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

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- 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 O₂ 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 O₂ and CO₂ regulation (Fig. 1) in combination with MitoXpress®-Intra, (Luxcel Biosciences) which enables real-time monitoring of cellular oxygenation. Data are presented using HepG2 cells and iPSc derived cardiomyocytes (Cor.4U®, Axiogenesis).

Materials & Methods

- Clear 96-well plate (Sarstedt)
- Antimycin (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 O₂ probe MitoXpress-Intra (Luxcel Biosciences) 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 O₂ 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

<table>
<thead>
<tr>
<th>MitoXpress-Intra</th>
<th>Time-resolved fluorescence, bottom optic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filters</td>
<td>Ex TR, Dichroic, LP TR, Emission 645-20</td>
</tr>
<tr>
<td>Gain</td>
<td>2300</td>
</tr>
<tr>
<td>Well Multichromatic</td>
<td>2 integration windows</td>
</tr>
<tr>
<td>Window 1</td>
<td>Start 30 µs, Time 30 µs</td>
</tr>
<tr>
<td>Window 2</td>
<td>Start 70 µs, Time 30 µs</td>
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<tr>
<td>General settings</td>
<td>No. of flashes 100, Setting time 0.1 s</td>
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<tr>
<td>Incubation</td>
<td>37°C</td>
</tr>
<tr>
<td>Atmospheric control</td>
<td>Reduction from room O₂ to 1 %, 50 min at 1 % O₂, increase O₂ back to room</td>
</tr>
</tbody>
</table>

Fig. 1: Example of ischemia-reperfusion atmospheric conditions in the CLARIOstar microplate reader with ACU. O₂ and CO₂ levels were regulated as defined in the reader software.

Fig. 2: Components of ischemia-reperfusion model
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.

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).

**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 parameters such as MMP and ROS can be performed during/after the ischemia reperfusion event.

**References**