Novel in vitro tumour-hypoxia model monitoring the effects of cellular oxygenation on cancer cell metabolism

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Introduction

Oxygen availability is a key factor in cellular signalling, particularly in tumour metabolism. Despite its importance, detailed analysis of the interplay between oxygenation, signalling, and metabolic reprogramming is still limited. Delineating the relationship between oxygenation and such metabolic reprogramming is key to a deeper understanding to these processes and to the development of more effective therapeutic interventions.

Current Limitations: Most *in vitro* assays investigate this relationship without specific knowledge of the actual cellular oxygenation levels driving the signalling events under study. Precise modelling of tumour hypoxia for detailed analysis of metabolism and signalling under hypoxic conditions has been limited due to the absence of methodologies capable of controlling and monitoring cellular oxygenation, while in parallel, probing other key metabolic parameters.

Here we describe a methodology which addresses this deficit through the use of novel fluorescence probe technologies and plate reader instrumentation capable of modulating ambient O_2.

MitoXpress® Intra is a cell-penetrating O_2-sensitive fluorescent probe allowing real-time kinetic monitoring of cellular oxygenation (i[O_2]).

pH-Xtra™ is a water-soluble pH-sensitive fluorescent probe for the kinetic analysis of extracellular acidification.

CLARIOstar® with Atmospheric Control Unit (ACU) is a high-sensitivity multimode plate reader equipped with an integrated atmospheric control unit.

Investigating the interplay between O_2 and metabolism

Cellular oxygenation is influenced by cellular metabolism and does not equal applied [O_2]

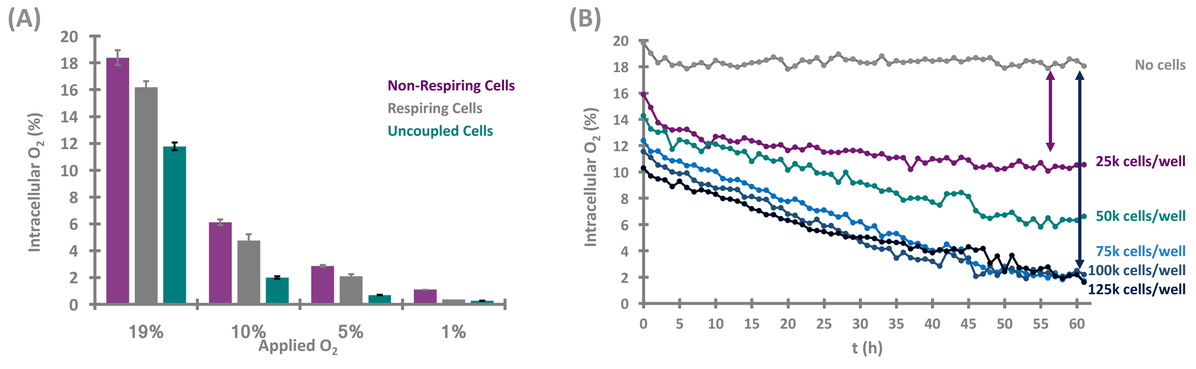

Fig. 1: (A) Intracellular vs ACU-applied [O_2] *in A549 lung carcinoma cells, measured with MitoXpress® Intra. Non-respiring cells were treated with 1 µM Antimycin A (inhibits the ETC), respiring cells were vehicle treated and uncoupled cells were treated with 2.5 µM FCCP to induce maximal respiration.* ***(B) Long term measurement of cellular oxygenation*** *in A549 lung carcinoma cells over 60h seeded at different densities, measured with MitoXpress® Intra at atmospheric oxygen concentrations in a 5% CO_2 atmosphere.*

- In non-respiring cells, intracellular [O_2] is close to ambient [O_2], while basal respiration in untreated cells reduces cellular oxygenation. Uncoupling cells increases the oxygen consumption and leads to significant reduction of the oxygenation experienced by the cells **(Fig. 1a)**.
- Cell density and proliferation affects cellular oxygenation **(Fig. 1b)**. Cellular oxygenation initially ranges from ~16-10% depending on seeding density. As cells proliferate, cells at high densities experience <5% O_2 **(Fig. 1b, arrow)**.
- Cellular oxygenation can not be inferred from applied [O_2]. Ignoring this discrepancy can lead to misjudgement of depth of hypoxia experienced in *in vitro* tumour models

Cellular oxygenation impacts glycolytic flux – Measuring glycolytic flux during hypoxia

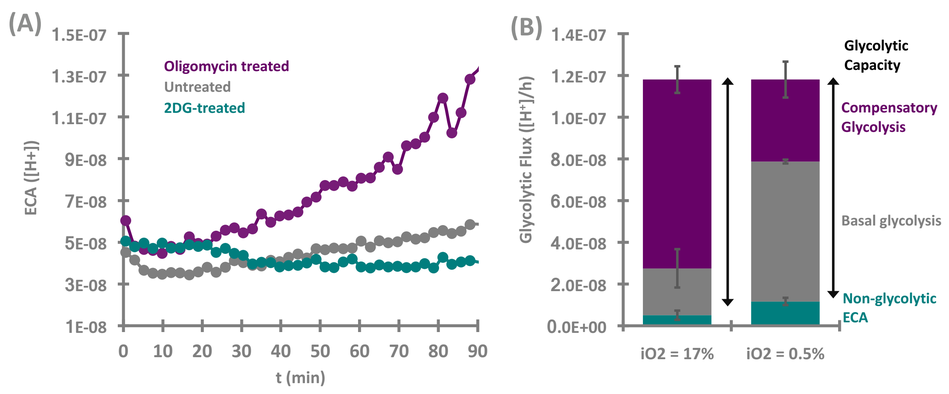

Fig. 2: (A) Extracellular acidification *of A549 cells measured using the pH-Xtra™ Glycolysis Assay multiplexed with MitoXpress® Intra.* ***(B)*** *Key glycolytic characteristic were calculated after treatment with Oligomycin, vehicle or 2-Deoxyglucose.*

- Multiplexed measurements using MitoXpress® Intra and the pH-Xtra™ Glycolysis Assay allows measurement of glycolytic flux during hypoxia.
- When cells experienced acute hypoxia (0.5% i[O_2]), basal glycolytic rates were increased ~3-fold compared to cells at atmospheric [O_2]. This ability to adapt rapidly to reduced [O_2] by increasing glycolysis demonstrates metabolic flexibility. However, the maximal glycolytic capacity remained constant, suggesting no immediate increase in compensatory glycolysis **(Fig. 2b)**.

Kinetic real-time measurement of ROS and cellular oxygenation during hypoxia

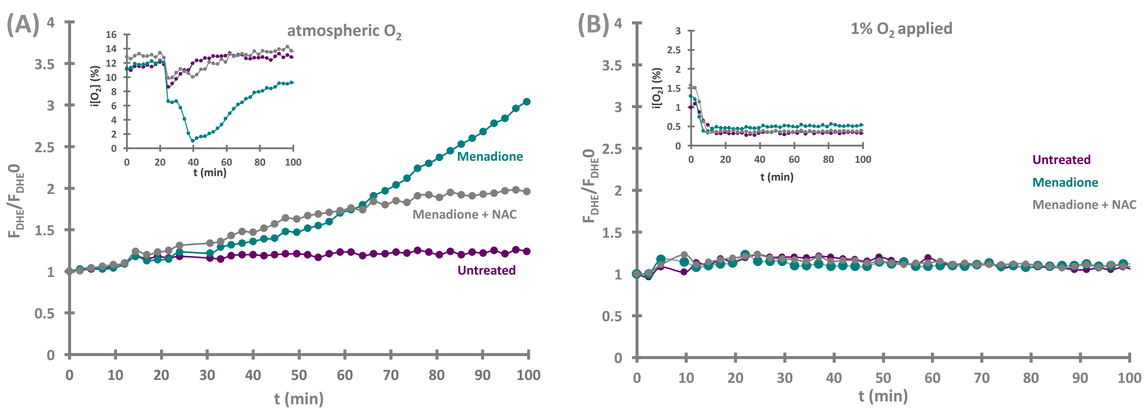

Fig. 3: Kinetic profile of ROS production at atmospheric (A) and 1% (B) [O_2] *measured using DHE as fluorescent superoxide sensitive ROS indicator in A549 cells. Inserts show intracellular [O_2] measured simultaneously using MitoXpress® Intra. 300µM Menadione was used to induce cellular ROS production; additional cells were pre-incubated with 2.5mM N-Acetylcystein (NAC) as antioxidant for 1.5h before and during incubation with Menadione as control.*

- Basal cellular oxygenation levels of around 13% rapidly decreased to <1% then increased back to ~8% post Menadione treatment. This rapid and partly transient response is not detectable using end-point readouts of [O_2] concentrations **(Fig. 3a)**.
- Addition of the redox-cycler Menadione causes a significant increase in ROS formation, which was reduced by the antioxidant N-Acetylcystein **(Fig. 3a)**.
- This effect was not observable at <1% cellular oxygenation **(Fig. 3b)**. In highly hypoxic tumours, cellular oxygenation levels close to 0.5% have been reported.[1] At this level of oxygenation, induction of ROS production by menadione was greatly reduced **(Fig. 3b).** The almost anoxic state of the cells offers a possible explanation for the lack of ROS formation observed.

Measuring metabolism during cycling hypoxia

Kinetic real-time analysis of ROS and cellular oxygenation during hypoxic cycling

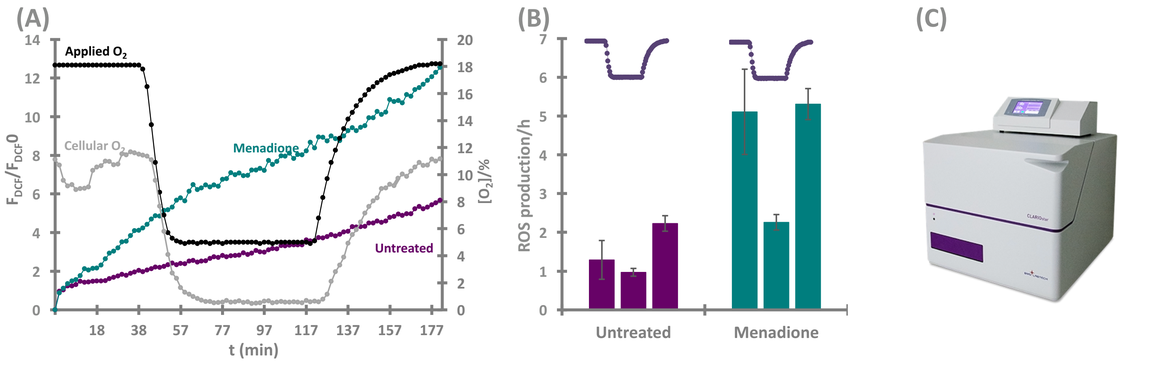

Fig. 4: (A) Kinetic measurement of ROS formation and intracellular [O_2] during a hypoxic cycle *in A549 cells. ROS formation was measured using the non-specific fluorescent ROS indicator H_2DCF-DA and normalized to the fluorescence intensity at t=0 min.* ***(B) Rates of ROS-formation*** *were determined by calculating the slope of a linear portion of the curve before, during and after reducing the applied [O_2].* ***(C)*** *BMG CLARIOstar® with ACU.*

- The MitoXpress® Intra signal is fully reversible, allowing repeated measurements at different applied [O_2].
- BMG CLARIOstar® with ACU allows precise modulation of [O_2] to provide the desired depth and duration of hypoxia. The chamber can then be vented in a controlled manner to model rapid reoxygenation.
- The combination of the oxygen ramping capability of the CLARIOstar® ACU with MitoXpress® Intra enabled cellular oxygenation measurements, facilitates precise control of cellular oxygenation.
- Respiration decreases cellular oxygenation, causing a significant difference between applied [O_2] and cellular oxygenation **(Fig. 4a)**.
- Simultaneous kinetic measurement of ROS-production during a cycle of applied hypoxia **(Fig. 4a)** suggests that both Untreated and Menadione treated ROS-production rates were decreased at lower oxygenation levels and restored after reoxygenation **(Fig. 4b)**.

Kinetic real-time analysis of MMP and cellular oxygenation during hypoxic cycling

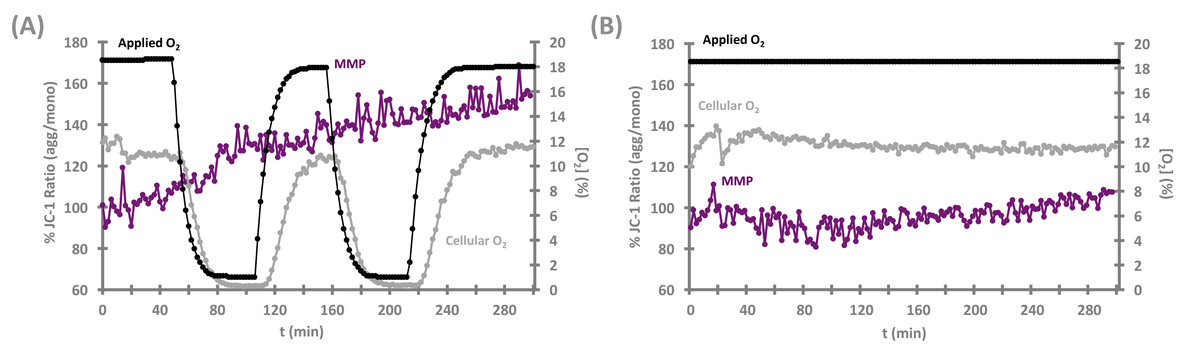

Fig. 5: Real-time kinetic measurement of MMP and intracellular [O_2] in a hypoxia cycling model. *A549 cells were loaded with MitoXpress® Intra and JC-1. Using the CLARIOstar with ACU, changes in MMP and cellular oxygen concentrations were monitored simultaneously during cycling of oxygen between 18% and 1%* ***(A)*** *or while maintaining 18% O_2* ***(B)****.*

- The method described above can be extended to multiple hypoxic cycles **(Fig. 5a)**, allowing precise modelling of hypoxic cycling.
- Multiplexed kinetic measurement of cellular oxygenation and MMP during hypoxic cycling revealed increased rates of mitochondrial hyperpolarisation when cellular oxygenation is reduced to <1% **(Fig. 5a)** compared to cellular oxygenation under atmospheric conditions **(Fig. 5b)**.

Conclusion

- Cellular oxygenation is a key factor in cellular signalling and other metabolic/mitochondrial parameters such as glycolysis, ROS generation and mitochondrial membrane potential.
- Dynamic bi-directional crosstalk exists between cellular metabolism and cellular oxygenation. Consequently, applied O_2 levels do not equal the O_2 levels experienced by cells. This is particularly relevant in tumours due to their complex spatial and temporal fluctuations in oxygen levels.
- Here we describe a method that illustrates the importance of accounting for cellular oxygenation. Ignoring cellular oxygenation levels in *in vitro* studies of cancer metabolism can lead to results lacking in physiological relevance and can introduce artefacts.
- This was achieved by precisely and rapidly modulating oxygen levels using a BMG CLARIOstar® with an ACU, while monitoring cellular oxygenation levels using MitoXpress® Intra.
- Combining MitoXpress® Intra with pH-Xtra™ and fluorescent ROS and MMP probes enables researchers to investigate the interplay between cellular oxygenation and key metabolic parameters during hypoxic events in real-time on a single micro plate.
- This approach can be extended to facilitate precise modelling of cycling hypoxia to investigate oxygen sensitive pathways with implications in drug development and the development of physiologically relevant *in vitro* tumour models.

The method introduced here offers a simple solution for precise modelling of tumour hypoxia with parallel detailed characterisation of the interrelationship of cellular metabolism and cellular oxygenation.

Acknowledgements
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References
1. McKeown, S R. “Defining Normoxia, Physoxia and Hypoxia in Tumours—implications for Treatment Response.” *The British Journal of Radiology* 87.1035 (2014): 20130676. *PMC*.