The Measurement of Cellular Bioenergetics at Defined Steady-State Oxygen Concentrations: A Novel Microplate Based Method

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Introduction

Although often overlooked, most cell-based *in vitro* assays are conducted under hyperoxic conditions, while the impact of oxygen concentration ($[O_2]$) on experimental outcome is typically ignored. It is known however that for cells cultured at lower than ambient $[O_2]$, there exists a potential for higher $[O_2]$ to significantly impact cellular bioenergetics and, by extension, data output. While there is a growing appreciation of this deficiency, technical limitations have prevented broad uptake of $[O_2]$-informed *in vitro* assay design. These limitations have been ameliorated by recent the advances in both instrumentation and assay technologies: integrated atmospheric control units (ACUs) now facilitate measurement at defined $[O_2]$ while advanced phosphorescent extracellular O_2 and fluorescent extracellular pH probes, allowing simultaneous multiparametric measurements of cellular oxygenation, electron transport chain activity and glycolytic flux. Described herein is a high-throughput method for measuring all three key metabolic parameters under defined $[O_2]$ thereby further addressing such technical limitations. The method utilizes open-flow respirometry, a technique that allows for the measurement of cellular respiration at steady state (e.g. O_2 supply = O_2 demand). Preliminary data on respiration rate and media acidification using two different cell types at predefined $[O_2]$ is presented, facilitating a more detailed interrogation for the effect of $[O_2]$ and media formulation on the balance between oxidative phosphorylation and glycolytic activity. This system is further delineated using known metabolic effectors including the mitochondrial modulators, oligomycin, antimycin A and FCCP. The method therefore complements existing technologies, and as a cost-effective, efficient means to measure cellular bioenergetics at an $[O_2]$ most relevant to a given cell type or experimental model. Using this system, the effects of $[O_2]$ and glucose concentration on oxidative phosphorylation and glycolytic status of MCF10A and MDA231 cells are examined.

Oxygen Consumption Rate Optimization and Methods

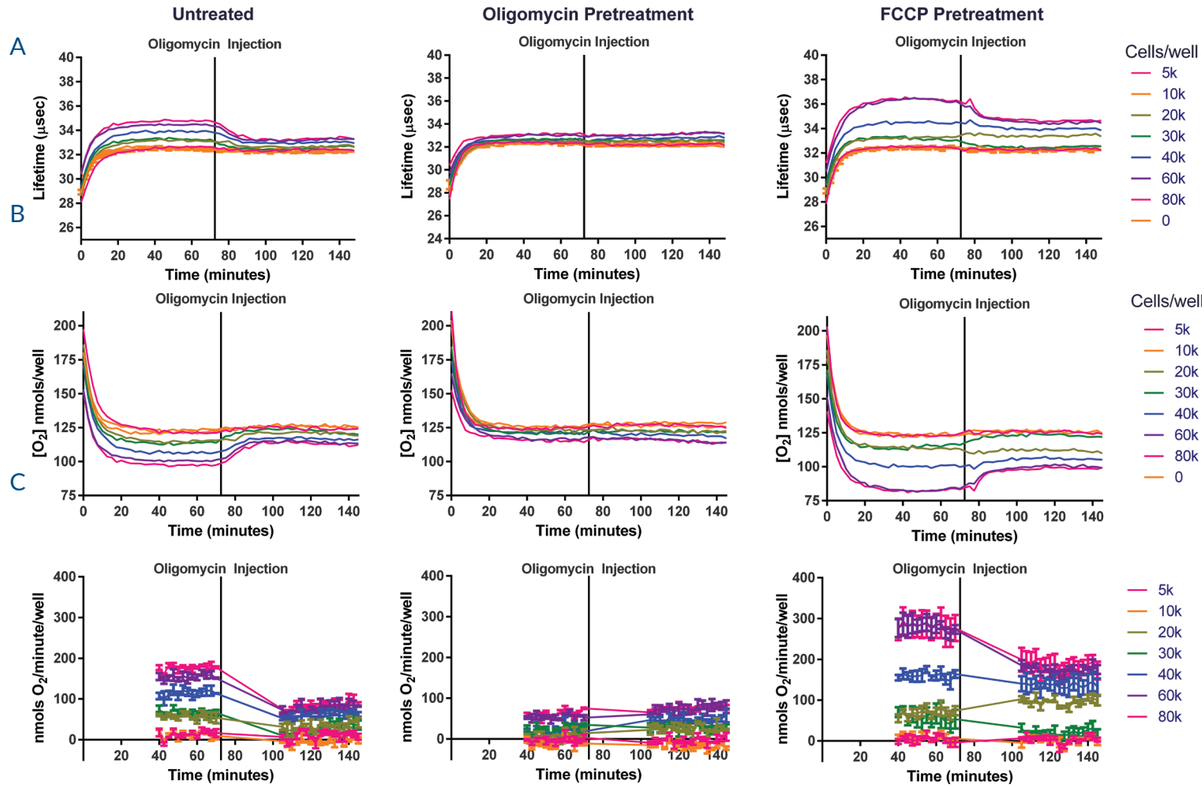

Figure 1 – Row A shows the lifetime readout of MitoXpress® Xtra at 10% O_2 at differing concentrations of HepG2 cells in a glucose-based buffer. As O_2 is depleted by the cells, lifetime of MitoXpress® Xtra increases. Control compounds are added to validate the response. Row B shows the calculated conversion of MitoXpress® Xtra to nmols O_2 based on the Stern-Volmer profile of MitoXpress® Xtra. Row C shows the cell dependent oxygen consumption rate (OCR) as calculated using the steady-state respiration equation outlined in Figure 2. Oxygen consumption rates were calculated at time points where the system was at equilibrium (O_2 supplied = O_2 consumed). The concentrations of the control compounds were 2 µg/ml for oligo and 2 µM for FCCP.

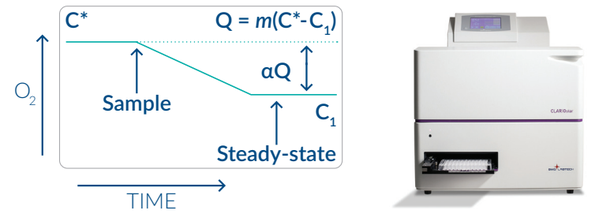

Figure 2 – Originally Built inside of a UV/VIS spec/fluorimeter, the principle behind open flow respirometry is easily applicable to BMG CLARIOstar with an ACU. In the microplate system, C* is defined as $[O_2]$ in wells that lack cells, C_1 is the $[O_2]$ in wells containing cells, at equilibrium. The rate of diffusion from the gas to liquid phase is defined as *m*.

General – All experiments were conducted using the CLARIOstar microplate plate reader equipped with Atmospheric Control Unit and bandpass filters optimized for use with MitoXpress® Xtra and pH-Xtra™

Open Flow Respirometry – The principle behind open-flow respirometry can be found above, with more information found in reference 1. Atmospheric control was maintained through the utilization of the atmospheric control unit affixed to the CLARIOstar. For OCR experiments conducted using HepG2 cells in serum free MEM, oxygen concentrations were set and the chamber was purged and equilibrated with N_2 and 5% CO_2 prior to the start of each experiment. Upon equilibration, microplates containing cells, MitoXpress® Xtra and pH-Xtra inserted into the plate reader and O_2 measurements taken every 2 minutes. Injections of oligomycin (2 µg/ml) and FCCP (2 µM) were made at equally spaced intervals allowing for the system to return to equilibrium following injections. OCR and ECAR experiments utilizing MCF10A and MDA231 cells were conducted in serum free, unbuffered DMEM containing 1 mM pyruvate and 1 mM glutamate with varied glucose concentrations (25 mM, 6.25 mM and glucose free, all pH 7.4 at 37°C). Oxygen experiments were set up as previously mentioned with the following exceptions: chamber was purged with N_2 alone, measurements were taken for both O_2 and pH at 2 minute intervals.

Cell Culture – HepG2, MDA231 and MCF10A cells were maintained in accordance with ATCC guidelines.

Data Analysis – All data were analyzed using a custom template built into MARS Data Analysis Software (v3.20 R2, BMG LABTECH) and exported to Graphpad Prism 6 (v.6.07).

Results and Discussion

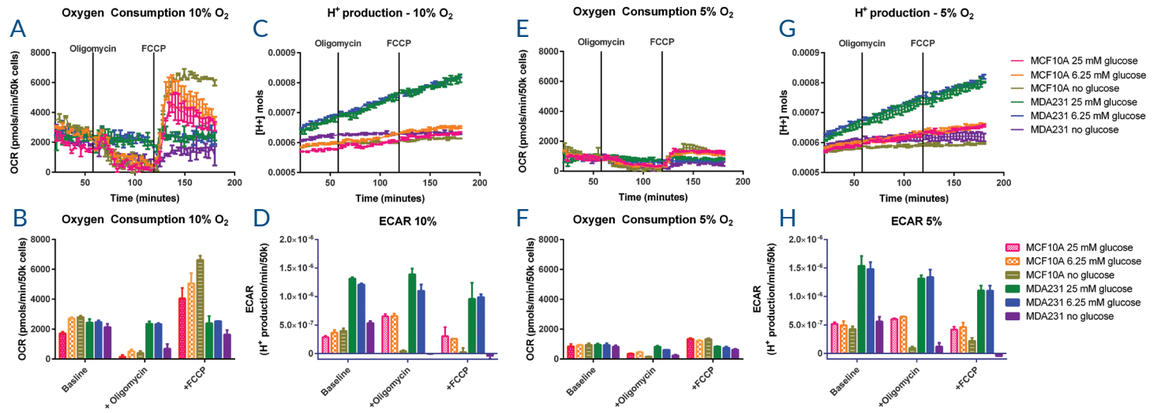

Figure 3 – Panels A and B show oxygen consumption rates for MDA231 and MCF10A cells at 10% O_2. Data are presented as OCR vs. time (A) and average OCR (B). Panels C and D show extracellular acidification (ECA) data for MDA231 and MCF10A cells at 10% O_2. Data are presented as H^+ production vs. time (C) and average rate of ECAR (D). Panels E through H are arranged identically to A through D, showing OCR data (E and F) and ECA data (G and H) obtained at 5% O_2. The x-axes on figures A,C,E and G have been truncated, omitting the first 20 minutes as the $[O_2]$ equilibrated. Data are presented as means ± standard error of wells run in triplicate.

In order to apply this system, the effects of $[O_2]$ and glucose concentration on MCF10A cells (normal breast tissue) and MDA231 cells (highly aggressive breast tumor) were studied. Oxygen consumption, and extracellular acidification rates at 10% O_2 show MCF10A cells to be primarily aerobic, exhibiting a strong response to both oligomycin (OCR and ECAR) and FCCP (OCR only) (Figures 3A-D). Glucose concentration had minimal effect on baseline OCR, or oligomycin treated rates, but did exhibit a concentration dependent increase in FCCP treated OCR (Figure 3B). In contrast, the aggressive MDA231 cells appear to be primarily glycolytic, showing no change in OCR or ECAR when treated with oligomycin in the presence of glucose (Figure 3A-3D). Additionally, the high basal OCR and lack of response to oligomycin and FCCP suggests that the mitochondria found in MDA231 cells either have a high degree of proton leak, or are altogether uncoupled. Interestingly, in the absence of glucose, mitochondrial function in MDA231 is restored. A decrease in OCR is observed upon treatment of these cells with oligomycin, suggesting that these cells are capable of oxidative phosphorylation when glucose is absent. Under all glucose conditions tested MDA231 cells did not exceed baseline OCR with the addition of FCCP.

When comparing OCR and ECAR measurements taken at 5% and 10% O_2, it was surprising to see such drastic differences in OCR in all cell types (Figure 3A and B, vs Figure 3E and F), with only a slight increase in ECAR (Figure 3C and D vs. Figure 3G and H). One theory is that while $[O_2]$ is low enough to limit maximal OCR (e.g. FCCP treated), enough O_2 remains to allow OXPHOS to continue. The remainder of cellular ATP demand is likely supplemented by glycolysis, as suggested by the increased ECAR. Interestingly, even at 5% O_2 an oligomycin response is still observed in MDA231 cells lacking glucose. This observation further demonstrates that while MDA231 cells prefer glucose as a substrate, they are capable of metabolic switching under extreme circumstances. Taken together, these data suggest that in the absence of glucose, MDA231 cells regain the use of their mitochondria as a means to generate ATP. These data also demonstrate the importance of media formulation and $[O_2]$ when studying the bioenergetics of different cell types.

References

1. Dimitriev, RI *et. al Anal. Chem.* 2012.
2. Hoffman D.L. and Brookes, P.S. *J. Biol. Chem.* 2009

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