The CLARIOstar® with ACU exposes cells to ischemia-reperfusion conditions and monitors their oxygenation

Andrea Krumm[1], C. Carey[2], R. Kettenhofen[3], M. Schwalfenberg[2], J. Hynes[2]
[1]Andrea Krumm, BMG LABTECH GmbH, Ortenberg, Germany, [2]Agilent Technologies, Santa Clara, USA, [3]Ncardia, Cologne, Germany

- **Oxygen ramping of atmospheric control unit facilitates control of ischemic and reperfusion insults in cells**
- **Intracellular probe tracks cellular oxygenation during ischemia-reperfusion cycle**
- **Parallel monitoring of ROS and MMP probes allow detailed metabolic characterization of ischemia-reperfusion**

Introduction

The lack of oxygen supply is associated with a number of life-threatening diseases such as stroke, myocardial infarction or renal failure whereby cells are temporarily deprived of O_2 and nutrient (ischemia). Significant cell damage can also occur during the reperfusion phase through oxidative stress and inflammatory responses. Investigating these pathologies *in vitro* requires an experimental set-up capable of rapid deoxygenation, rapid reperfusion, and parallel monitoring of critical biological parameters including cellular oxygenation and ROS. The ischemia-reperfusion model presented here uses a microplate reader with software-controlled programmable O_2 and CO_2 regulation (Fig. 1) in combination with MitoXpress Intra, (Agilent Technologies) which enables real-time monitoring of cellular oxygenation. Data are presented using HepG2 cells and iPS derived cardiomyocytes (Cor.4U®, Axiogenesis).

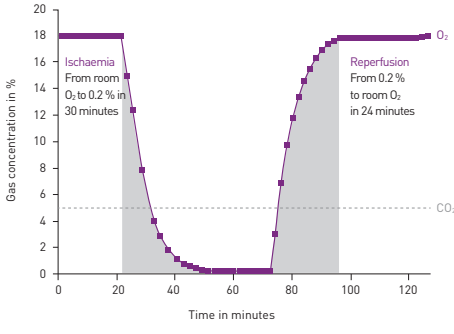

Fig. 1: Example of ischemia-reperfusion atmospheric conditions in the CLARIOstar microplate reader with ACU. O_2 and CO_2 levels were regulated as defined in the reader software.

Materials & Methods

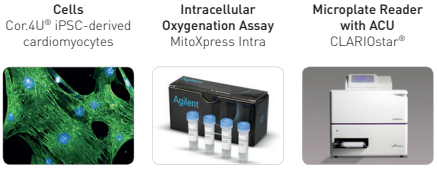

Fig. 2: Components of ischemia-reperfusion model

- Clear 96-well plate (Sarstedt)
- Antimycin A (1µM) and FCCP (2.5µM)
- Dihydroethidium (DHE) (Sigma Aldrich)
- JC-1 (Cayman Chemical)

Experimental Procedure

HepG2 cells were plated at a density of 25,000 cells/well and returned to culture overnight. Cor.4U cells (Axiogenesis) were plated and maintained as per manufacturer's instructions. Cellular Oxygenation: Cells were loaded overnight with the intracellular O_2 probe MitoXpress Intra (Agilent Technologies) as per manufacturer's instructions and measured on the CLARIOstar microplate reader using the settings detailed below. Preconfigured measurement protocols and data analysis templates for automatic O_2 concentration calculation are available on BMG LABTECH software allowing real-time monitoring of cellular oxygenation. Mitochondrial membrane potential (MMP): Cells were loaded with JC-1 (Cayman Chemical) 30 min prior to measurement as per manufacturer's instructions and measured ratiometrically using the settings detailed below. A dissipation of MMP reduces J-aggregate formation causing a reduction on aggregate:monomer ratio Reactive Oxygen Species (ROS): Cells were loaded with 2.5µM DHE (Sigma Aldrich) for 30 min prior to measurement and measured using the settings detailed below.

Instrument Settings

MitoXpress Intra			
Optic settings	Time-resolved fluorescence, bottom optic		
	Filters	Excitation	Ex TR
		Dichroic	LP TR
		Emission	645-20
	Gain	2300	
	Well Multichromatic: 2 integration windows		
	Window 1	Start 30 µs, Time 30 µs	
	Window 2	Start 70 µs, Time 30 µs	
General settings	No. of flashes	100	
	Settling time	0.1 s	
Incubation	37°C		
Atmospheric control	Reduction from room O_2 to 1 %, 50 min at 1 % O_2, increase O_2 back to room		

Rev. 06/2018
Keywords: ACU, Oxygen consumption, ROS, Mitochondrial membrane potential, Multiplexing, Real-time, Oxygen, MitoXpress

Mitochondrial membrane potential with JC-1				
Optic settings	Fluorescence intensity, top optic			
	Mono-chromator	Excitation	485-15	485-15
		Dichroic	541	511
		Emission	595-10	535-10
	Gain	1950		
General settings	No. of flashes	100		
	Settling time	0.1 s		
Reactive oxygen species with DHE				
Optic settings	Fluorescence intensity, endpoint, top optic			
	Mono-chromator	Excitation	510-15	
		Dichroic	560	
		Emission	615-25	
	Gain	1700		
General settings	No. of flashes	60		
	Settling time	0.1s		

Results & Discussion

The CLARIOstar microplate reader equipped with software-controlled programmable O_2 and CO_2 regulation was used in combination with MitoXpress Intra Intracellular Oxygen Assay to induce a defined ischemia/reperfusion event *in vitro* using a liver and cardiac model (HepG2 and Cor.4U cells respectively) Fig.3 shows the precise atmospheric control achievable, with O_2 reduced to 1%, maintained at this concentration for a pre-defined period and then rapidly increased to 18%. Parallel monitoring of MitoXpress Intra reveals the importance of real-time oxygenation monitoring, as cellular respiration significantly impacts oxygen concentrations at the cell monolayer. Antimycin treated HepG2 cells (no respiration), reflect instrument conditions (ACU) however respiring cells experience much lower resting oxygen concentrations and deeper more sustained hypoxia. This disparity between atmospheric and cellular O_2 increases further when respiration is increased through FCCP treatment (uncoupled cells). Using real-time oxygenation monitoring, ACU parameters can therefore be modulated to achieve the desired cellular ischemia-reperfusion profile.

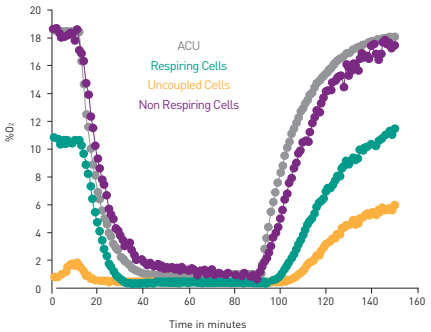

Fig. 3: Ischemia-reperfusion proof-of-concept using HepG2 cells. Ischemia-reperfusion insult induced by modulating O_2 in the measurement chamber. Cellular oxygenation is monitored in respiring, non-respiring (Antimycin treated), and uncoupled (FCCP treated) cells.

The approach was also evaluated using iPS-derived cardiomyocytes (Cor.4U cells) with parallel monitoring of MMP and ROS (Fig. 4). Non-respiring cells reflect ACU conditions, while respiring cells experience significantly reduced O_2 concentrations.
The convenient multiplexing function of the CLARIOstar was used to measure MMP and cellular oxygenation in parallel. ROS measurements were also performed on the same text plate using DHE. Antimycin treatment blocks respiratory activity increasing cellular oxygenation to ambient levels (Fig. 4A) while also causing MMP dissipation (Fig. 4B) and increased ROS production returning (Fig. 4B).

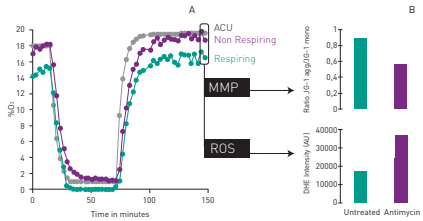

Fig. 4: Multiparametric analysis of Cor4U cells during *in vitro* ischemia-reperfusion validating multiplexed measurement of MitoXpress Intra and JC-1/DHE. Cell oxygenation traces describe depth and duration of Cor.4U ischemia-reperfusion **(A)** with parallel monitoring of MMP and ROS **(B)**.

Conclusion

The CLARIOstar microplate reader with ACU facilitates precise programmable control of both O_2 and CO_2, enabling the simulation of a hypoxic insult of defined depth and duration, while active venting enables rapid controlled reperfusion. Real-time oxygenation monitoring is realised using MitoXpress Intra in conjunction with pre-configured data analysis templates. Critically, this allows ACU parameters to be modulated so that, at the cellular level, the desired depth and duration of hypoxic insult, and the required reperfusion rates are achieved. Multi-parametric analysis of key cellular parameter such as MMP and ROS can be performed during/after the ischemia reperfusion event.

References

1. Hynes J, et al. 2015. *Methods Mol Biol.*,**1264**:203-17.
2. Chapple S.J., et al 2016. *Free Radic. Biol. Med.*, **92**: 152-162

CLARIOstar®