Measuring mitochondrial function and glycolytic flux in 3D cell cultures

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- **3D RAFT™ cell cultures were used in 96 well microplate format**
- **MitoXpress Xtra Oxygen Consumption Assay (HS Method) and pH-Xtra Glycolysis Assay were used to determine mitochondrial function and glycolytic flux**

Introduction

While, historically, 2D cultures have been the mainstay of *in vitro* assays, there is a developing interest in transferring to 3D models in an effort to increase the biological relevance of the measurement. By more closely reflecting conditions within the tissue, the hope is that such 3D models will help bridge the gap between *in vitro* and *in vivo* measurements thereby increasing the usefulness of the in vitro assay. Of equal importance to the aim of increasing biological relevance is the parameter measured. One of the most informative is to measure cell metabolism, whereby perturbed metabolism or mitochondrial function is probed without disrupting the 3D structure. This can be achieved in microtitre plate format using the *MitoXpress Xtra HS* and *pH-Xtra* products from Agilent Technologies.

MitoXpress Xtra HS measures oxygen consumption and therefore informs specifically on mitochondrial function, while pH-Xtra measures extracellular acidification and is therefore a convenient measure of glycolytic flux. Here we demonstrate the application of these probe technologies to 3D cultures generated using the *RAFT*™ system from TAP Biosystems. *RAFT*™ facilitates the convenient production of consistent collagenbased structures. This, in conjunction with *MitoXpress Xtra HS* and *pH-Xtra* facilitates detailed microplate-based measurements of metabolic activity of 3D cultures without disrupting the integrity of the 3D structure. Measurements are conducted on the FLUOstar Omega microplate reader from BMG LABTECH.

Materials & Methods

- 3D RAFT™ cultures including black walled clear bottom 96-well cell culture microplates,
- MitoXpress Xtra-Oxygen Consumption Assay (HS Method), (Agilent Technologies, MX-200-4)
- pH-Xtra Glycolysis Assay, (Agilent Technologies, PH-200-4)
- FLUOstar® Omega microplate reader, (BMG LABTECH)
- DMEM and culture media were obtained through usual distribution channels

Plate Preparation

3D RAFT cultures were prepared with either A549 or HepG2 cells (data not shown) at the indicated density in 240 µl DMEM/Collagen solution on a 96-well plate. RAFT cultures were formed as per manufacturer's protocol (Fig. 1).

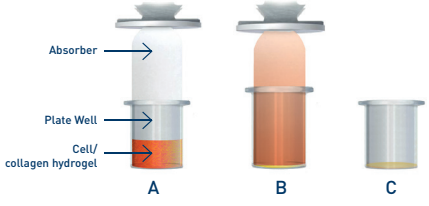

Fig. 1: RAFT Process Principle.

(Fig. 1A) Cells and neutralized collagen were mixed and pipetted into one well. After 15 min incubation at 37°C a hydrogel is formed. Medium was absorbed from the hydrogel (Fig. 1B) to increase concentration of collagen and cells to *in vivo* conditions. The process is completed (Fig. 1C) in less than 1 hour and results in a structure that is about 120 µm thick.

Oxygen Consumption Measurements

Culture medium was removed after a desired culture period and 100 µl of MitoXpress Xtra stock solution prepared in prewarmed DMEM was added. 1 µl of compound (100x) was added to appropriate wells. All wells were then sealed by adding 100 µl prewarmed HS mineral oil to prevent the back diffusion of ambient oxygen. The plate was then measured kinetically on a FLUOstar Omega for 90-120 minutes at 37°C.

Extracellular Acidification Measurements

Two hours prior to measurement the RAFT cell culture plate was placed in a CO_2 free incubator at 37°C, 95% humidity, in order to remove CO_2 from the plate material. Media was removed and 2 wash steps were performed using the Respiration Buffer (0.5 mM KH_2PO_4, 0.5 mM K_2HPO_4, 20 mM Glucose, 4.5 g/L NaCl, 4.0 g/L KCl, 0.097 g/L $MgSO_4$, 0.265 g/L $CaCl_2$). Finally 150 µl of Respiration Buffer containing pH-Xtra™ probe at the recommended concentration was added to each well. The plate was then measured kinetically.

FLUOstar Omega/CLARIOstar® instrument settings

Measurement method:	Time-resolved fluorescence (Omega: TR-F Optical attachment installed)
Measurement mode:	Plate Mode Kinetic
Measurement time:	120 min (data points every 2 min)
Measurement temperature:	37°C

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	Excitation/ Emission filters	Integration start/time (µs)
MitoXpress®-Xtra	1 TREx L + 655-50 (Omega) + 645-20 (CLARIOstar)	30/30
	2 TREx L + 655-50 (Omega) + 645-20 (CLARIOstar)	70/30
pH-Xtra™ Glycolysis Assay	1 TREx L and 615-BP10	100/40
	2 TREx L and 615-BP10	300/40

Results & Discussion

Sample oxygen consumption profiles are presented in Figure 2.

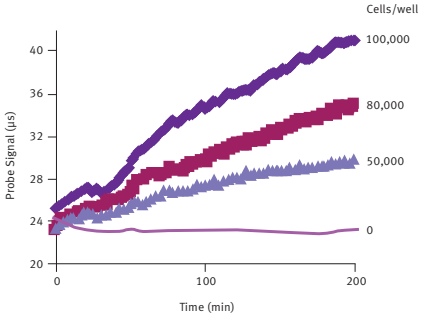

Fig. 2: Oxygen consumption profiles from A549 RAFTTM cultures of increasing cell density.

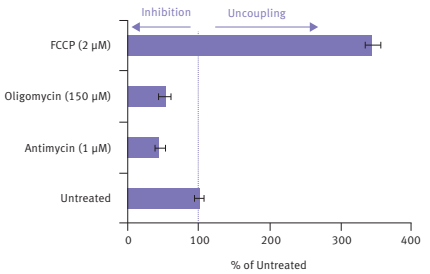

Fig. 3: Relative effect of drug treatment on cultures plated at 80,000 cells/well.

Increasing the density of cells in the 3D matrix results in increased rates of oxygen consumption, with strong signal changes observed across the cell concentrations tested. Fig 3 shows the effect of treatment on cells within the 3D structure where ETC activity has been pharmacologically modulated. Sample acidification profiles are presented in Figure 4 with significant acidification observed for untreated cells.

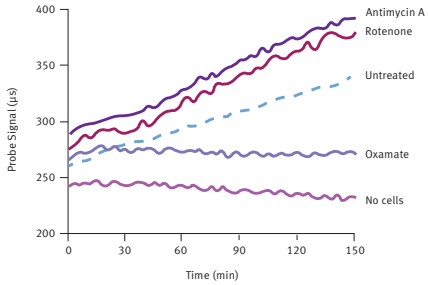

Fig. 4: Extracellular acidification profile from A549 RAFT cultures, using treated and untreated cells.

Acidification is inhibited almost completely on treatment with oxamate indicating that the acidification derives from the production of lactic acid. Treatment with antimycin however causes a significant increase in acidifications the cell increases glycolytic flux in order to maintain cellular ATP supply.

Conclusion

MitoXpress Xtra – Oxygen Consumption Assay and pH-Xtra Glycolysis Assay, provide a convenient, sensitive and high throughput measure of mitochondrial function, metabolism and cellular energy flux, when combined with the FLUOstar Omega multimode plate reader. These data illustrate the applicability of these assays to the study of cellular function in a complex multicellular 3D RAFT™ culture system without disrupting the integrity of the 3D structure. The FLUOstar Omega is enabled with menu selection for easy instrument set-up and data analysis, including the ability to input calibrations to generate O_2 and H^+ scales.