

Investigating Cardiomyocyte Dysfunction through Combined Analysis of Beating, Metabolic Flux and Cellular Oxygenation

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Introduction

Cardiotoxicity:- Cardiotoxicity and related cardiac impairment remains one of the main reasons for both drug withdrawal [1] and FDA black box warning [2] and are a significant cause of compound attrition in preclinical development.

ATP Demand and Mitochondria:- Cardiac tissue requires an uninterrupted supply of respiratory substrates to meet the very high ATP demand imposed by continuous beating. Over 95% of this ATP is generated by oxidative phosphorylation (OxPhos) with the necessary mitochondrial network taking up approximately one third of cardiomyocyte cell volume. Energy starvation and mitochondrial dysfunction are therefore significant factors in the progression of cardiotoxicity and so, detection of such metabolic dysfunction is an important aspect of cardiotoxicity screening. This is best achieved by monitoring the two main ATP generating processes; OxPhos and Glycolysis (Fig. 1).

Cardiac Metabolism and Oxygenation:- *In vivo*, the most important respiratory substrates for ATP production are pyruvate and fatty acyl CoA, however cardiomyocyte metabolism is particularly adaptable and substrates such as amino acids, lactate and ketone bodies can also be used. Examples of this adaptability include HIF mediated metabolic responses to hypoxia and ischemia, and a the shift from fatty acid oxidation (FAO) to glucose metabolism that occurs in hypertrophic cardiac tissue. These adaptations highlight the importance of information on substrate preference and oxygenation when designing and interpreting *in vitro* cardiomyocyte analyses.

Contractility and Mitochondrial Function:- As cardiac contraction is the main ATP consumer, the coupling of contractility to ATP production, and by extension, mitochondrial activity, is critically important to normal cardiomyocyte function, particularly as the mitochondrial reticulum regulates intracellular calcium homeostasis and a multitude of critical signalling pathways. The ability to relate beating to altered metabolic activity would therefore be of significant utility.

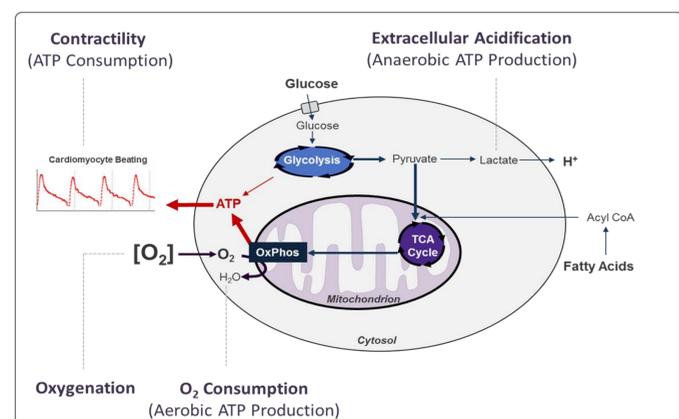


Fig 1: A simplified schematic of the inter-relationship between cardiomyocyte metabolism and beating activity. OxPhos produces the majority of the ATP needed, with pyruvate and Acyl CoA being the main respiratory substrates. By measuring beating, ETC activity (via O₂ consumption) and glycolytic flux (via extracellular acidification), cellular oxygenation a more complete picture of cardiomyocyte function can be established.

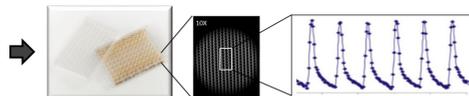
The interplay between cardiomyocyte beating and metabolism

Here we examine the feasibility of combining microelectrode-based iPS cardiomyocyte (Cor.4U[®]) contractility measurements with a microplate-based bioenergetics assessment to better characterise cellular responses to drug treatment.

Mitochondrial Dysfunction & Contractility

Combining Contractility and Metabolism Measurements

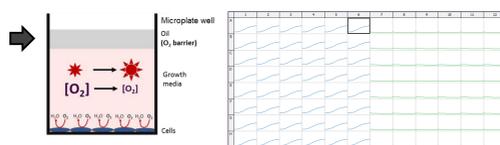
Contractility is measured by culturing iPS derived Cor.4U[®] cardiomyocytes (Axiogenesis) on E-plates (opposite) and measuring on the xCELLigence Cardio system (ACEA). This allows interrogation of the impact to drug treatment on cardiomyocyte beat rate.



Cell Metabolism is measured using the MitoXpress-Xtra HS oxygen consumption assay (opposite) to assess **mitochondrial function** and the pH-Xtra extracellular acidification (ECA) assay to assess **glycolytic flux**.



Cellular oxygen consumption or acidification causes an increase in probe signal allowing plate reader-based analysis of cell metabolism.



Both probes can be measured using dual-read time resolved fluorescence [3]. This allows measurement on E plates such that, if necessary, **metabolism and contractility can be measured sequentially on the same test plate**.

The impact of Mitochondrial Impairment on Cor.4U[®] iPS Cardiomyocyte Beating

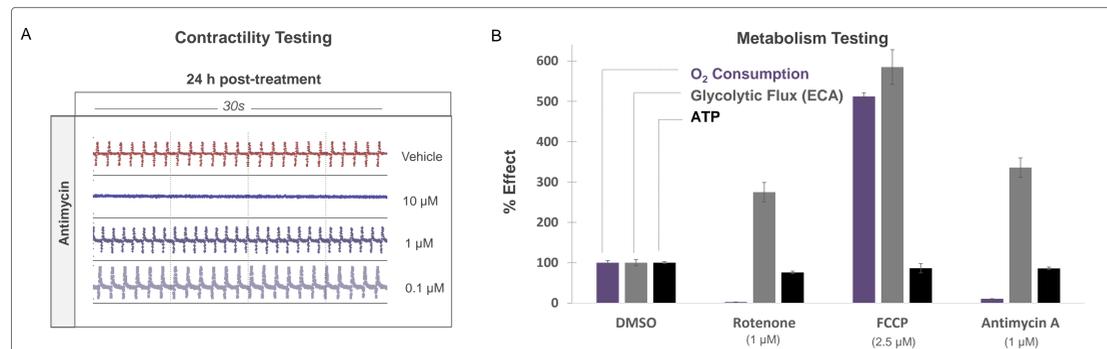


Fig 2: Impact of mitochondrial impairment on Cor.4U cardiomyocyte beating. Beating is maintained in the presence of mitochondrial inhibitors through increased glycolytic ATP supply. 30s xCELLigence traces 24 h post treatment (A). OCR, ECA and ATP measured at fixed concentration (B). Data presented relative to untreated control.

Antimycin and Rotenone treatment causes an immediate inhibition of mitochondrial function while FCCP treatment causes an immediate uncoupling of OxPhos (Fig. 2). Despite this, iPS derived Cor.4U[®] **cardiomyocyte beating** continues in the presence of inhibitory concentrations of Antimycin, Rotenone and FCCP (Fig. 2A) and is maintained for over 24h.

Analysis of O₂ consumption (using MitoXpress-Xtra) and ECA (using pH-Xtra) shows a decrease in O₂ consumption due to reduced ETC activity and an accompanying increase in ECA (Fig. 2B) suggesting that ATP depletion is ameliorated through increased glycolytic flux.

Together, this suggests that increased glycolytic flux supplies sufficient ATP to facilitate cardiomyocyte beating despite complete impairment of OxPhos. This is consistent with previous observations on specific cell lines [4].

Coupling of Contractility and Metabolism

Impact of Increased Beat Rate on Metabolism

Treatment with the β -adrenoreceptor agonist isoproterenol causes an increase in Cor.4U cardiomyocyte beat rate. This increase can be observed by xCELLigence testing while MitoXpress-Xtra and pH-Xtra assess the impact of treatment on cell metabolism.

Fig.3A shows the measured increase in beating caused by isoproterenol.

Fig.3B shows the effect of isoproterenol on cell metabolism with immediate increases in O₂ consumption suggesting increased aerobic ATP production in response to increased ATP demand.

ECA is not increased significantly (not shown) suggesting that OxPhos rather than glycolysis is supplying the additional ATP requirements.

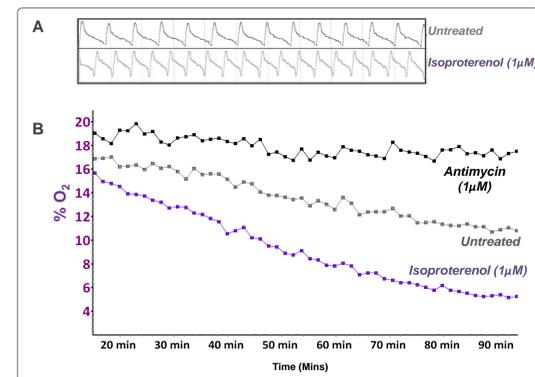


Fig. 3: Impact of isoproterenol on beat rate and metabolism measured on a FLUOstar Omega (BMG Labtech).

Impact of Increased Beat Rate on Cellular Oxygenation

MitoXpress[®]-Intra is an intracellular oxygen probe and facilitates the measurement of cellular oxygenation [5,6]. Fig. 4 shows the effect of pharmacologically altering Cor.4U beat rate on cardiomyocyte oxygenation.

Basal metabolism has reduced O₂ concentrations from ambient (~21%) to ~14%, while treatment with the ETC inhibitor Antimycin blocks O₂ consumption causing intracellular O₂ levels to return to ambient levels.

Treatment with the β -adrenoreceptor agonist isoproterenol increases cardiomyocyte beat rate which in turn causes an increase in oxygen consumption (Fig. 3).

This causes a significant but temporary reduction in O₂ availability with values of ~6% observed for >15 min despite cells being cultured and measured at 21% O₂.

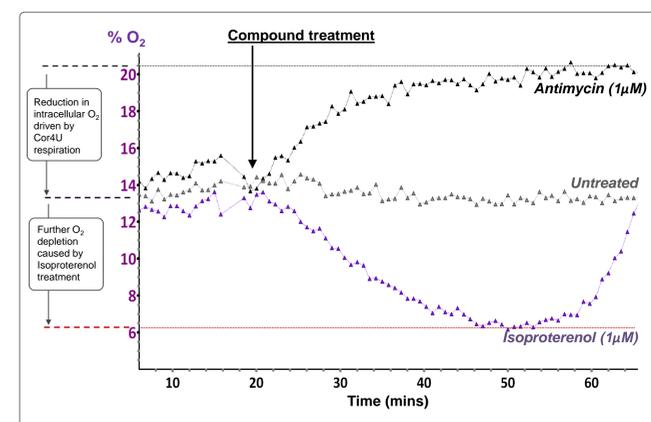


Fig. 4: Impact of isoproterenol on cardiomyocyte oxygenation measured on a FLUOstar Omega with ACU (BMG Labtech).

Conclusions

- Due to the dual-read TRF measurement approach used, MitoXpress-Xtra based measurements of O₂ consumption and pH-Xtra based measurements of ECA can be performed on xCELLigence E-plates (ACEA) using conventional TRF plate readers.
- This allows contractility and cell metabolism measurements to be performed in sequence on the same test plate.
- The combined use of microplate-based contractility and metabolism measurements has been demonstrated as an means to generate a more complete picture of cardiomyocyte response to drug treatment and allows the delineation of inter-relationships between cardiomyocyte beating and cell metabolism.
- Complete impairment of OxPhos through treatment with ETC inhibitors did not immediately impair Cor.4U cardiomyocyte beating. Increased ECA suggests that ATP supply is maintained through increased glycolytic flux allowing beating to continue for >24h post treatment.
- The β -adrenoreceptor agonist Isoproterenol increased beat rate and caused a significant increase in O₂ consumption but little change in ECA. This suggests that increased ATP demand is being met through OxPhos rather than glycolysis.
- This combined analysis of critical cardiomyocyte functions therefore delivers a more holistic and informative *in vitro* cardiotoxicity screen in that it related cellular function to the metabolic activity driving that function. In so doing it provides additional mechanistic information as to the cause of observed alterations in cardiomyocyte metabolism or contractility.

Methods

Preparation:

Cor.4U cells (Axiogenesis) were plated onto fibronectin coated 96 well plates and placed in culture for 2-3 days, performing media changes as per manufacture instructions. Cells were plated at 4-5x10⁴ cells/well for pH-Xtra and MitoXpress-Xtra assay.

Measurement:

MitoXpress-Xtra (HS method): Fresh media containing MitoXpress[®] reagent (Luxcel Biosciences), 150 μ l/well was added prior to measurement. Compounds were added directly and all wells were sealed with pre-warmed HS oil. Plates are measured at (37°C) for 2.5-3 hour kinetically (Ex380nm, Em650nm and dual-read TR-F Lifetime measurement - FLUOstar Omega, BMG Labtech)

pH-Xtra Glycolysis measurement: The sample plate is placed in CO₂ free incubator 3 hours prior to measurement, in order to remove CO₂. Samples are washed 3 times using Respiration Buffer (1mM phosphate) prepared using the buffer tablet provided. 150 μ l of Respiration Buffer containing pH-Xtra[®] reagent (Luxcel Biosciences) was added to sample wells. Compounds were added directly, and the plate was measured kinetically for 2.5 hours, on a pre-warmed plate reader (37°C). (Ex380nm, Em615nm and dual-read TR-F Lifetime measurement - FLUOstar Omega, BMG Labtech).

MitoXpress-Intra measurement: Cells were loaded with MitoXpress-Intra reagent (Luxcel Biosciences) overnight (14 hours) in 96-well plate the day prior to measurement. Cells are washed twice and 150 μ l of fresh media was added. The plate was measured kinetically at 37°C. (Ex380nm, Em650nm and dual-read TR-F - FLUOstar Omega with ACU, BMG Labtech).

xCELLigence RTCA Cardio measurement: iPS-Cardiomyocytes were plated on 96 well E-Plates and impedance measurements were recorded at selected time points (60s sweep at a sampling rate of 77 Hz). Drug treatment was initiated once the culture showed 40-60 synchronic beats/min. The data were normalized to baseline beating rate.

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