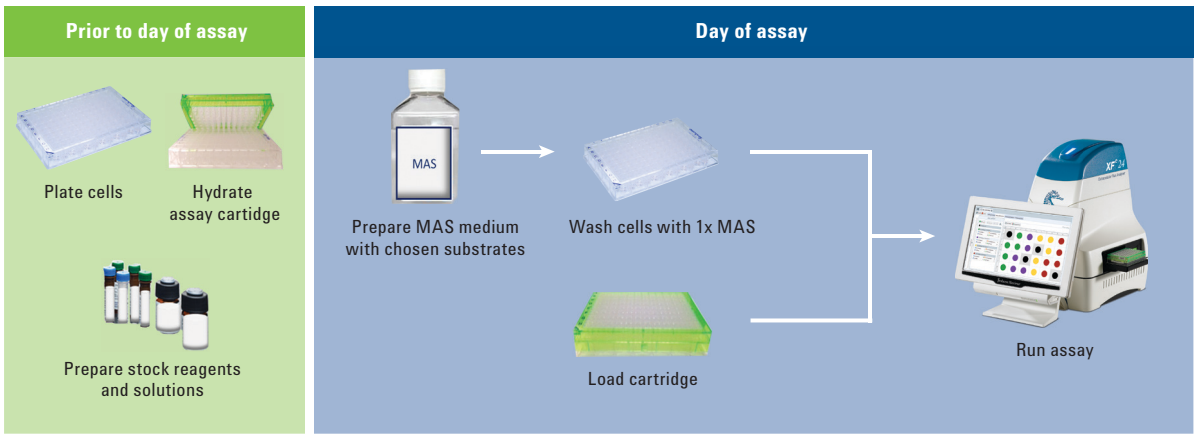

Figure 2. XF PMP Assay workflow.

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).

Reagent preparation

All substrates and inhibitors used in this assay (Table 1) were freshly prepared, as indicated in *Assessing Mitochondrial Respiratory Complexes Using Cells Permeabilized with XF Plasma Membrane Permeabilizer (PMP)*[5]. XF PMP reagent (p/n 102504-100) was used as according to the manufacturer's instructions.

Data analysis

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

Interpretation of Results

XF PMP-treated cells, as described in this method, examine mitochondrial respiratory complex activity via uncoupler-stimulated respiration using various substrates in a single well. As such, this method is most suitable when an assessment of substrate oxidation is limited by sample size.

In this method, permeabilized cells initially receive a complex I-linked substrate such as pyruvate or glutamate. Rotenone injected using Port A inhibits complex I and halts NADH-linked respiration (Figure 3). Next, succinate injected through Port B drives respiration from electrons fed directly into the ubiquinone pool via succinate dehydrogenase (complex II) and by-passing the complex I inhibition. Addition of antimycin A via Port C inhibits complex III and abolishes this rate. Lastly, the injection of ascorbate with TMPD in Port D bypasses the block at complex III, and delivers electrons directly to cytochrome C oxidase (complex IV).

Table 1. Substrates and inhibitors.

Substrate	Final conc.	Add with...	Relevant inhibitors
Pyruvate[a]	10 mM	1 mM malate 2 mM DCA*	2 µM rotenone 2 µM UK5099
Succinate[b]	10 mM	2 µM rotenone	2 µM antimycin A 2 µM myxothiazol 20 mM malonate
Ascorbate[c]	10 mM	100 µM TMPD 2 µM antimycin A	20 mM azide

[a] NADH-linked (Complex I) substrates
[b] Q-linked (Complex II or III) substrates
[c] Cytochrome oxidase-linked (Complex IV) substrates
* Optional: DCA will relieve potential kinase inhibition of pyruvate dehydrogenase. Adapted from Divakaruni; *et al.* 2014[6].

To correlate changes in respiration to specific mitochondrial respiratory complexes, permeabilized human fibroblasts were initially offered pyruvate (with malate), and then treated with either of two compounds (Figure 4). UK5099, an inhibitor of mitochondrial pyruvate uptake, slows the rate of pyruvate driven respiration, but has no effect on the oxidation of succinate or ascorbate/TMPD. Myxothiazol, an inhibitor of complex III, completely blocks oxidation of both pyruvate and succinate, as both require a functioning Q-cycle. When electrons are directly fed to complex IV, however, a decrease in respiration is not observed.

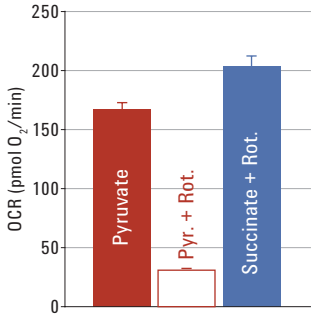

Figure 3. Complex linked respiration. Human fibroblasts (Coriell Institute, GM04078) are offered pyruvate, succinate, or rotenone, as indicated. Data are mean ±SEM.

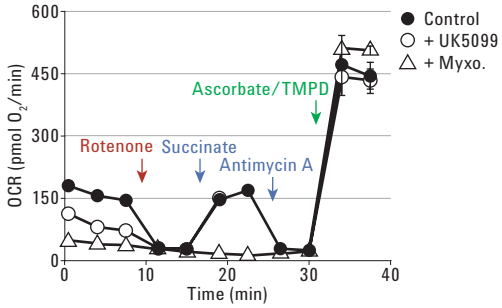

Figure 4. The *in situ* respiratory complex assay. Human fibroblasts (Coriell Institute, GM04078) are offered pyruvate, succinate, or rotenone, as indicated. Data are mean ±SEM.

Summary

Using intact cells to measure mitochondrial respiration can help to elucidate the relationship between mitochondrial function and disease etiology. The identification and quantification of components that can affect mitochondrial function requires control of substrate provision to the mitochondria.

The traditional methods of either isolating mitochondria from cells or using substrate-supplemented media with intact cells have several disadvantages. Moreover, unlike detergent-based permeabilization methods such as digitonin or saponin, XF PMP is far less prone to cell lifting or mitochondrial outer membrane damage[7].

XF PMP forms large pores in the cellular plasma membrane without causing damage to the mitochondria, allowing the user to choose which substrates are offered. By exploiting the fact that respiratory substrates feed differentially into mitochondrial pathways (Figure 2), careful experimental design can isolate the mechanism associated with an observed metabolic defect.

This Technical Overview describes a method that uses XF PMP-treated cells to gain experimental control over substrate provision when there is limited sample availability, or if only a general overview of respiratory complex is required. Refer to *Using PMP to Measure Substrate Specific ETC/OxPhos Activity in Permeabilized Cells*[4] for an alternative method of interrogating respiratory complex activity.

Agilent Seahorse XF technology, in combination with the XF PMP reagent, provides a powerful research tool to study cell physiology, disease etiology and pathology, as well as the mechanism of action for potential drug candidates.

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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