Measuring changes in cellular metabolism by monitoring extracellular acidification and oxygen consumption in real-time

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- **CLARIOstar® with atmospheric control unit (ACU) provides a key element in an open-flow respirometry system**
- **OCR and ECA measured simultaneously in real-time under physiologically relevant atmospheric conditions**

Introduction

Dysregulation of cellular energy metabolism is a common factor in a variety of disorders including diabetes and obesity as well as cancer to name a few. The current technologies used to assess mitochondrial function via extracellular acidification (ECA) and oxygen consumption rate (OCR) are often designed in such a way that they ignore the impact of O_2 concentration on cellular bioenergetics.
Here we describe the implementation of the CLARIOstar with ACU in conjunction with advanced phosphorescent dyes to measure O_2 consumption and glycolytic flux in an open-flow respirometry system. This system enables measurement of respiration rate at steady state, where O_2 supplied equals O_2 consumed. The phosphorescent oxygen and pH probe (Cayman Chemical) combined with the CLARIOstar and ACU provides a 'push button' multiplexed system with the potential for monitoring additional cell health outcomes. Experiments can be performed under user defined [O_2], using either adherent cells or cell suspensions where relevant, with throughput comparable to existing technologies.

Assay Principle

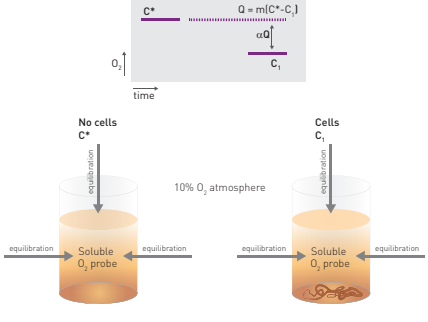

Fig. 1: Open-flow in a 96-well plate. Respiration rate is calculated from the indicated formula where Q = respiration (nmols O_2/min/well), m = linear diffusion rate of O_2 from gas phase to liquid phase, C* = zero respiration wells at steady state., C_1 = experimental sample wells at the same time point.

Open-flow respirometry is a useful, physiologically relevant approach that allows for the measurement of mitochondrial respiration rates at dynamic steady-state oxygen concentrations[1,2]. To improve throughput, this system was translated to a 96-well plate format. This requires the use of at least 2 negative control wells that exhibit zero respiration (Figure 1). Results from experimental samples are subtracted from controls to calculate respiration rate.

Materials & Methods

- Phosphorescent oxygen and pH probe (Cayman Chemical)
- CLARIOstar with ACU (BMG LABTECH)
- Black, clear bottom, 96-well plates (Greiner)
- H9C2 cells and HepG2 cells
- Other reagents from commercially available sources

The indicated cell types were plated at a density of 7 X 10^4 cells/well. Reagent preparation, cell seeding, instrument set up, and assay protocols were all performed as indicated in kit literature.
For dose-response experiments, serial dilutions of antimycin A were prepared in DMSO to create a 10 point half-log dilution series with a maximum concentration of 10 µM (final DMSO concentration = 0.01%).

The measurement settings are also provided in the CLARIOstar test protocol, available at:
www.bmglabtech.com/cayman/

The test protocol includes use of onboard reagent injectors to add control compounds. Oligomycin is used to decrease OCR and increase ECA. FCCP is used to increase OCR. These control compounds are included in the test kit.

Instrument settings

	MitoXpress® Xtra	pH-Xtra™
Optic settings	Time-resolved fluorescence, Top optic	
	Excitation: Ex TR	
	Dichroic: LP TR	
	Emission: 645-20	Emission: 615-18
	Multichromatic (4)	
	Integration settings	
	Integration window 1	
	Start: 30 µs, Time: 30 µs	Start: 100 µs, Time: 100 µs
	Integration window 2	
	Start: 70 µs, Time: 30 µs	Start: 300 µs, Time: 100 µs
General settings	Settling time: 0.2 s	
	Number of flashes per well: 100	
Kinetic settings	Number of cycles: 60	
	Cycle time: 280 s	
Atmospheric control	Temperature: 37 °C	
	Oxygen: 10 %	
Injections	Volume: 5 µl, pump speed: 430 µl/s	
	1 (Oligomycin): injection cycle 20	
	2 (FCCP): injection cycle 40	

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Calculation of ECA and OCR were performed in MARS, BMG LABTECH's data analysis software. A data analysis template is available at the web address indicated above. Subsequent analysis of dose-response and Z' data were also performed in MARS.

Results & Discussion

When the phosphorescent oxygen and pH probes are used with the CLARIOstar with ACU, real-time changes in ECA and OCR can be observed (Figure 2). After an initial 20 min equilibration, a steady-state signal for OCR can be observed. The expected changes in OCR and ECA in response to control compounds are also observed following their injection for the triplicate samples displayed.

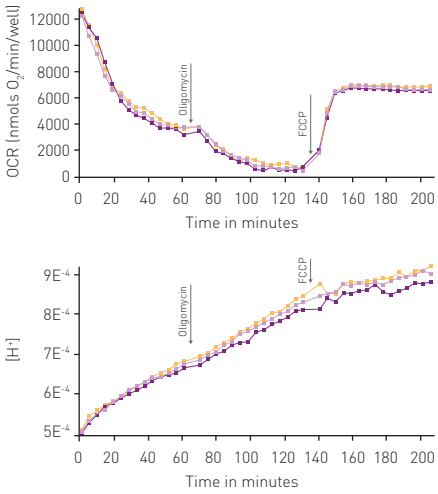

Fig. 2: Simultaneous measurement of OCR and [H⁺] in response to oligomycin and FCCP. OCR and ECA was measured for H9C2 cells using the adherent cell protocol. Arrows indicate injection of oligomycin and FCCP, respectively. The first 40 minutes of the OCR trace is indicative of plate equilabration to the oxygen environment and should not be used as respiration data.

As an example of the capabilities of this test for compound screening a dose response to the known mitochondrial inhibitor antimycin A was prepared. Figure 3 shows the anticipated concentration dependent effect of anitmycin A on OCR. From this plot an IC_{50} = 120.8 nM is determined. Similar results can be obtained for ECA analyses (data not shown).

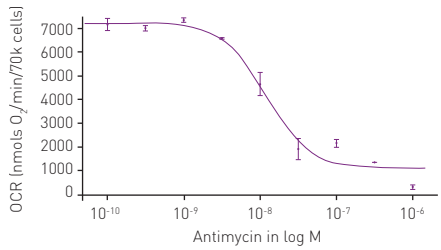

Fig. 3: Oxygen consumption in 10% O_2 environment. Duplicate wells containing HepG2 cells were treated with the indicated concentrations of antimycin A. Following equilibration baseline OCR could be calculated. The effect of Antimycin exhibits a response that conforms to a 4-parameter fit (r^2=0.97574).

Finally, we calculated the Z' statistic for this assay (Figure 4). The value of above 0.59 is indicative of an excellent assay that is well suited to screening purposes.

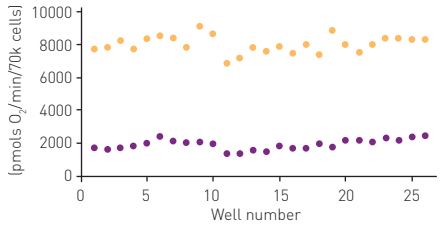

Fig. 4: OCR Z-prime data. Baseline OCR data from vehicle (●) treated HepG2 cells was compared with the OCR data from the same cells following injection of 10 µg/mL oligomycin (●).

Conclusion

The phosphorescent oxygen and pH probe (Cayman Chemical) and the CLARIOstar with ACU provide a robust and efficient means of measuring cellular bioenergetics at [O_2] that are physiologically relevant. Additional utility can be derived from the ability to easily use non-adherent cells and to perform additional multiplexing to examine additional cellular responses.

References

1. Cole R.P. *et al* (1982) Mitochondrial function in the presence of myoglobin *J. Appl. Physiol.* **53**, 1116-1124
2. Hoffman, D.L. *et al* (2007) Respsonse of mitochondrial reactive oxygen species generation to steady-state oxygen tension: implications for hypoxic cell signaling *Am. J. Heart. Circ. Physiol.* **292**, H101-108

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