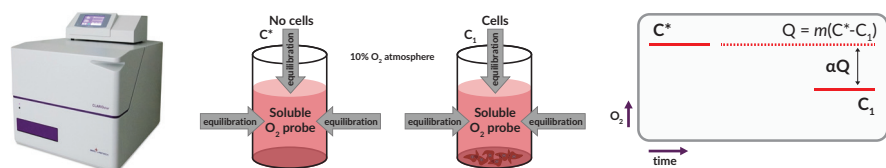


Introduction

Although often overlooked, the vast majority of cell-based *in vitro* assays are conducted under hyperoxic and hyperglycemic conditions, with the impact of elevated parameters on experimental outcomes typically ignored. These are important considerations, as higher [O₂] (hyperoxia) can significantly impact cellular bioenergetics and, by extension, cellular response to drug treatment. However, technical limitations have, to date, limited broad uptake of [O₂]-informed *in vitro* assay design. Here we interrogate the impact of these parameters on cellular responses to drug-induced mitochondrial dysfunction through the development of a novel high-throughput microplate assay, interrogating metabolic perturbation at defined [O₂]. The method, available as Cayman Chemical's Mitochondrial Stress Test Kit (Item No. 601450), harnesses integrated atmospheric control, advanced luminescent intracellular O₂, and extracellular pH probes to achieve simultaneous measurement of cellular oxygenation, oxygen consumption rate (OCR), and extracellular acidification (ECA), providing detailed information on the interplay between oxygenation, oxidative phosphorylation (OXPHOS), and glycolytic flux. This is achieved using an open-flow approach whereby respiration is measured unsealed at steady state (O₂ supply = O₂ demand), allowing direct control of sample [O₂] and enabling sequential compound addition to determine dependence on glycolysis and OXPHOS-derived ATP. The method is validated using a panel of classical metabolic modulators including oligomycin, antimycin A, FCCP, and etomoxir, with measurements performed at decreasing O₂ and glucose concentrations. Switching cells to more physiologically relevant [O₂] increases dependence on glycolytic ATP (e.g., MCF10A cells show a 60% increase in ECA between 10% and 5% O₂). By contrast, reducing glucose concentrations in the presence of pyruvate and glutamine increases dependence on OXPHOS-derived ATP and impacts spare respiratory capacity. Testing with a compound panel (troglitazone, flutamide, nimesulide, fenofibrate) reveals that this metabolic recalibration makes cells more susceptible to the downstream implications of drug-induced mitochondrial dysfunction. This method therefore complements existing technologies, offering an efficient means to measure cellular bioenergetics and screen for mitochondrial dysfunction at an [O₂] most relevant to a given cell type or experimental model.

Methods



General – All experiments were conducted using Cayman's Mitochondrial Stress Test Kit (Item No. 601450) and BMG LABTECH's CLARIOstar® microplate reader equipped with atmospheric control unit (ACU) and bandpass filters optimized for use with MitoXpress® Xtra and pH-Xtra™. OCR and ECA experiments were conducted in HepG2, MCF10A, MDA231, or H9C2 cells (where indicated) in serum-free, unbuffered DMEM at varying concentrations of glucose.

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 ACU 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 N₂ and 5% CO₂ prior to the start of each experiment. For other cell types, experiments were carried out in unbuffered DMEM at 5% O₂, with the CO₂ channel disabled. Upon equilibration, microplates containing cells, MitoXpress® Xtra, and pH-Xtra™ were inserted into the plate reader and measurements were taken every two 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.

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

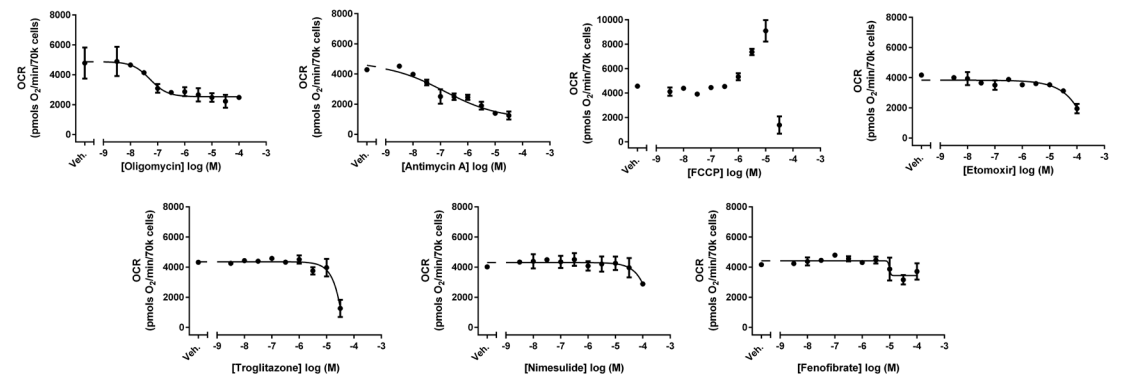
Data Analysis – All data were analyzed using a custom template written for MARS Data Analysis Software (v3.20 R2, BMG LABTECH) and then exported to Graphpad Prism 6 (v6.07). The script, test protocol, and analysis template are available for free download at www.bmg-labtech.com/cayman.

References
1. Cnifley, R.L., Zhandov, A.V., Jasonek, G., et al. *Anal. Chem.* **84**(6), 2930–2938 (2012).
2. Hoffman, D.L. and Brookes, P.S. *J. Biol. Chem.* **284**(24), 16236–16245 (2009).
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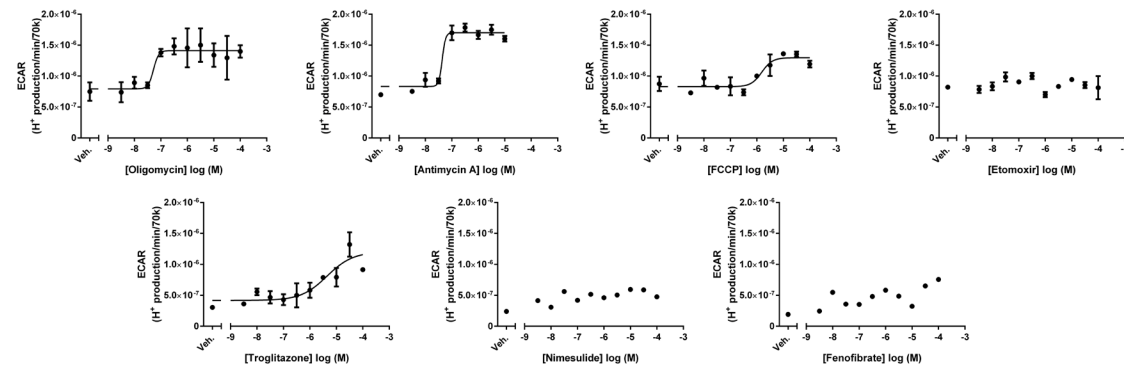
Results and Conclusions

Under all glucose conditions tested, MDA231 cells did not exceed baseline OCR with the addition of FCCP. Comparisons between OCR and ECAR measurements at 5% and 10% O₂ show drastic differences in OCR in all cell types (Figure 2A and B versus Figure 2E and F), with only a slight increase in ECAR (Figure 2C and D versus Figure 2G and H). One theory is that while [O₂] is low enough to limit maximal OCR (e.g., FCCP treated), enough O₂ 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% O₂ 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.

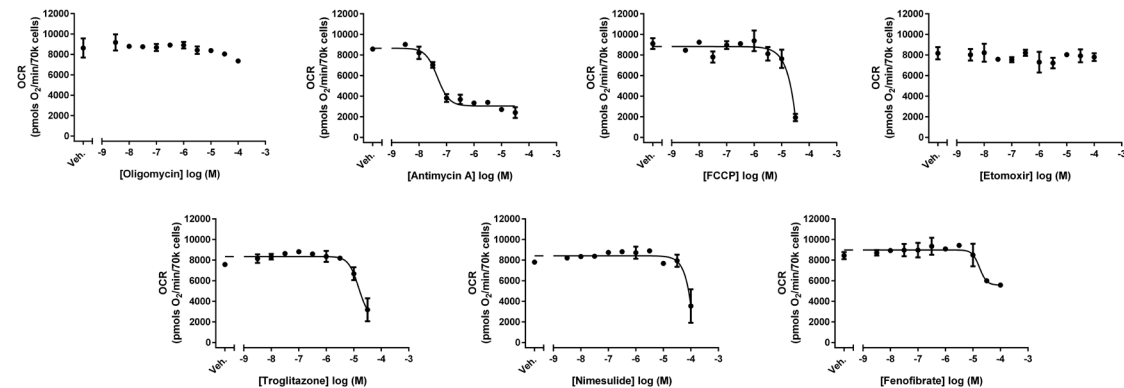
Screening for Mitochondrial Toxicity – Using Cayman's Mitochondrial Stress Test Kit (Item No. 601450), the compounds indicated below were screened for effects on mitochondrial function. Conditions examined: baseline OCR, baseline ECAR, and effects on uncoupled respiration.



Basal OCR Screening – All control compounds (oligomycin, antimycin A, FCCP) functioned as expected with troglitazone influencing basal OCR at high concentrations.



Basal ECAR Screening – Basal ECAR values were obtained simultaneously with basal OCR values. All compounds that influence OCR levels (inhibitors and uncouplers) were shown to increase ECAR values, consistent with inhibition of OXPHOS.



FCCP Treated OCR Values – Following FCCP treatment, all compounds affecting the electron transport chain were shown to inhibit uncoupled OCR. Oligomycin inhibits the ATP synthase, and therefore any inhibitory effects observed under basal conditions are ablated.

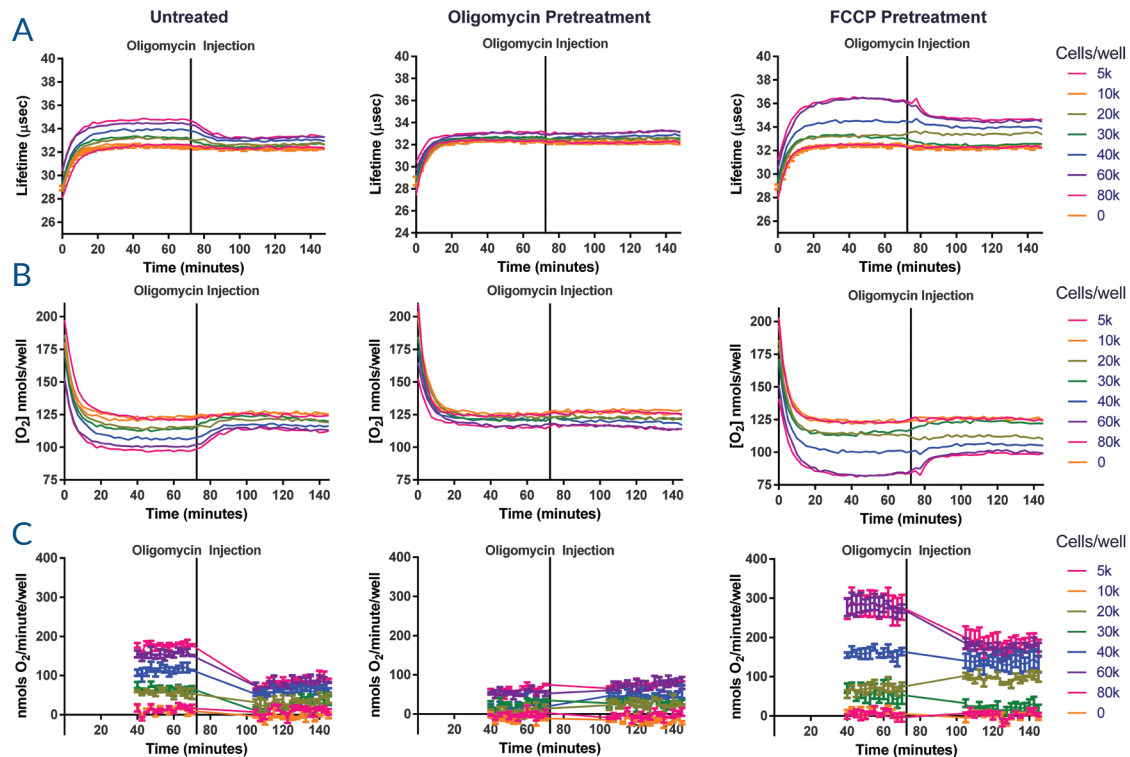


Figure 1 – Row A shows the lifetime readout of MitoXpress® Xtra at 10% O₂ to differing concentrations of HepG2 cells in a glucose-based buffer. As O₂ is depleted by the cells, lifetime of MitoXpress® Xtra increases. Control compounds are added to validate the response. Row B shows the calculated conversion of MitoXpress® Xtra to nmols O₂ based on the Stern-Volmer profile of MitoXpress® Xtra. Row C shows the cell-dependent OCR as calculated using the steady-state respiration equation outlined in Figure 2. OCRs were calculated at time points where the system was at equilibrium (O₂ supplied = O₂ consumed). The concentrations of the control compounds were 2 µg/ml for oligomycin and 2 µM for FCCP.

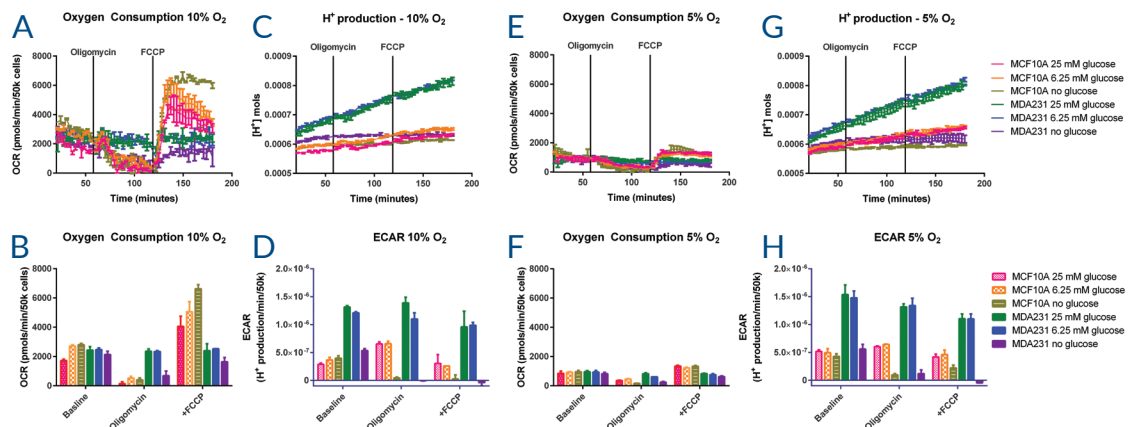


Figure 2 – Panels A and B show OCRs for MDA231 and MCF10A cells at 10% O₂. Data are presented as OCR versus time (A) and average OCR (B). Panels C and D show ECA data for MDA231 and MCF10A cells at 10% O₂. Data are presented as H⁺ production versus time (C) and average rate of ECA (D). Panels E through H are arranged identically to A through D, showing OCR data (E and F) and ECA data (G and H) obtained at 5% O₂. The x-axis on figures A, C, E, and G has been truncated, omitting the first 20 minutes as the [O₂] equilibrated. Data are presented as means ± standard error of wells run in triplicate.

Metabolic Switching – One application focused on the effects of [O₂] and glucose concentration on MCF10A cells (normal breast tissue) and MDA231 cells (highly aggressive breast tumor). OCR and extracellular acidification rates (ECAR) at 10% O₂ show MCF10A cells to be primarily aerobic (OCR and ECAR) (Figure 2A-D), with glucose concentration having minimal effect on baseline OCR or oligomycin treated rates. Upon uncoupling FCCP (Figure 2B), a concentration-dependent increase in OCR was observed. In contrast, the aggressive MDA231 cells were primarily glycolytic, showing no change in OCR or ECAR when treated with oligomycin in the presence of glucose (Figure 2A-D). Additionally, the high basal OCR and lack of response to oligomycin and FCCP suggest that the mitochondria found in MDA231 cells either have a high degree of proton leak or are altogether uncoupled. 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 MDA231 cells are capable of OXPHOS when glucose is absent.