

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 O₂ has a wide ranging influence on cellular functions including metabolic poise and differentiation. Insufficient O₂ leads to the development of hypoxic environments which are important in solid tumours.

Here we use a novel method of measuring O₂ within a cell monolayer and look at the effects that reduced ambient O₂ coupled with O₂ 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 O₂-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 O₂ (0.1-20%) and CO₂ in the measurement chamber.



Real Time Monitoring of Cellular Oxygenation

1. Intracellular O₂ levels cannot be inferred from applied O₂

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.

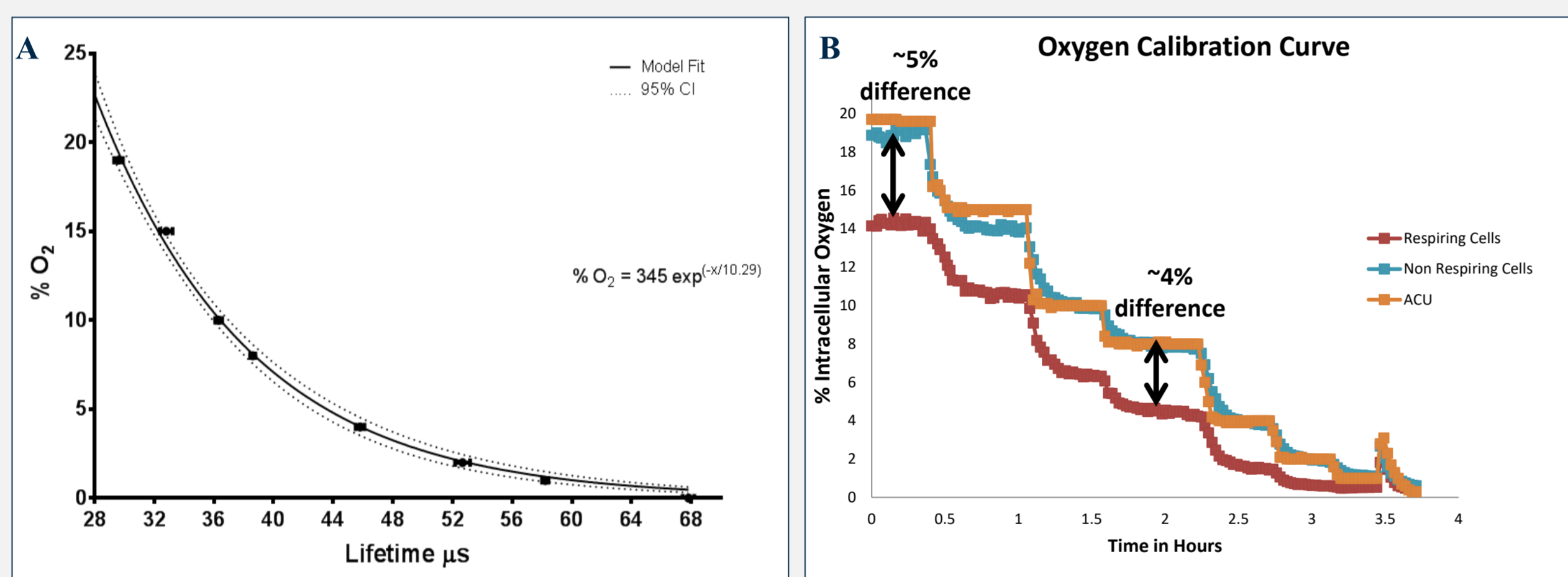


Figure 1: A non-respiring monolayer was used to create a calibration curve by plotting measured lifetime against the applied O₂ and a one phase exponential fit was applied to this curve (A). Using the equation from the model fit allows for the quantification of the oxygen levels within the cells. (B) Illustrates the impact of reducing applied O₂ 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. Intracellular oxygenation 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 icO₂ levels.

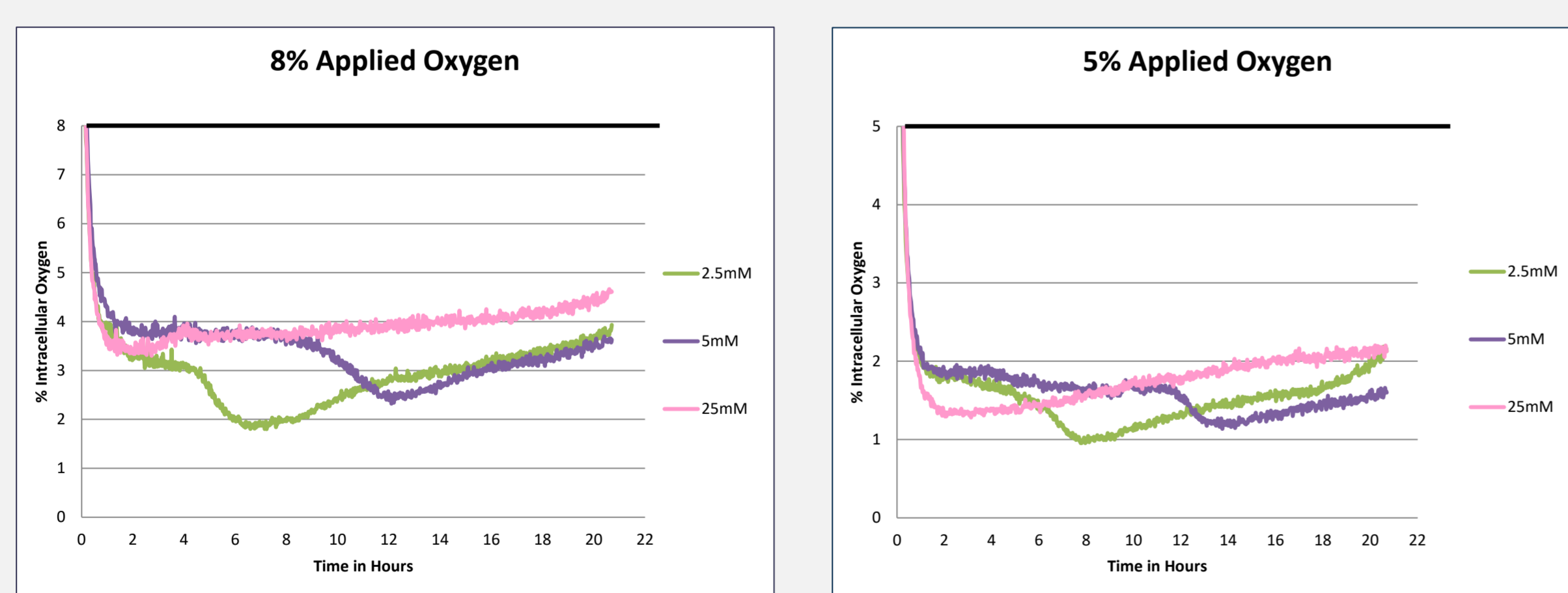


Figure 2: icO₂ levels of HT1080 cells were monitored over a 20hr period at 8 and 5% applied O₂. Cells were grown on standard DMEM (25mM glucose) and immediately before assaying were changed to DMEM containing 2.5, 5 or 25mM glucose. On more physiologically relevant O₂ concentrations (8 & 5%) we see biphasic oxygen profiles on the lower glucose concentrations indicative of glucose depletion resulting in increased mitochondrial OxPhos and a subsequent enhanced reduction in the icO₂ levels observed.

3. Substrate availability and utilisation affects icO₂

As demonstrated in figure 2 the concentration and utilisation of substrates (glucose) in the media can have a profound effect on the intracellular oxygen level the cells experience. To investigate this further we repeated the experiment with cells on 2.5mM glucose DMEM which we expected would run out of glucose after ~5hrs. Cells either had their media replenished, others were treated with antimycinA and rotenone and the remainder were left un-disturbed. Figure 3 demonstrates that when fresh media is added to the cells (green line) that a drop in icO₂ can be inhibited but occurs eventually once glucose again becomes depleted.

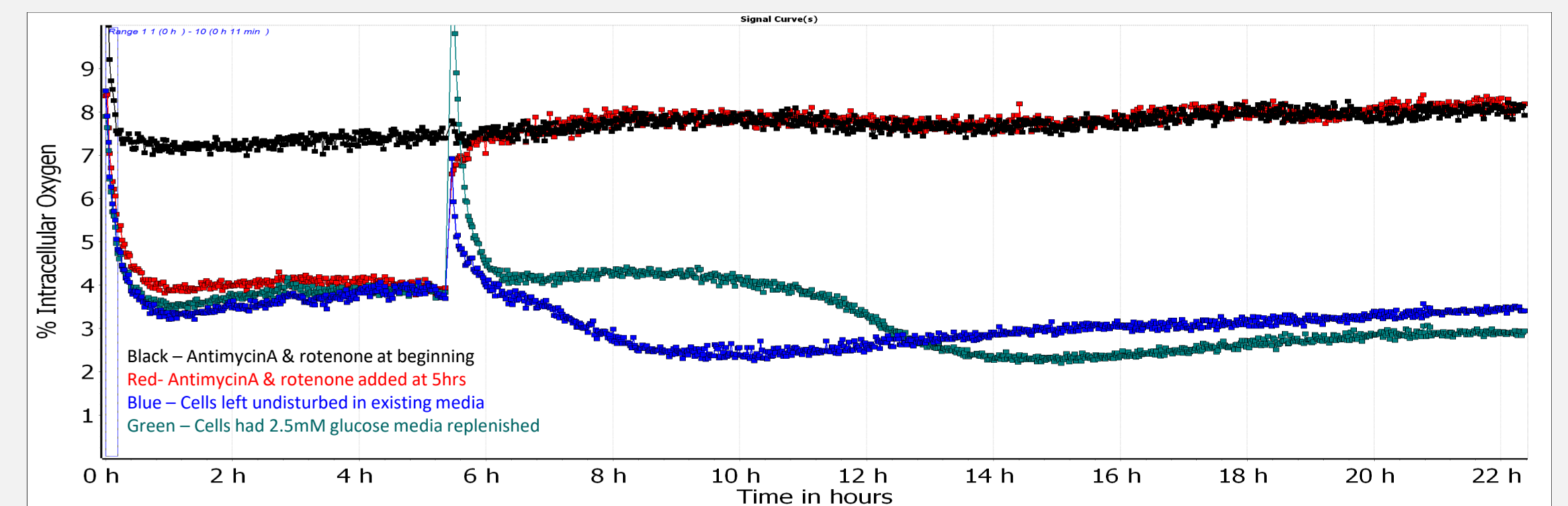


Figure 3: icO₂ levels in HT1080 cells were monitored at 2.5mM glucose and 8% applied oxygen. After 5 hours fresh media was added to some of the wells (green) while others were left untouched (blue). The cells that received fresh media failed to experience the same drop in oxygen that the untouched cells did at 6hrs. However, the media did become depleted 10hrs and the cells went through their characteristic drop in icO₂.

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

The HT1080 cells have a luciferase reporter gene under the control of hypoxia response element (HRE). icO₂ levels were measured overnight followed by a luciferase assay to assess Hif activity. We found that cells exposed to 5% ambient O₂ have intracellular levels approaching ~1% which is seen as ~40% luciferase activity (normalised to DMOG control (Figure 4)). This finding reinforces the need to monitor oxygen intracellularly because the dynamic deviation between applied and actual O₂ could impair the physiological relevance of our experimental observations. Interestingly, we don't see significant luciferase activity in cells on 5mM glucose at 1% applied oxygen.

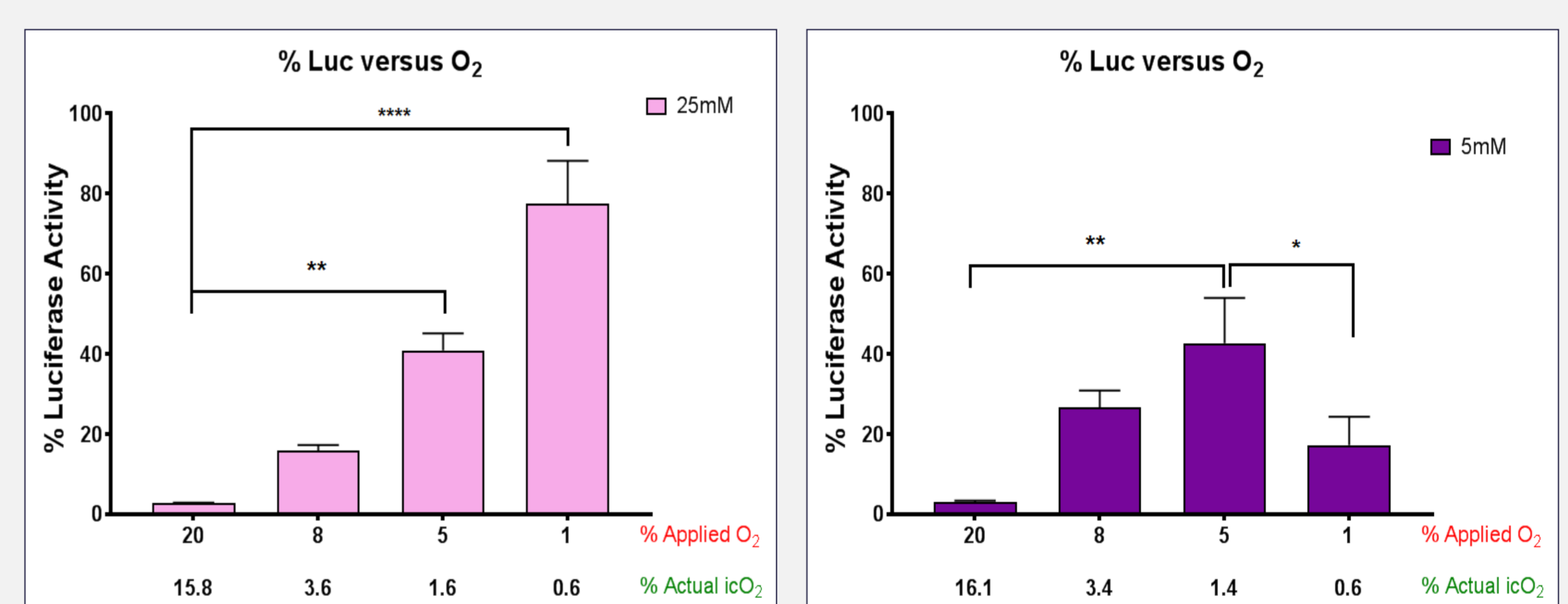


Figure 4: As ambient/icO₂ levels of HT1080 HRE cells are decreased we observe an increase in the % luciferase activity indicative of hif stabilisation on 25mM glucose (left). When glucose levels are dropped to 5mM glucose (right) the icO₂ profiles are comparable but for the 1% applied oxygen treated 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 O₂ 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 O₂ 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 O₂. 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.