High-throughput *in vitro* ischemia-reperfusion model with real-time monitoring of cellular oxygenation and reactive oxygen species generation

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Introduction

Ischaemia-reperfusion (IR) injury: IR injury is a feature of multiple diseases including myocardial infarction, renal failure and stroke, occurring when tissue blood supply is restricted and subsequently restored. While reperfusion is essential for tissue survival, it is also associated with significant ROS-mediated damage, triggering inflammatory responses and ultimately cell death.

***In vitro* IR models:** The rational development of targeted therapeutic interventions require both a detailed understanding of the mechanisms underlying reperfusion injury and a convenient means of assessing the efficacy of putative therapeutics. This need has driven the development of a number of in vitro IR models including pharmacological ATP depletion, oil overlays to induce autohypoxia, glucose oxidase addition to cause sample deoxygenation, and N_2 purged hypoxia chambers to induce a slow reduction in dissolved oxygen.

Limitations of current *in vitro* models: The utility of these models has however been limited as they do not facilitate the induction of the rapid, controlled, transient, ischemic shock and reperfusion necessary to replicate an IR injury condition *in vitro*. Critically, neither do they facilitate real-time cellular oxygenation monitoring to allow accurate IR characterisation or parallel measurements of critical IR injury parameters such as reactive oxygen species (ROS) and mitochondrial membrane potential (MMP).

Here we present a model which addresses these limitations through the combined use of iPS-derived cardiomyocytes (Cor.4U®), plate reader with integrated atmospheric control facilitating rapid [O_2] modulation (CLARIOstar®) and a novel intracellular probe capable of reporting cellular oxygenation in real time (MitoXpress®-Intra).

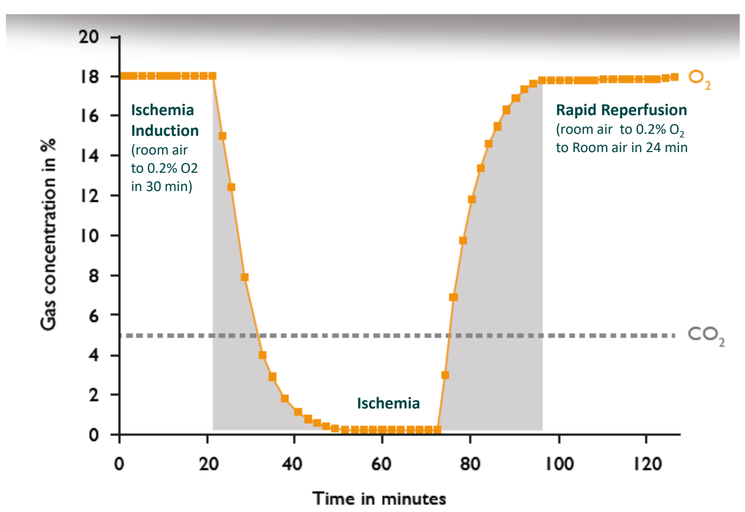


Fig. 1: Sample oxygen ramping and rapid re- of the measurement chamber of a CLARIOstar (BMG Labtech) with integrated ACU. CO_2 can be maintained at 5% during measurement.

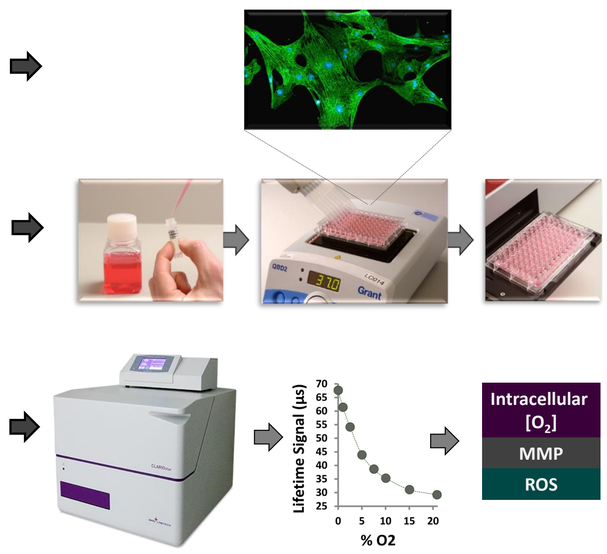
Real-time monitoring of Cardiomyocyte Oxygenation

Measuring Cellular Oxygenation

Cor.4U cells are 100% pure (approx. 60% ventricular) , fibroblast free human induced pluripotent stem (iPS) cell-derived cardiomyocytes.

MitoXpress-Intra is a O_2-sensitive cell-penetrating nanoparticle probe. It is chemically stable, and is taken up by cells during an overnight loading period. Oxygen quenches the phosphorescent emission of the probe, such that measured signal (Ex/Em: 380nm/650nm) is proportional to intracellular oxygen concentration ([iO2]), thereby allowing real-time monitoring of cellular oxygenation.

CLARIOstar with Atmospheric Control Unit (ACU) is a high-sensitivity multimode plate reader equipped with time-resolved fluorescence capabilities and in integrated atmospheric control unit enabling gaseous O_2 and CO_2 control in the measurement chamber (0.1 - 20%)



Media Equilibration & Impact of Mitochondrial Activity on Cellular Oxygenation

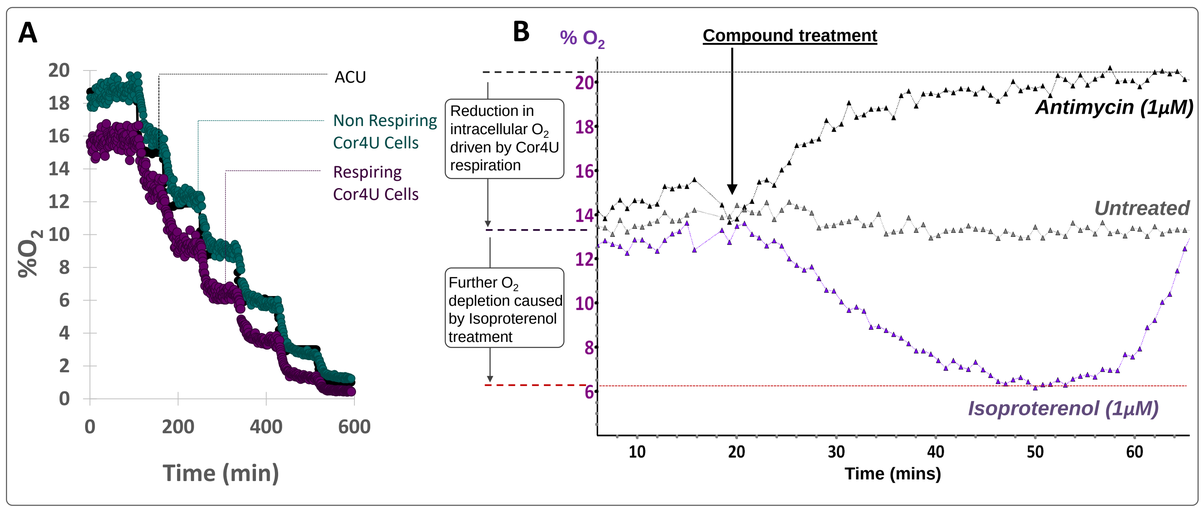


*Fig. 2: (A) Impact of reducing **ACU-applied O_2 concentrations** (19, 15, 12, 9, 6, 3, 1, 0%) on the oxygenation levels of **Respiring** and **Non-Respiring** (Antimycin treated) Cor.4U cells measured using MitoXpress-Intra. **(B)** Impact of pharmacological respirometric modulation on Cor.4U oxygenation: Antimycin blocks electron transport chain activity while Isoproterenol increases ETC activity to meet increased ATP demand.*

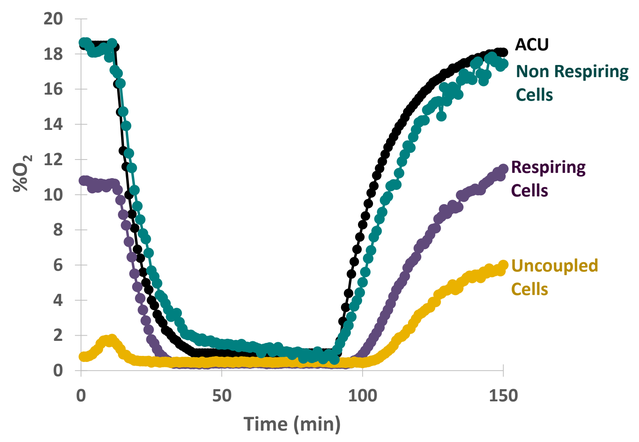
MitoXpress-Intra facilitates the measurement of cellular oxygenation [**1,2**].

Fig. 2A illustrates the time taken for the cell monolayer to equilibrate to ACU-applied [O_2] and shows the differences in oxygenation caused by electron transport chain activity at each [O_2]. These differences illustrate that cellular respiration drives the monolayer to lower [O_2] than those imposed with the ACU.

Fig. 2B again demonstrates that basal metabolism has reduced O_2 concentrations from ambient (~21%) to ~14%, while treatment with the ETC inhibitor Antimycin blocks O_2 consumption causing intracellular O_2 levels to return to ambient levels. Treatment with the β-adrenoreceptor agonist isoproterenol increases cardiomyocyte beat rate which in turn causes an increase in oxygen consumption. This causes a significant but temporary reduction in O_2 availability with values of ~6% observed for >15 min despite cells being cultured and measured at 21% O_2.

Ischemia-Reperfusion

Ischemia-Reperfusion Proof of Concept



- Measurement chamber O_2 modulated using **ACU** to model IR insult.
- Cellular oxygenation monitored using MitoXpress-Intra
- **Non-respiring cells** shows rapid deoxygenation and re-oxygenation
- **Respiring cells** experience **significant additional hypoxia, increasing the depth and duration of the ischemic insult.**
- Respiration can also impact the speed and level of re-perfusion **Uncoupled cells** show an even more pronounced effect

*Fig. 3: Ischemia reperfusion proof-of-concept using highly confluent HepG2 cells. Ischemia-reperfusion insult induced by modulating **[O_2] in the measurement chamber**. Cellular oxygenation is then monitored in **Respiring**, **Non-Respiring** (Antimycin treated), and **Uncoupled** (FCCP treated) cells.*

Multiparametric Analysis

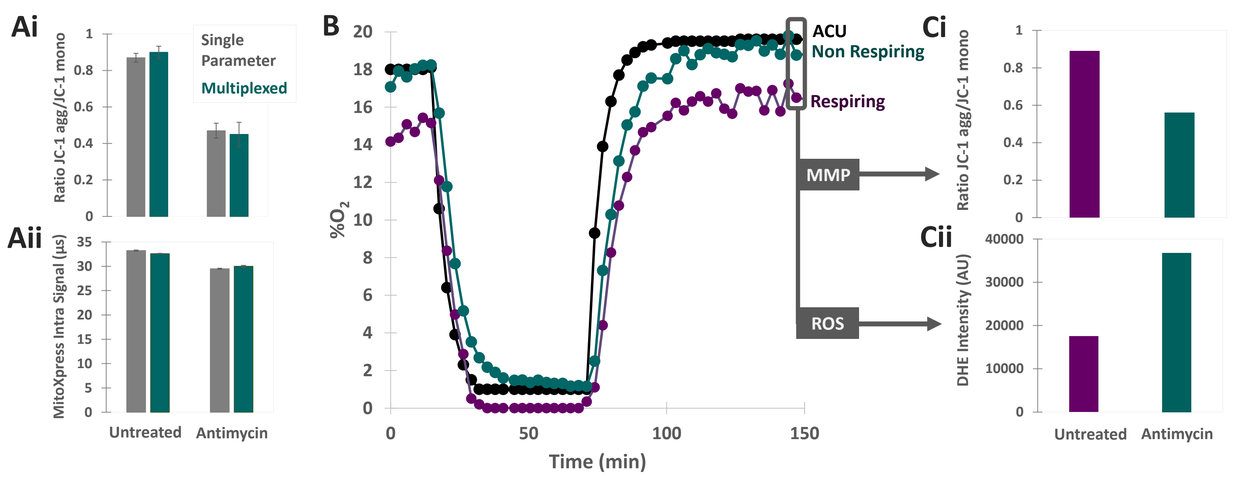


*Fig. 4: Multiparametric analysis of Cor.4U cells during In Vitro Ischemia-reperfusion validating multiplexed measurement of JC-1 and MitoXpress-Intra (**A**), illustrating cell oxygenation traces that describe the depth and duration of Cor.4U ischemia- reperfusion (**B**) with parallel monitoring of MMP and ROS (**C**).*

Fig. 4A compares Cor.4U cells co-loaded with JC-1 (MMP probe) and MitoXpress-Intra. Cells show similar probe signals, (Ai: JC-1, Aii MitoXpress-Intra) illustrating that both MMP and cellular oxygenation can be measured from the same cells simultaneously.

Fig. 4B shows a sample *In Vitro* Ischemia Reperfusion profile of Cor.4U cells whereby the oxygenation of **Respiring** and **Non–Respiring** (Antimycin treated) Cor.4U cells is measured using MitoXpress-Intra. These data illustrate the rapid sample reperfusion achievable on the CLARIOstar (BMG Labtech) and the impact of cell metabolism on cellular oxygenation under measurement conditions. MMP and ROS are measured simultaneously (Fig. 4C).

Fig. 4C presents sample multiparametric MMP (JC-1, Ci) and ROS (DHE, Cii) data taken at 150 min, illustrating that Antimycin treatment has reduced JC1-ratio and has increased DHE signal. These data can be interrogated in real-time during ischemia-reperfusion.

Conclusions

- The combination of the **oxygen ramping** capability of the CLARIOstar ACU with MitoXpress-Intra enabled **cellular oxygenation measurements** facilitates precise control of an ischemic insult whereby instrument [O_2] can be modulated (21% to 0.1% O2) to provide the desired depth and duration of hypoxia. The chamber can then be vented in a controlled manner to model rapid reperfusion.
- Measurement of cellular oxygenation facilitates real-time monitoring of the depth and duration of the hypoxic insult experiences by test cells and the specific rate of cellular reperfusion. **These measurements are essential to a proper IR characterisation** due to the **significant impact cell respiration can have on cellular oxygenation**.
- **Multiplexing MitoXpress-Intra and JC-1** facilitates parallel monitoring of **MMP** and **cellular oxygenation** in the same cells with **parallel monitoring ROS** (DHE) also demonstrated. This facilitates detailed metabolic characterisation of the short-term metabolic implications of reperfusion offering a means by which the efficacy of model therapeutic interventions can be investigated.

Methods

***Cell plating:** Cor.4U cells (Axiogenesis) were plated and maintained as per manufacturers instructions.*

Measurement:

MitoXpress-Intra: Cells are loaded overnight in fresh culture media containing the recommended concentration of MitoXpress-Intra (Luxcel Biosciences). Prior to measurement, cells are washed with 150µL of pre-warmed media and finally the desired volume of culture is added to test wells (typically 80-150µL). Plate is measured kinetically at 37°C (Ex380nm/Em650nm with dual-read TR-F Lifetime measurement (delay 1: 30µs delay 2: 70µs, window: 30µs) on a CLARIOstar (BMG Labtech). Preconfigured measurement protocols and data analysis templates are available on BMG Labtech software. Lifetime calculation and conversion to oxygen concentrations are performed automatically.

JC-1: Cells are loaded in fresh culture media for 30 min prior to measurement. Cells are then washed with 150µL of pre-warmed media and measured at Ex535nm/Em594nm (J-aggregate) and Ex485nm/Em535nm (monomer). J-aggregate:Monomer ratios are then calculated.

DHE: Cells were loaded with DHE for 30 min prior to measurement and measured at Ex450nm/Em535nm

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*

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