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# *In vitro* ischemia-reperfusion model with real-time monitoring of cellular oxygenation and reactive oxygen species generation

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

## Introduction

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.

A number of *in vitro* models have been developed to aid the development of effective therapeutic interventions including pharmacological ATP depletion and enzymatic deoxygenation. The utility of these models has however been limited as they do not facilitate the rapid, controlled, transient, ischemic shock and reperfusion necessary to replicate 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 parameters such as reactive oxygen species (ROS) and mitochondrial membrane potential (MMP).

The ischemia-reperfusion model presented here addressed these limitations using a microplate reader with **software-controlled programmable $O_2$ and $CO_2$ regulation** (BMG LABTECH, Fig. 1) in combination with MitoXpress®-Intra Intracellular Oxygen Assay, (Luxcel Biosciences) which enables **real-time monitoring of cellular oxygenation.** Data are presented using HepG2 cells (Fig. 3) and hiPS derived cardiomyocytes (Cor.4U®, Axiogenesis, Fig.2.)

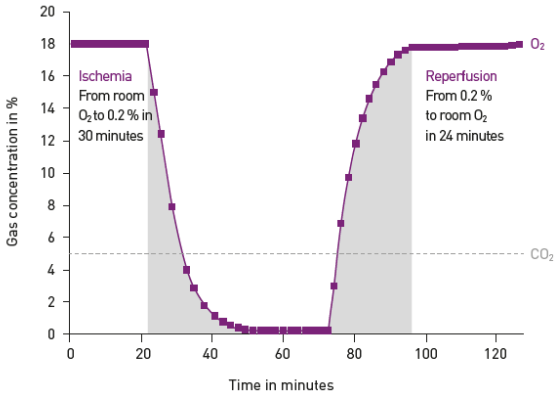
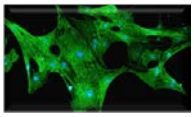



**Fig. 1 Sample ischemia-reperfusion conditions** in the CLARIOstar® microplate reader with ACU. $O_2$ and $CO_2$ levels were regulated as defined in the reader software.

## Model Components & Measuring Oxygenation

### hiPS Derived Cardiomyocytes



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

### Cellular Oxygenation Assay

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.

### Microplate reader with ACU

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%)

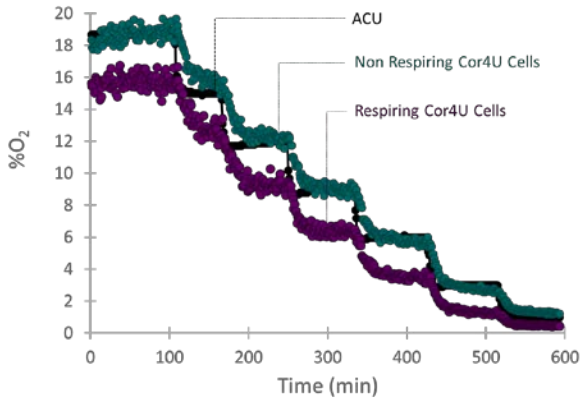



**Fig 2. Respiration impacts oxygenation:** Impact of reducing ACU-applied O2 concentrations (19, 15, 12, 9, 6, 3, 1, 0%) on the oxygenation levels of Respiring and Non-Respiring (Antimycin treated) Cor.4U ®cells measured using MitoXpress®-Intra Intracellular Oxygen Assay.

## Ischemia-Reperfusion

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 importance of MitoXpress® Intra enabled 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. The 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.

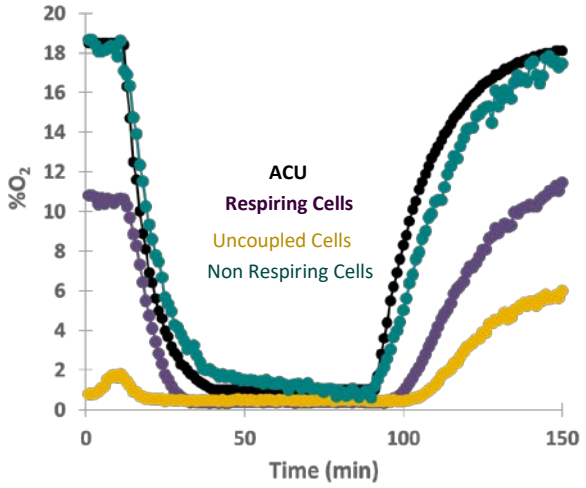



***Fig. 3**: **Ischemia reperfusion proof-of-concept** using HepG2 cells. Ischemia-reperfusion insult induced by modulating $O_2$ in the measurement chamber. Cellular oxygenation is monitored in respiring, non-respiring (Antimycin treated), and uncoupled (FCCP treated) cells.*

The approach was also evaluated using hiPS-derived cardiomyocytes (Cor4U® cells) with parallel monitoring of mitochondrial membrane potential (MMP) and reactive oxygen species (ROS). Non-respiring cells reflect ACU conditions, while respiring cells experience significantly reduced $O_2$ concentrations (Fig. 4). This highlights again both the utility of the rapid programmable $O_2$ ramping achievable on the ACU and the importance of real-time oxygenation monitoring describing the depth and duration of the hypoxic insult and the speed of reperfusion.

As the CLARIOstar® facilitates multiparametric measurements, cells were co-loaded with MitoXpress®-Intra and JC-1 to enable multiplexed measurement of both MMP and cellular oxygenation. Parallel ROS measurements were also performed on the same test 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. 4C).

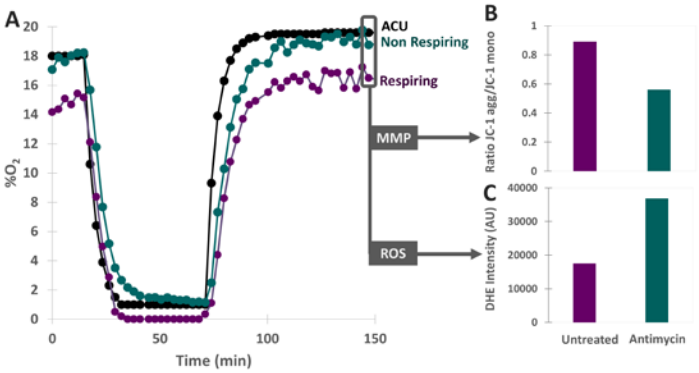



**Fig. 4:** Multiparametric analysis of Cor.4U® cells during *in vitro* ischemia-reperfusion validating multiplexed measurement of MitoXpress®-Intra and JC-1/DHE. Cell oxygenation traces describe depth and duration of Cor.4U® ischemia-reperfusion (**A**) with parallel monitoring of MMP and ROS (**B**).

## Conclusion

The combination of the rapid oxygen ramping with MitoXpress®-Intra enabled cellular oxygenation measurements facilitates precise control of an ischemic insult can be modulated to provide the desired depth and duration of hypoxia.

Measurement of cellular oxygenation facilitates real-time monitoring of the depth and duration of the hypoxic insult and the 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.

Multi-parametric analysis of key cellular parameter such as MMP and ROS 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.

## 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*

***Acknowledgements**: The research presented here was carried out as part of the MetaCell-TM project. MetaCell-TM is funded by the EU Horizon 2020 Fast Track to Innovation Pilot (H2020-FTIPilot-2016-1-737978-MetaCell-TM)*