Application Note

Cell Metabolism

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Metabolic Profiling of Cells in 3D Cultures using MitoXpress Xtra and pH-Xtra Assays

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Abstract

Metabolism is a key regulator of cellular function and energy homeostasis, and plays an important role in numerous diseases and therapeutic approaches. Technologies enabling researchers to measure cellular metabolism in more complex *in vitro* models are therefore in demand.

This application note presents two distinct workflows that use the Agilent MitoXpress Xtra oxygen consumption and pH-Xtra glycolysis assay to interrogate basal and perturbed cellular metabolism in intact RAFT 3D collagen structures on fluorescent plate-readers.

These simple workflows combine the advantages of plate-reader-based metabolism assays with those of the more physiological extracellular environment created by collagen 3D cultures. These workflows can also help to overcome some of the challenges previously associated with metabolism assays in 3D cultures, therefore, they have the potential to further narrow the gap between *in vitro* and *in vivo* metabolism research.

Introduction

Recent years have seen a growing appreciation of the breadth of roles played by cell metabolism in fundamental biological processes. Changes in metabolic equilibrium are now understood as key features of a multitude of disease states, immune response, adverse drug effects, and are known to impact therapeutic efficacy, particularly within an immune-oncology context. Consequently, there is a growing need for tools that facilitate a further unraveling of the interplay between metabolic reprogramming or perturbation, and the etiology of specific disease states.

An extra consideration when undertaking such *in vitro* metabolic assessments is the suitability of the cell model being used. There is a desire in some contexts to perform these analyses in suitable 3D cell cultures that better reflect the complex metabolite gradients and cell-cell interactions that pertain *in vivo*. Methods that facilitate the convenient study of metabolism within such 3D constructs are therefore of significant utility.

This application note demonstrates how the MitoXpress Xtra oxygen consumption and pH-Xtra glycolysis assays can enable such metabolic analyses of cells within intact 3D collagen structures using a conventional microplate workflow.

Assay workflow

Agilent's soluble sensor workflows (Figures 1 and 2) are simple mix-and-measure protocols that are easily performed in 3D collagen structures such as those formed using Lonza's RAFT kits.

Before performing either of these assays, cells are plated into the collagen structures, then grown and treated as desired. The workflow for these metabolism assays using RAFT cultures is very similar to the workflow when measuring conventional 2D culture plates, while ensuring extra care is taken not to disrupt the collagen structure during media removal, washing, and probe addition. The assays can be performed separately on parallel plates, or in separate wells of the same plate when the workflow described in this application note is used.

The MitoXpress Xtra oxygen consumption assay facilitates the kinetic measurement of aerobic metabolism using fluorescence plate readers. The soluble, oxygen-sensitive MitoXpress Xtra probe is mixed with assay medium, added to the wells, then overlaid with a layer of HS oil. As cellular respiration occurs, the concentration of oxygen in the sample decreases. This causes an increase in the MitoXpress Xtra signal over time, providing a measure of relative cellular oxygen consumption.

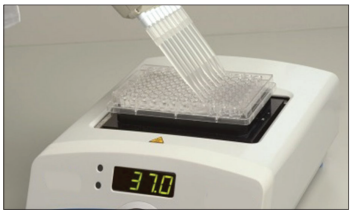
Figure 1. The Agilent MitoXpress Xtra assay workflow.

pH-Xtra is a soluble pH-sensitive probe designed to assess glycolysis in samples by measuring changes in the pH in the assay buffer, which is indicative of the rate of lactate excretion. In contrast to MitoXpress Xtra, the well does not need to be sealed with a layer of HS oil before measurement. The assay does, however, require the use of a low buffered assay medium (included in the kit) to detect changes in pH. The pH-Xtra signal is proportional to the extracellular acidification and can be further transformed to the pH- or $[H^+]$-scale for a more accurate assessment of differences in glycolysis between samples.

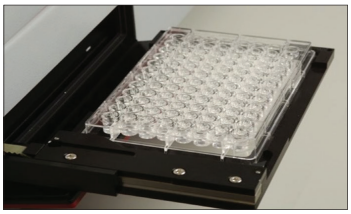
Agilent pH-Xtra workflow

- Place the plate in a CO_2-free incubator for 2 hours.

- Reconstitute assay buffer tablet.
- Adjust to pH 7.4.
- Reconstitute pH-Xtra in the assay buffer.



- Remove the spent culture medium.
- Wash the wells with the assay buffer.
- Add pH-Xtra in assay buffer.



- Measure the pH-Xtra signal kinetically.

Figure 2. The Agilent pH-Xtra glycolysis assay workflow.

Material and methods

Cell culture and RAFT formation

HepG2 and A549 cells were maintained in DMEM (Gibco, #41965039) supplemented with 10% FBS (Sigma, #F0804), 1 mM pyruvate (Sigma, #S8636), and 1% Pen/Strep solution (Sigma, #P4333).

On the day before the assay, 3D Raft Cultures at the indicated cell numbers were formed using Lonza's RAFT kit (Lonza, #016-0R94) as per manufacturer's instructions using 240 µL of DMEM/collagen solution per well in 96-well plates.

MitoXpress Xtra assay

Note: All media, reagents, and the plate-reader should be prewarmed to 37 °C.

1. Agilent MitoXpress Xtra (part number MX-200-4) reagent was reconstituted in growth medium as described in the MitoXpress Xtra user guide.
2. The plate was placed on a plate-block heater set to 37 °C, and the spent growth medium in each well was gently replaced with 100 µL of growth medium containing MitoXpress Xtra without disturbing the collagen structure.
3. Where applicable, test compound at the indicated concentration or vehicle was added to the wells.
4. Wells were sealed by overlaying them with 100 µL of prewarmed HS oil (included in the kit) using a repeater pipette to limit oxygen back-diffusion into the sample.
5. Dual-read time-resolved fluorescence (TRF) intensity was immediately measured for 90 to 120 minutes on a suitable fluorescence plate reader equipped with suitable filters and TRF detection as described in the assay user guide.

pH-Xtra assay

Note: All media, reagents, and the plate-reader should be prewarmed to 37 °C.

1. Three hours before measurement, the RAFT plate was placed in a CO_2-free incubator at 37 °C to remove CO_2 from the plate material. Respiration buffer and Agilent pH-Xtra (part number PH-200-4) reagent were reconstituted as described in the pH-Xtra user guide.
2. The plate was placed on a plate-block heater set to 37 °C, and the spent growth medium was removed. The wells were washed gently two times with 100 µL of respiration buffer. After the last wash, 100 µL of respiration buffer containing pH-Xtra was added to each well.

3. Where applicable, test compound at the indicated concentration or vehicle was added to the wells.
4. Dual-read time-resolved fluorescence intensity was immediately measured for 90 to 120 minutes on a suitable fluorescence plate reader equipped with suitable filters and TRF detection as described in the assay user guide.

Data reduction and analysis

Data analysis was performed as described in the MitoXpress Xtra and pH-Xtra assay user guides.

Briefly, the data analysis templates for MitoXpress Xtra and pH-Xtra assays for the plate-reader software was used to calculate the fluorescence lifetime for both sensors. For pH-Xtra, further conversion to the analyte scale ($[H^+]$) was performed. Rates were obtained by performing linear regression over the linear part of the kinetic data, also using the data-analysis templates.

Instrument settings and data analysis resources

Suitable plate-readers from many vendors with the capacity to measure time-resolved fluorescence, such as BioTek's Cytation 1/5 or Synergy H1/Neo2, can be used with the MitoXpress Xtra and pH-Xtra assays.

Dedicated instrument protocols for the plate-reader used here and other selected models, with preset optimum measurement settings and predefined data analysis templates, are available from the Agilent cell analysis page or cellanalysis.support@agilent.com.

Results and discussion

Oxygen consumption and extracellular acidification profiles were measured as outlined previously to illustrate the capacity of MitoXpress Xtra and pH-Xtra to assess metabolism and detect perturbations of metabolism in cells within 3D structures. Typical results are shown in Figures 3 and 4.

Figure 3A shows MitoXpress Xtra signal (measured as fluorescence lifetime) changes during a kinetic measurement in RAFT cultures containing different numbers of A549 cells/well. The MitoXpress Xtra signal changed more rapidly with increasing cell density, reflecting the faster depletion of oxygen caused by cellular respiration in wells containing more cells.

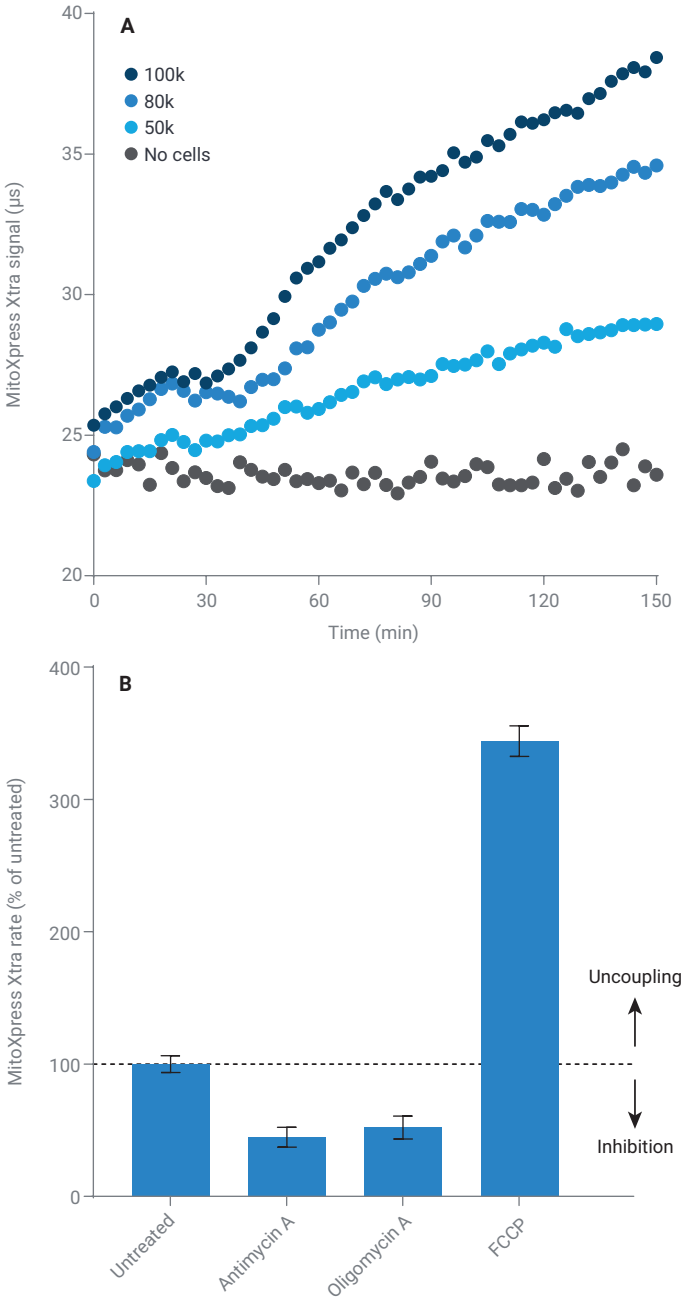


Figure 3. (A) Agilent MitoXpress Xtra assay profiles from A549 3D RAFT cultures of increasing cell density and (B) relative effect of acute drug treatment on cultures plated at 80,000 cells/well.

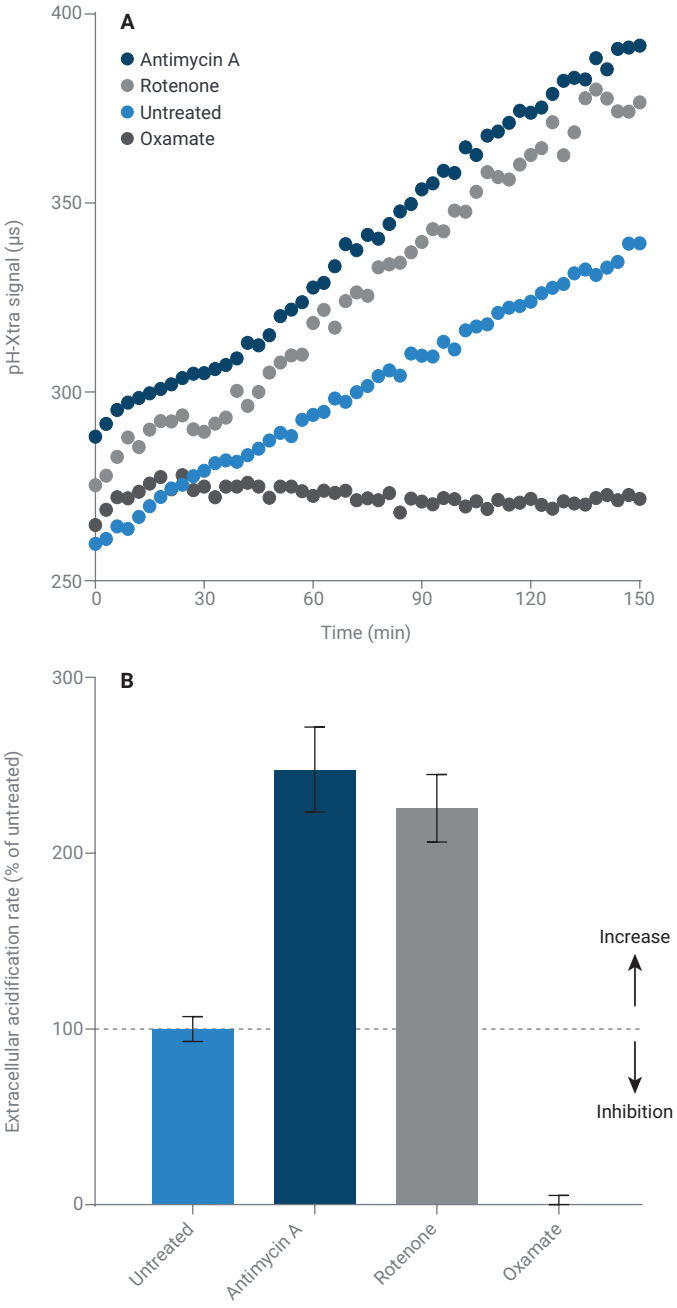


Figure 4. (A) Agilent pH-Xtra glycolysis assay profiles from HepG2 RAFT cultures (80,000 cells/well) acutely treated with drugs. (B) Relative rates of extracellular acidification derived from these traces.

Figure 3B shows rates of MitoXpress Xtra signal change when cells were treated with drugs that acutely impair mitochondrial respiration. These rates are indicative of the relative oxygen consumption in the samples. Upon treatment with inhibitors of mitochondrial ATP generation, such as Oligomycin and Antimycin, oxygen consumption is reduced compared to vehicle-treated cells. When mitochondrial oxygen consumption was uncoupled from ATP generation through treatment with FCCP, oxygen consumption increased significantly, seen as a more rapid rate of signal increase

This is further demonstrated for measurements of extracellular acidification of cells in RAFTs shown in Figure 4.

The change of pH-Xtra signal throughout the experiment (Figure 4A) and the rates of extracellular acidification derived from this data (Figure 4B) were significantly inhibited upon treatment with oxamate, an inhibitor of extracellular acidification caused by glycolysis. In contrast, treatment with Antimycin A and Rotenone both caused an increase in acidification, as the cells increase glycolytic flux to maintain cellular ATP supply upon inhibition of oxidative phosphorylation by these compounds.

Together, these data demonstrate the ability of MitoXpress Xtra and pH-Xtra assays to profile cellular metabolism and detect perturbations of cellular respiration and glycolysis in cells within a 3-dimensional collagen structure.

Conclusion

The mix-and-measure approach of the Agilent MitoXpress Xtra and pH-Xtra assay workflows presented here facilitates the convenient assessment of cellular metabolism in intact RAFT 3D collagen cultures using a fluorescent plate-reader. The data presented are representative examples demonstrating the use of these workflows to profile cellular metabolism in cancer cell lines by measuring oxygen consumption and extracellular acidification. Moreover, treatment with typical small molecule modulators of mitochondrial ATP generation and glycolytic flux further reveal the utility of these assays to examine drug-induced effects. The described workflows are therefore shown to combine the convenience and information content of plate-reader-based metabolism assays with the biological relevance of 3D culture, thereby providing valuable insights into cellular function within such 3D collagen constructs.

Additional resources

MitoXpress Xtra Assay user guide

https://www.agilent.com/en/products/cell-analysis/mitoxpress-ph-xtra-consumables/mitoxpress-xtra-oxygen-consumption-assay#support

pH-Xtra user guide

https://www.agilent.com/en/products/cell-analysis/mitoxpress-ph-xtra-consumables/ph-xtra-glycolysis-assay#support

MitoXpress Xtra Quick Start Guide - Five Step Workflow

https://www.agilent.com/cs/library/usermanuals/public/user-guide-mitoxpress-xtra-quick-start-guide-cell-analysis-5994-1822en-agilent.pdf

Downloadable instrument settings and data analysis templates for selected plate-readers are available from:

https://www.agilent.com/en/products/cell-analysis/plate-reader-compatible-assays

The Agilent MitoXpress Xtra and pH-Xtra data visualization tool is available from:

https://www.agilent.com/en/products/cell-analysis/mitoxpress-ph-xtra-consumables/mitoxpress-xtra-oxygen-consumption-assay#support

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DE.2785300926

Printed in the USA, August 12, 2020
5994-2195EN

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