Application Note: 3D Cultures

Metabolic Profiling (OCR/ECA) in RAFT™ 3D cell cultures using MitoXpress®-Xtra & pH-Xtra™

Introduction

3D cell culture facilitates the development of complex intra-cellular interactions thereby helping to narrow the gap between *in vitro* and *in vivo* biological systems. Adoption of 3D technologies has however been limited, in part due to difficulties associated with producing reproducible 3D cultures. Difficulties can also arise due to an incompatibility with certain *in vitro* assay technologies. Here we demonstrate the use of **RAFT™** technology as a means of conveniently producing consistent collagen-based 3D matrices and show how detailed measurements of **mitochondrial function** and **cell metabolism** can be achieved without disrupting the integrity of the 3D structure. This is achieved using two simple mix-and-measure assays; **MitoXpress®-Xtra HS** (*Cat# MX-200*), measuring oxygen consumption and **pH-Xtra™** (*Cat# PH-100*) measuring extracellular acidification [1,2,3]. Oxygen consumption provides detailed information on mitochondrial function, specifically on the activity of the electron transport chain (ETC), while extracellular acidification (ECA) informs on glycolytic flux. Measurements are conducted on **standard 96-well microtitre plates**, on a fluorescence plate reader, and facilitate a deep insight into the metabolic behaviour of the 3D culture and into how metabolism is perturbed by a particular compound or environmental condition.

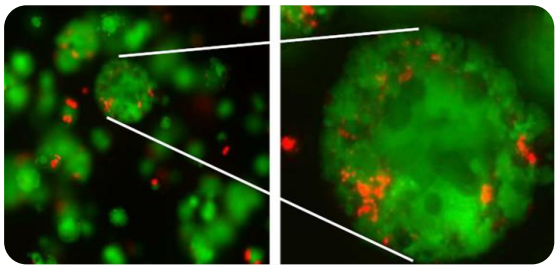

Fig1: A live cell fluorescent image of MCF7 cells forming tumoroid structures after 11 day RAFT™ culture. Calcein staining (green) identifies 'live' cells while PI staining (orange) identifies dead cells.

Method

Plate Preparation

- *3D RAFT™ cultures were prepared with either **A549** or **HepG2** cells at the indicated density in 240µl DMEM / Collagen solution on a 96-well plate. RAFT™ cultures were formed as per manufacturer's protocol (www.raft3dcellculture.com).*

Oxygen Consumption Measurements

- *For oxygen consumption measurements **MitoXpress®-Xtra** stock was prepared in 16ml of pre-warmed DMEM and culture media was replaced in each well with 150µl of this solution. Where applicable, 1µl of compound stock (150X) was added to each well.*
- *Wells were then sealed by overlaying with 100µl pre-warmed HS mineral oil to inhibit oxygen back diffusion into the sample. This is best done using a repeater pipette.*
- *The plate was then measured kinetically on a FLUOstar Omega (BMG Labtech) for 90-120mins with ~2 minute interval exciting the probe at 380nm and measuring emission at 650nm. Ratiometric measurements were performed using the following delay and gate settings. Delay 1: 30µs, Gate 1: 30µs, Delay 2: 70µs, Gate 2: 30µs.*

Extracellular Acidification Measurements

- *Three hours prior to measurement the RAFT™ 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.*
- *Spent 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 150µl of Respiration Buffer containing **pH-Xtra™** probe at the recommended concentration was added to each well*
- *The plate was then measured kinetically on a FLUOstar Omega (BMG Labtech) for 90-120mins with ~2 minute interval exciting the probe at 380nm and measuring emission at 615nm. Ratiometric measurements were performed using the following delay and gate settings. Delay 1: 100µs, Gate 1: 30µs, Delay 2: 300µs, Gate 2: 30µs.*

Sample Data

Sample **oxygen consumption** profiles are presented in Figure 2A for RAFT™ cultures prepared and measured as outlined above. By increasing the density of cells in the 3D matrix, increased rates of oxygen consumption are generated due to the activity of the ETC, with strong signal changes observed across the cell concentrations tested. Figure 2B shows the effect of treatment on cells within the 3D structure where ETC activity has been pharmacologically modulated.

When ETC activity is uncoupled from ADP phosphorylation through treatment with FCCP, oxygen consumption increases significantly, seen as a more rapid rate of signal increase. In contrast; treatment with the ATPase inhibitor Oligomycin and the Complex III inhibitor Antimycin cause inhibition of oxygen consumption. While treatment with the ETC modulators causes the expected response, the levels of inhibition are lower than typically observed in 2D cultures.

Sample **acidification profiles** are presented in Figure 2C with significant acidification observed for 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 acidification as the cell increases glycolytic flux in order to maintain cellular ATP supply.

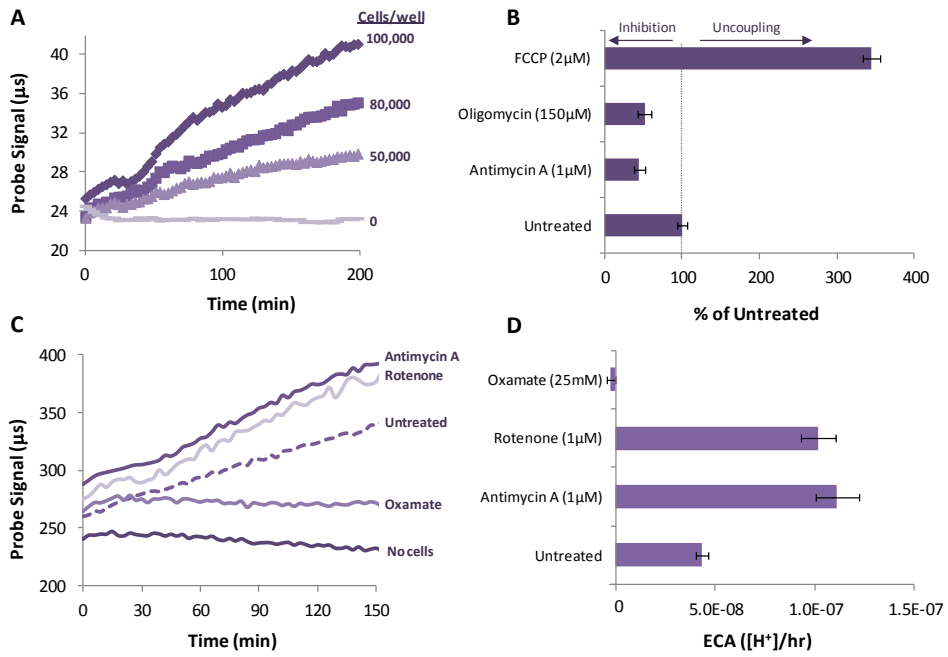

Fig 2: Oxygen consumption profiles from **A549** RAFT™ cultures of increasing cell density (A) and relative effect of drug treatment on cultures plated at 80,000 cells/well (B). Extracellular acidification profiles from **HepG2** RAFT™ cultures treated with Antimycin and Oxamate (C). Acidification rates can be presented in hydrogen ion scale thereby providing more accurate comparisons (D). Treatment with oxamate inhibits LDH resulting in a reduction in acidification. Antimycin treatment causes an increase in acidification as cells increase glycolytic flux to maintain [ATP]. All measurements are conducted immediately post treatment.

These data illustrate the capacity of MitoXpress®-Xtra and pH-Xtra™ to profile and detect perturbed metabolism within RAFT™ cultures without disrupting the integrity of the 3D structure.

References

[1] Hynes J, *et al,* Current Protocols in Toxicology 2.16.1-2.16.2, 2009.

[2] Hynes J, *et al,* Analytical Biochemistry 390, 21-28, 2009.

[3] Hynes J, *et al,* Toxicology In Vitro, 27, 560-569, 2013.

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