Modelling tumour hypoxia through parallel analysis of cellular oxygenation, glycolysis flux and mitochondrial function.

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Introduction

Cancer cells show complex, dynamic behaviour allowing survival even in the most unfavourable conditions of substrate and oxygen stress. Advances in technology have helped further our understanding of the complex underlying molecular processes underpinning cancer, but there are still many unanswered questions. Standard cell culture conditions have historically been centred around hyperglycaemic and hyperoxic conditions. However, this does not accurately reflect the in-vivo scenario. There is now a growing appreciation for the need to implement more physiologically relevant culture conditions using oxygen conditions (1-8%) and glucose concentrations (~5mM).

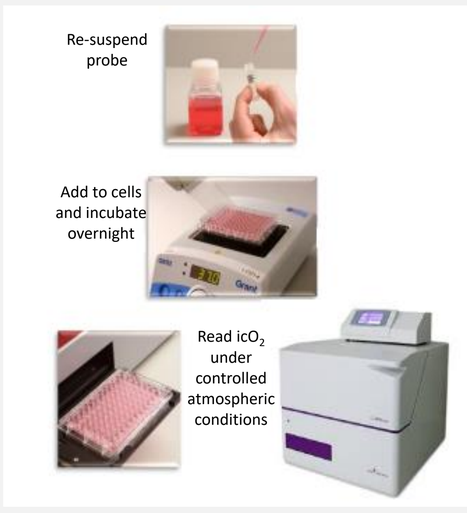
The need to control oxygen concentrations in-vitro is important as O_2 has a wide ranging influence on cellular functions including metabolic poise and differentiation . Insufficient O_2 leads to the development of hypoxic environments which are important in solid tumours.

Here we use a novel method of measuring O_2 within a cell monolayer and look at the effects that reduced ambient O_2 coupled with O_2 consumption of HT1080 fibrosarcoma cells on various glucose concentrations has on the intracellular oxygen levels and the hypoxic response.

MitoXpress®-Intra

MitoXpress®-Intra is an O_2 -sensitive cell-penetrating nanoparticle taken up by cells through endocytosis during an overnight loading incubation. Molecular oxygen quenches the phosphorescent emission of the probe. The measured signal is proportional to intracellular oxygen concentration (icO2), thereby allowing real-time monitoring of cellular oxygenation within the monolayer.

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



Real Time Monitoring of Cellular Oxygenation

1. Intracellular O_2 levels cannot be inferred from applied O_2

Intracellular oxygen levels significantly influence cellular physiology, redox state and metabolism. Altered oxygen availability is involved in several pathological states, such as cardiovascular diseases, cancer and stroke. MitoXpress® Intra enables researchers to gather quantitative real-time information on the oxygen concentration within a cell population in a non-invasive and high-throughput manner, thus providing access to a critical parameter in the study of hypoxia which is beyond the capacity of extracellular sensing methodologies.

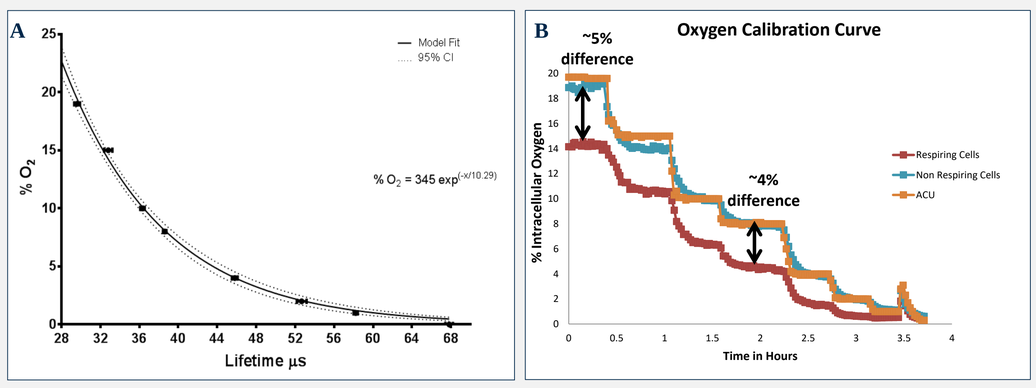


Figure 1: A non-respiring monolayer was used to create a calibration curve by plotting measured lifetime against the applied O_2 and a one phase exponential fit was applied to this curve (A). Using the equation from the model fit allows for the quantification of the oxygen levels within the cells. (B) Illustrates the impact of reducing applied O_2 concentrations (19, 15, 12, 8, 4, 2, 1, 0%) on the oxygenation levels of respiring (red) and non-respiring (antimycinA and rotenone treated -green) HT1080 cells measured using MitoXpress-Intra.

2. Oxygen deprivation is a dynamic process

We demonstrated in figure 1 that, depending on the cells metabolic poise, the depth of oxygenation experienced is impacted significantly by the cells respiratory capacity. We next extended this observation by varying the glucose concentrations in the media and we see that respiration induced oxygen deprivation is a dynamic process that is effected by the composition of the media. When glucose levels are reduced they become exhausted more rapidly and drive the cells to be even more aerobic which is seen as a further decrease in the icO_2 levels.

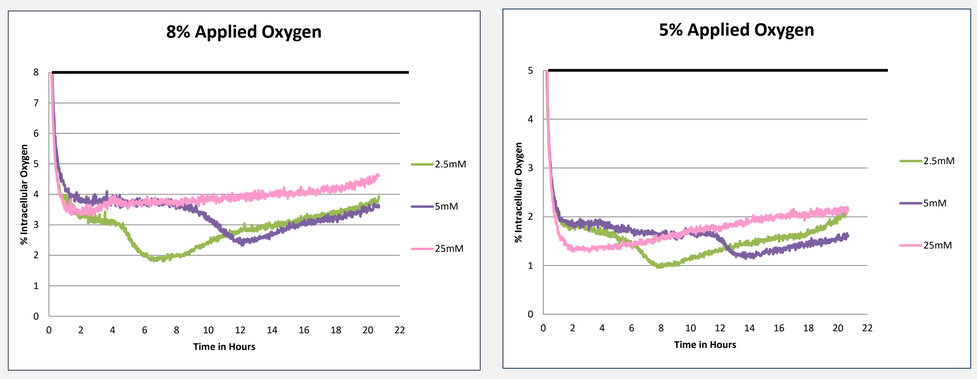


Figure 2: icO_2 levels of HT1080 cells were monitored over a 20hr period at 8 and 5% applied O_2. Cells were grown on standard DMEM (25mM glucose) and immediately before assaying were changed to DMEM containing 2.5, 5 or 25mM glucose. On more physiologically relevant O_2 concentrations (8 & 5%) we see biphasic oxygen profiles on the lower glucose concentrations indicative of glucose depletion resulting in increased mitochondrial OxPhos and a subsequent enhanced reduction in the icO_2 levels observed.

3. Assessing the effect of oxygen and glucose on hypoxic response using a HRE luciferase reporter

The HT1080 cells used have a luciferase reporter gene under the control of hypoxia response element (HRE) of the target gene EPO. The icO_2 levels were measured overnight followed by a luciferase assay to assess Hif activity. We found that cells exposed to 5% ambient O_2 have intracellular levels approaching ~1% which is seen as ~40% luciferase activity when compared to a DMOG treated control (Figure 3 orange arrows). If we had assumed icO_2 levels to reflect ambient levels then extrapolating from the curve in Figure 3 we should have only observed ~10% luciferase activity (purple line). This finding reinforces the need to monitor oxygen intracellularly because the dynamic deviation between applied and actual O_2 could impair the physiological relevance of our experimental observations.

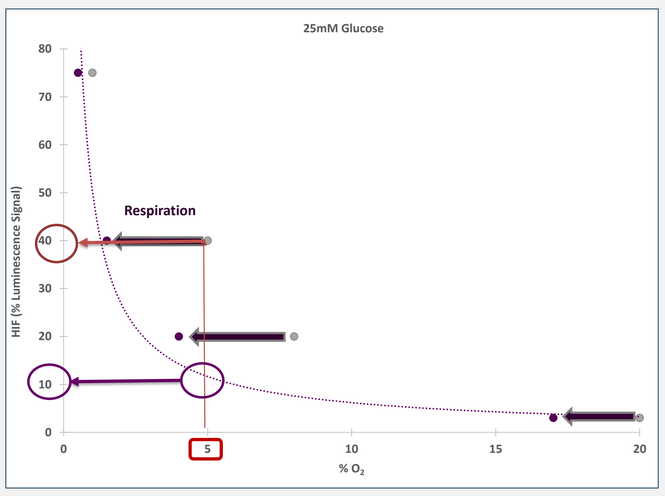


Figure 3: icO_2 levels of HT1080 HRE cells on 20, 8, 5 and 1% applied O_2 and at the end of a 20hr period luciferase activity was measured and data normalised to a DMOG treated control. As oxygen decreases we see an increase in luciferase activity as expected with hypoxic stabilisation of Hif. We also see large differences between applied and actual icO_2 which impact the luciferase activity measured. At 5% applied oxygen (not considered hypoxic) we see luciferase activity of ~40% due to the lower than expected icO_2 levels.

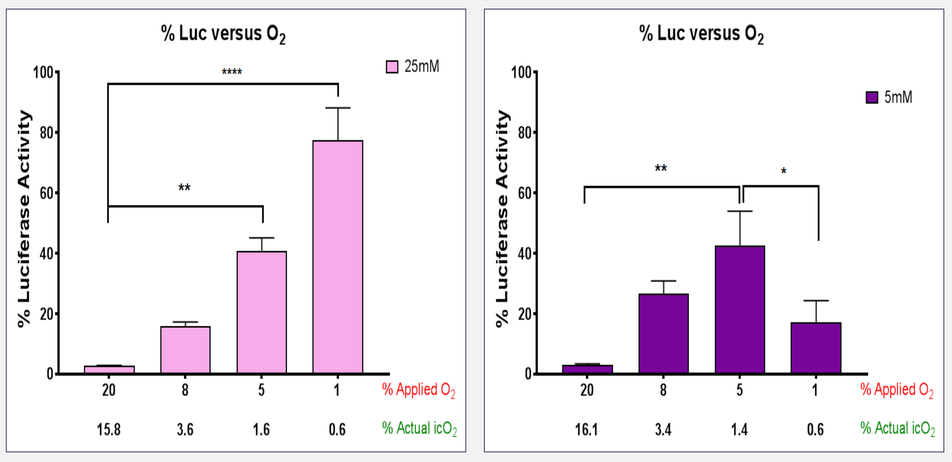


Figure 4: As ambient/icO_2 levels of HT1080 HRE cells are decreased we observe an increase in the % luciferase activity indicative of hif stabilisation on 25mM glucose (left). When glucose levels are dropped to 5mM glucose (right) the icO2 profiles are comparable but for the 1% applied oxygen treated cells we see a decrease in luciferase activity suggesting a lack of hif stabilisation despite an hypoxic microenvironment.

Key Findings

1) Metabolic poise and significant electron transport chain (ETC) activity can cause differences in the levels of oxygenation experienced by the cells.
2) In an oxidative cell line like the HT1080 cells cellular respiration drives the monolayer to lower O_2 levels than those imposed with the ACU.
3) Oxygenation within the cell monolayer in a static culture is extremely dynamic and is affected by media composition and depletion of substrates.
4) Knowing the degree of discrepancy between applied and actual O_2 is imperative when evaluating cellular responses to hypoxia and hif stabilisation.

These results highlight the importance of measuring and knowing the levels of oxygen within the cell and clearly show that levels *cannot* be inferred from applied O_2. If we continue to ignore the significance of this it will result in a flawed understanding of the relationship between oxygen concentration, Hif stabilisation and associated metabolic adaptations.

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