Application Note

Cell Metabolism

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Trusted Answers

Optimization of a Multimode Detection Model for Measuring Real-Time Cellular Respiration and Mitochondrial Function

Using fluorescent biosensors

Author

Wendy Goodrich

Agilent Technologies, Inc.

Abstract

Characterization of cellular metabolism is aided by tools designed to provide ease-of-use, higher throughput, and multiplexed data markers for analysis. One of these tools is a group of simple mix and measure assays compatible with a variety of cellular matrices. These assays use soluble, fluorescent nanosensor probes to measure oxygen (O_2) consumption rates (OCR), extracellular acidification rates (ECAR), and intracellular O_2 concentration (%O_2) that are useful to inform on cellular oxygenation and the activity of the electron transport chain (ETC) and glycolytic flux. These probes can be detected using relative fluorescence, standard time-resolved fluorescence, or advanced lifetime (µs) time-resolved fluorescence with reduced background and increased signal dynamic range dependent on the detection mode. Optimization of biosensor recognition was done in microplate format using multiple cell lines and drug compound treatments. In particular, the advanced lifetime (µs) time-resolved fluorescence mode (TRF) is highlighted for generating drug compound dose response against OCR (µs/t); presenting accurate comparisons of acidification rates converted to hydrogen ion scale (ECA H^+]/t); and, converting lifetime detection to intracellular O_2 concentration (%O_2) in live-cell 2D monolayers.

Introduction

The Agilent MitoXpress Xtra oxygen consumption assay, the Agilent pH-Xtra glycolysis assay, and the Agilent MitoXpress Intra intracellular oxygen assay are a family of fluorescent probes designed to aid in the study of real-time analysis of cellular metabolism in a variety of biological matrices. The probes are chemically stable and inert, water soluble, and can be multiplexed. The amount of fluorescence signal has an inverse relationship to intracellular or extracellular O_2 or proportional to extracellular H^+ in the sample. Intracellular O_2 levels (%O_2), extracellular O_2 consumption rate (OCR), and quantification of H^+ levels and acidification rates (ECAR) are calculated from the changes in lifetime signal over time. Optimal instrument settings to detect probe signal can be achieved using a validation procedure in the assay user manuals. These detection parameters can then be applied to verification of cell lines, cell seeding densities, and general cell-based experiments using compound controls. Independent testing should be conducted to optimize each cell line individually.

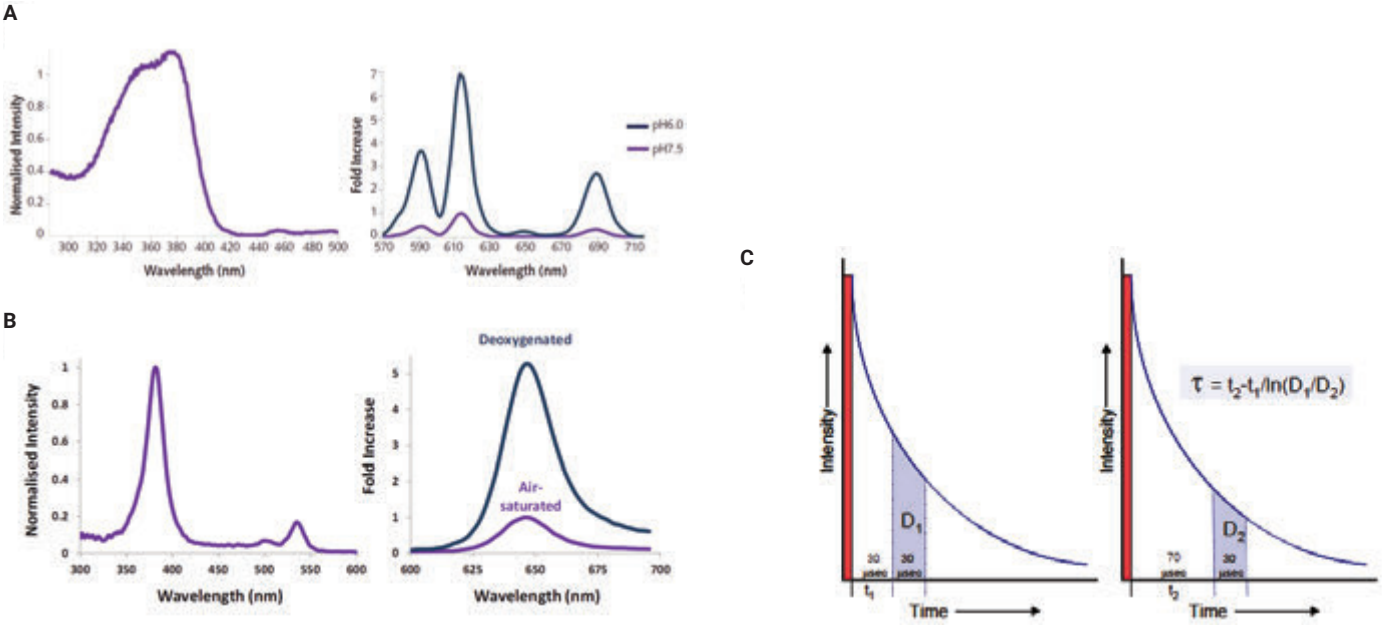


Figure 1. Excitation and emission spectra of the Agilent pH-Xtra glycolysis assay demonstrating normalized excitation (A, left) and 3- to 6-fold increase in emission peak signal in response to increased acidification (A, right). A 6-fold delta at 620 nm provides an optimal window for detecting signal change in response to pH levels. (B, left). The Agilent MitoXpress Xtra oxygen consumption assay and the Agilent MitoXpress Intra intracellular oxygen assay probes show emission peaks at a normalized intensity of 380 nm. The probe is quenched by oxygen, and thus there is an inverse relationship between the signal and O_2 levels. This is seen by a 4-fold increase of signal in deoxygenated conditions at emission peak 645 nm (B, right) compared to ambient. (C) The dual time-resolved fluorescent lifetime detection mode that uses two reads at different times over the decay of the probe increases stability and dynamic range in signal acquisition. These 'dual reads' are used in the equation shown to calculate a lifetime signal (µs, represented as τ).

Materials and methods

Agilent BioTek instrumentation

Agilent BioTek Cytation 1 cell imaging multimode reader:
Shown with optional dispenser and CO_2/O_2 gas control module, Cytation 1 combines automated digital microscopy and conventional multimode detection in one instrument. This unique design, provides both quantitative phenotypic cellular information with well-based quantitative data. Temperature control and shaking are standard.

Agilent BioTek Cytation 5 cell imaging multimode reader:
Cytation 5 combines automated digital widefield microscopy with conventional multimode microplate reading in a unique design. The imaging components and multimode optical modules are independent and physically separate within the instrument, ensuring optimized performance. CO_2/O_2 control, shaking, and temperature control. Reagent injectors are available to support live-cell assays.

Agilent BioTek Synergy H1 multimode reader:
Synergy H1 is a configurable multimode microplate reader equipped with monochromator-based optics for flexibility, filter-based optics for sensitivity, or both. The Agilent BioTek Hybrid Technology offers a modular platform to expand as your laboratory's needs change. Top and bottom fluorescence intensity, UV-visible absorbance, and luminescence detection are available. CO_2/O_2 control, dispensers, and temperature control are available.

Agilent BioTek Synergy Neo2 hybrid multimode reader:
Synergy Neo2 multimode microplate reader with hybrid technology offers filter- and monochromator-based optics including multiple PMTs ensuring ultrafast, high performance in all detection modes. Live-cell assays are fully supported with CO_2/O_2 control, incubation to 65 °C, dispensers, and direct bottom illumination.

Resources are available for running the assays on the instrumentation described, including preconfigured Agilent BioTek Gen5 software protocols, custom filter cubes, and user tutorials that provide detail on the software parameters.

Materials

- Agilent MitoXpress Xtra Oxygen Consumption Assay part number MX-200-4
- Agilent MitoXpress Intra Intracellular Oxygen Assay part number MX-300-4
- Agilent pH-Xtra Glycolysis Assay part number PH-200-4
- Corning COSTAR 96-well microplate (#3903)
- Glucose oxidase (GOx) powder (Sigma part number 49180) reconstituted in sterile water
- Respiration buffer (1 M glucose+DMEM media to final 40 mM glucose concentration)
- Phosphate buffered saline (PBS) (Sigma P4417) at pH 7.25 and 6.2 adjusted w/ 0.1 mM NaOH or HCl
- Respiration media (DMEM (Sigma D5030, powder), 20 mM HEPES, 1 mM sodium pyruvate, 20 mM glucose, 10% FBS, 10% Pen-Strep)
- DMEM culture media (depending on cell type) + additives (10% FBS, 10% P/S)
- DMEM culture media + additives + 20 mM glucose (wet testing media)
- HEK293 cells stably transfected with antibiotic resistant marker (proprietary)
- HepG2 cells grown and cultured from stock
- Rotenone (2.5 µM final) vehicle DMSO
- FCCP (Cayman Chemical Item No. 15218) in vehicle DMSO
- Antimycin A (Cayman Chemical Item No. 19433) in vehicle DMSO
- Phenformin (50 µM final) in vehicle sterile water

Methods

All methods used prewarmed plates, media, and buffers. High sensitivity oil used for the MitoXpress Xtra probe is prewarmed in a bead bath set at 37 °C. Probes are reconstituted in 1 mL sterile water and assayed at RT. Compounds are kept at –20 °C and brought to RT.

Signal and assay optimization:

The MitoXpress Xtra and MitoXpress Intra signal and assay optimization was run as defined in the user manual with reagent volumes and well locations shown in Table 1. The oil overlay is run for the MitoXpress Xtra probe only. The procedure was used to optimize gain for the probe signal in addition to validate lifetime read parameters, and kinetic time intervals for both full and partial plate reads.

Table 1. Map and reagent volumes for performing signal optimization of the Agilent probes on Agilent BioTek instrumentation. Signal optimization to validate detection gain and probe read height parameters is recommended before implementing live-cell analysis.

Map and Reagent Volumes						
MitoXpress Xtra, MitoXpress Intra	1	2	3	4	5	6
A	140 µL DMEM + 10 µL probe + (100 µL HS Oil) [21% O_2]			140 µL DMEM + 10 µL probe [21% O_2]		
B	130 µL DMEM + 10 µL probe + 10 µL GOx + (100 µL HS Oil) [0% O_2]			130 µL DMEM + 10 µL probe + 10 µL GOx [0% O_2]		
C	150 µL DMEM + (100 µL HS Oil)			150 µL DMEM		
	High Sensitivity (HS) Oil for MitoXpress Xtra probe only			No Oil		
pH-XTRA	1	2	3	4	5	6
A	140 µL PBS (pH 7.25) + 10 µL probe			140 µL Resp buffer + 10 µL probe		
B	140 µL PBS (pH 7.25) + 10 µL probe			130 µL Resp buffer + 10 µL probe +10 µL GOx 1		
C	140 µL PBS (pH 6.2) + 10 µL probe			130 µL Resp buffer + 10 µL probe +10 µL GOx 2		
D	140 µL PBS (pH 6.2) + 10 µL probe			130 µL Resp buffer + 10 µL probe +10 µL GOx 3		
E	150 µL PBS (pH 7.25) only			130 µL Resp buffer + 10 µL probe +10 µL GOx 4		
F				150 µL Resp buffer only		

Results and discussion

Results are shown using a Synergy H1 in standard TRF mode (Figure 2), and lifetime mode (Figure 3). Parameters for standard TRF mode included a 45 minute kinetic read with a 50 second interval between reads, TRF settings of 30 µs delay and 100 µs integration, and an auto gain on a high well with a scale of 60,000. The high well was a GOx control without cells diluted in cell media containing probe.

Results in Figure 4 are shown for the Synergy Neo2 using lifetime (µs) detection mode and parameters of 10 measurements/well at a gain value of 100 using an Agilent BioTek filter cube (part number 1035123). Signal-to-background calculations for the top and bottom optics position on the Synergy Neo2 using the MitoXpress Xtra probe were compared by dispensing 12 replicates of 10 µL of probe in 140 µL of 20 mM glucose media to wells A1 through A12 (PC), and 12 replicates of 140 µL of 20 mM glucose media to wells C1 through C12 (BLK) followed by a dispense of 100 µL of high sensitivity oil to all wells.

pH-Xtra: Signal optimization was performed using the volumes and reagents shown in Table 1 with an auto gain setting on a high well scaled at 60,000 relative units.

pH Scale to H^+ conversion

pH-Xtra: Using the volumes and reagents shown in Table 1, a 45-minute kinetic read using the fastest interval time on a Synergy H1 was done at 30 °C using lifetime parameters of a 100_30 and 300_30 delay and integration times respectively. Data are shown in Figure 5.

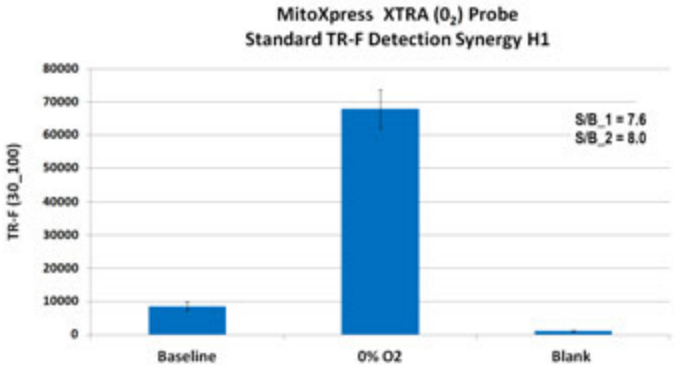


Figure 2. Assay window results in standard TRF mode. S/B_1 calculation is done for 0% O_2/19% O_2 and S/B_2 for 19% O_2/Blank (no probe). Chemically induced 0% O_2 is achieved using glucose oxidase (GOx).

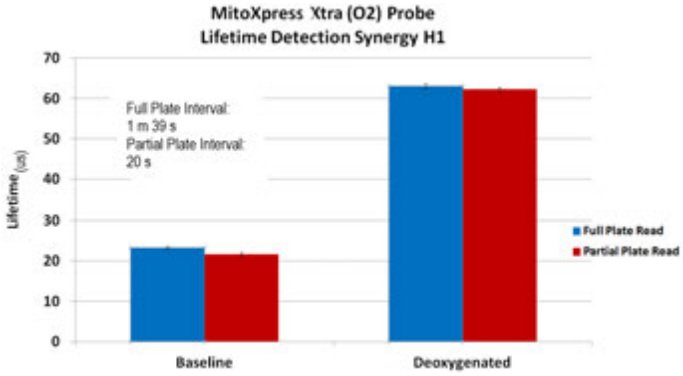
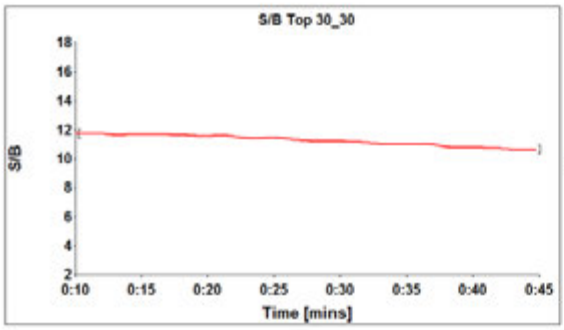
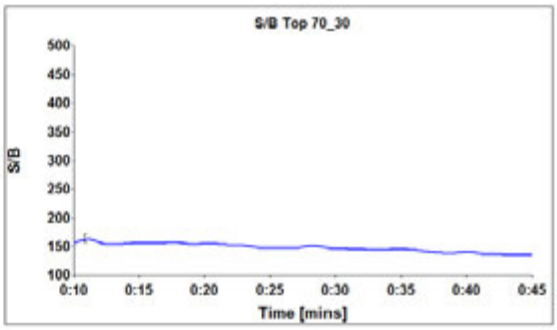


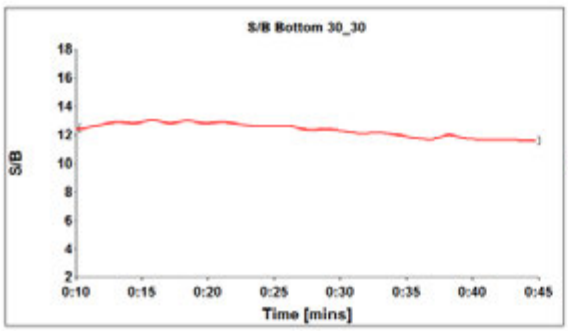
Figure 3. Comparison of lifetime signal stability for partial and full plate reads for Agilent MitoXpress Xtra. The full plate kinetic interval of 1 minute 39 seconds resulted in a 0.0274/sec change in assay window, compared with 0.14385/sec in partial plate mode using a 20 second kinetic interval. Lower interval times result in more data points, but higher interval times result in more stable assay windows. Although these data support using either kinetic interval time, it is recommended that a kinetic interval that can accommodate a full plate, in this example a minimum of 1 minute 39 seconds, be used for all analyses regardless of how many wells are read.





S/B R1 30_30	Mean	Std Dev	CV
Top	11.27	0.38	3.35
Bottom	12.36	0.49	3.98
Δ	1.09	0.11	0.63

S/B R2 70_30	Mean	Std Dev	CV
Top	148.07	7.76	5.24
Bottom	216.93	18.30	8.44
Δ	68.86	10.54	3.20



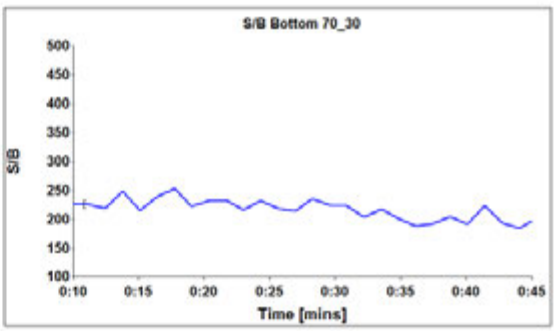
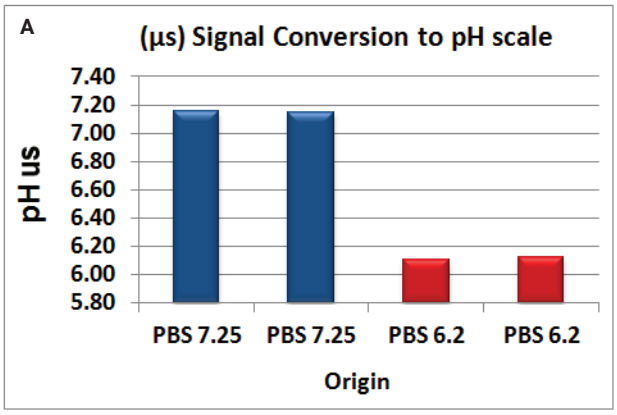
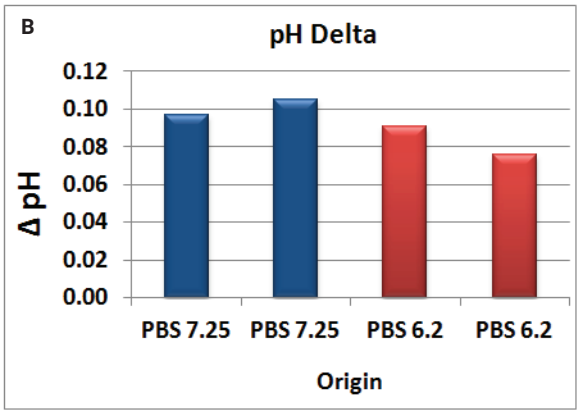
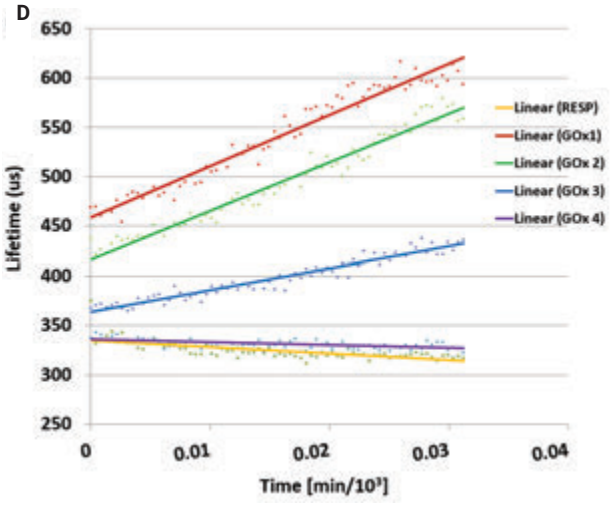


Figure 4. Comparison of top and bottom optics position on an Agilent BioTek Synergy Neo2 (n = 12) for each of the dual lifetime TRF reads of the MitoXpress Xtra probe. The extracellular assay requires an oil overlay. Results support that either optics position will produce similar signal response differentiation. As shown, the top optics position may result in less variability, and the bottom optics position in a wider assay window. Signal window can be further optimized by increasing measurements per well (mw) or adjusting the gain and probe read heights.





C		pH Calibration (1687.2 - µs) / 199.12		10^{-pH}	min = total kinetic runtime	Expected - Actual
45 min kinetic		Lifetime (µs)	pH scale	$[H^+]$	ECAR $[H^+/min]$	Δ pH
	manual	*214.07*	*7.40*	*4.00E-08*		
Full Plate Read	PBS 7.25	262.90	7.15	7.03E-08	2.503E-09	*0.10*
	PBS 7.25	264.47	7.15	7.16E-08	2.578E-09	*0.10*
	PBS 6.2	470.74	6.11	7.78E-07	2.548E-08	*0.09*
	PBS 6.2	467.73	6.12	7.51E-07	2.542E-08	*0.08*
Partial Plate Read	PBS 7.25	261.73	7.16	6.94E-08	1.639E-09	*0.09*
	PBS 7.25	265.03	7.14	7.21E-08	1.688E-09	*0.11*
	PBS 6.2	466.73	6.13	7.42E-07	1.759E-08	*0.07*
	PBS 6.2	467.79	6.12	7.52E-07	1.745E-08	*0.08*



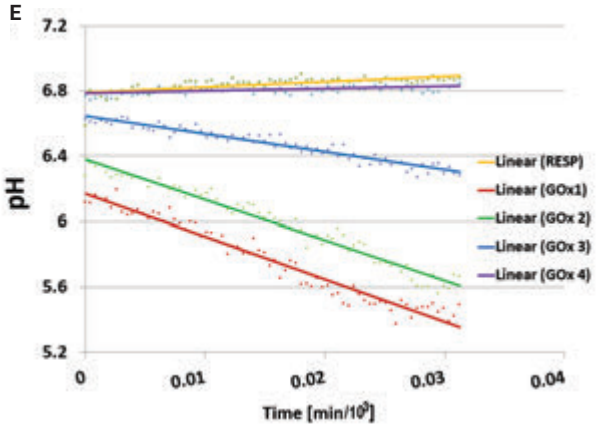


Figure 5. (ABC) Lifetime signal reproducibility between full and partial plate reads for pH-Xtra probe on the Agilent BioTek Synergy H1 at 30 °C over a 45-minute kinetic read using intervals of 1 minute and 39 seconds (full) and 38 seconds (partial). Using a default conversion function, pH is calibrated from lifetime values and H^+ is quantified from pH. Extracellular acidification rate (ECAR) is calculated from H^+ on average pH over the 45-minute kinetic read. Kinetic interval time difference has little effect on results. It is recommended that a kinetic interval that can accommodate a full plate, in this example a minimum of 1 minute 39 seconds, be used for all analyses regardless of how many wells are read. ΔpH between expected and actual pH values are all <1.6% of the origin although slightly higher than within error range (±1.5%). Delta pH can be improved by performing instrument specific pH calibration to adjust calculation variables. Glucose Oxidase (GOx) is a potent oxidoreductase enzyme and is used as a control for achieving maximal signal response from the Agilent probes. Lifetime profiles of GOx serial diluted 1:10 in respiration buffer (D) converted to pH scale (E) demonstrates the analogous relationship between probe signal change and acidification rate, where the highest glucose oxidase concentration (GOx 1) yields the greatest signal and acidification increase (red profiles GOx1).

Cellular metabolic analysis

MitoXpress Xtra: HEK293 and HepG2 cells were cultured in DMEM supplemented with 10% FBS and 1% pen/strep/glucose. Cells were trypsinized at 75% confluence and diluted with culture media to cell densities shown in corresponding Figures 6, 7, and 8. Cells were dispensed at 200 µL/well to 96-well microplates. The plates were incubated overnight at 37 °C, 5% CO_2. The following day the media was aspirated, 150 µL of prewarmed probe in media stock was dispensed to each well, and the plates were placed on a plate heater that was prewarmed to 30 °C. One microliter of the following compound treatments were added to respective wells: 150x FCCP (two-fold serial dilutions 0 to 20 µM final); 150x antimycin (two-fold serial dilutions 0 to 1 µM final); 150x rotenone (1 µM final); and phenformin (50 µM final). Blanks (150 µL media), Signal control (15x probe in media), and PC (10 µL 15x probe, 10 µL 15x GOx (solubilized 1 mg/mL in 1 mL sterile water) to 130 µL media per well) were run on all plates. One hundred microliters of prewarmed high sensitivity oil available in the assay kit was then dispensed to each well. Plates were read without lids.

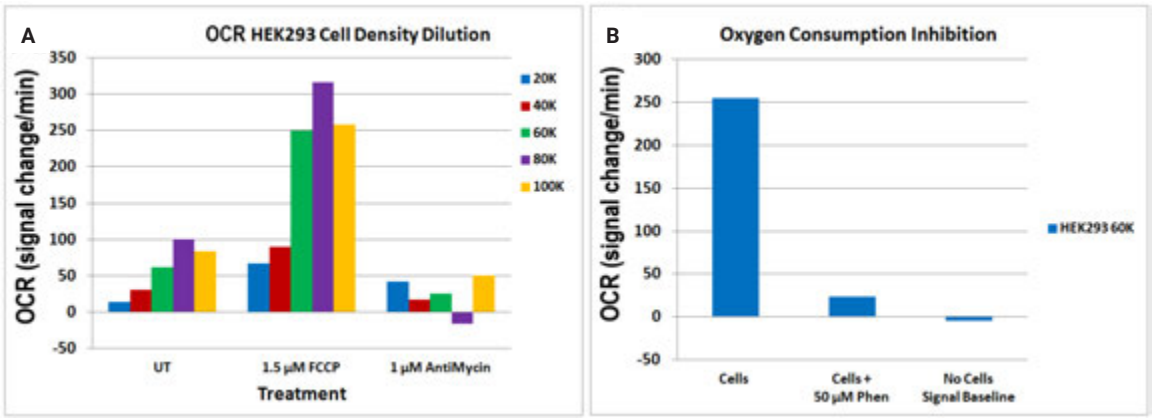


Figure 6. (A) Cell density and inhibition optimization of HEK293 cells using MitoXpress Xtra probe and oxygen consumption rate (OCR = MeanV RFU/min). Cell growth rates should be considered and densities optimized for each cell line. HEK293 cells becomes nonlinear at 100 K cells/well in basal and FCCP treated cells. 1 µM of antimycin inhibits oxygen rates at all densities. Densities of 6×10^4 to 8×10^4 are optimal for this cell line. (B) HEK293 cells seeded at 6×10^4/well and treated with a mitochondrial respiratory complex I inhibitor phenformin (50 µM) results in a 10 fold decrease in oxygen consumption rate compared to untreated cells.

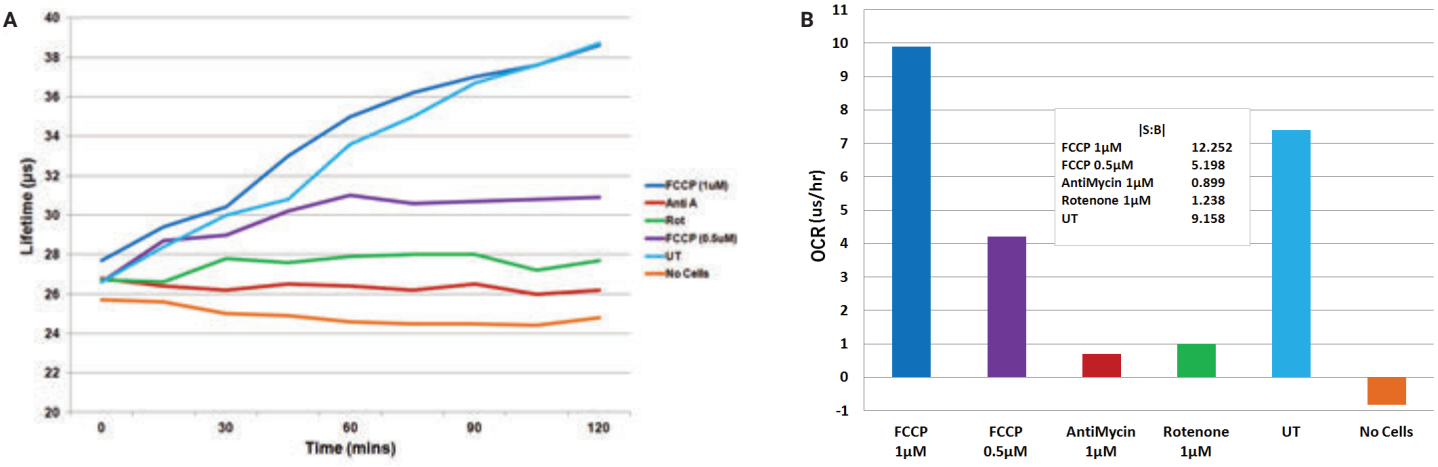


Figure 7. (A) Kinetic lifetime detection of extracellular oxygen consumption of HepG2 cells treated with uncoupling and inhibitor compounds at an interval of 2 minutes 19 seconds. FCCP is a mitochondrial oxidative phosphorylation uncoupler that drives cellular respiration to a maximal rate increasing the depletion of oxygen. Antimycin blocks cellular respiration via inhibition of complex III of the mitochondrial electron transport chain (ETC) resulting in low to no reduction of oxygen. Slope on lifetime was calculated from the linear portion (10 to 80 minutes) of the 2-hour kinetic run for the OCR calculation. (B) OCR profiling of the uncoupling and inhibition agents in comparison to untreated cells.

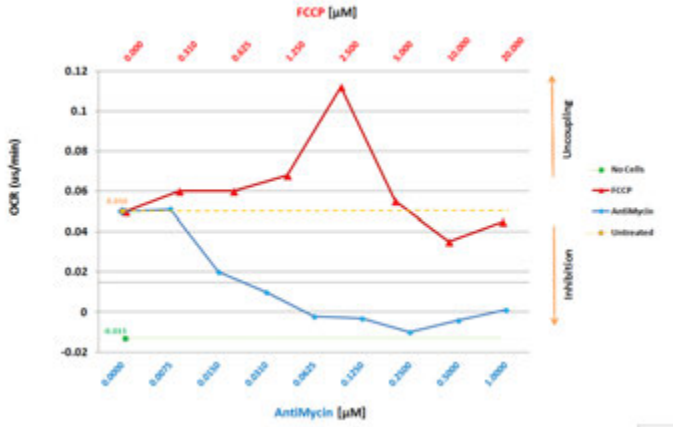


Figure 8. Dose-dependent oxygen consumption rate (OCR) in HepG2 cells (70K/well) as calculated from Agilent BioTek Synergy Neo2 lifetime measurement from the linear portion (9:16 to 130 minutes) of a 3-hour kinetic run. Antimycin inhibits oxygen consumption of HepG2 cells in a dose dependent manner. (Top) FCCP stimulation of maximal respiration is indicated at 2.5 µM falling to below basal levels at higher concentrations indicating probable toxicity.

MitoXpress Intra: HepG2 cells were cultured in DMEM supplemented with 10% FBS, and 1% pen/strep/glucose. Cells were trypsinized at 75% confluence and seeded at 7×10^4 cells/well in 200 µL of culture media then incubated ON at 37 °C, 5% CO_2. On Day 2, probe was reconstituted 1:11 in culture media. Spent cell media was aspirated and replaced with 100 µL/well of probe in media stock, then incubated ON at 37 °C, 5% CO_2. On Day 3, spent media was aspirated and cells were washed twice with Respiration media. After the final aspiration, 150 µL of fresh respiration media was added to the wells. Controls were run on each plate as described for the MitoXpress Xtra probe, with the exception that no oil is added to the plate. One microliter of test compounds were then added. A kinetic read was commenced at 37 °C on a Cytation 5. Plates were read without lids. Data are shown in Figure 9.

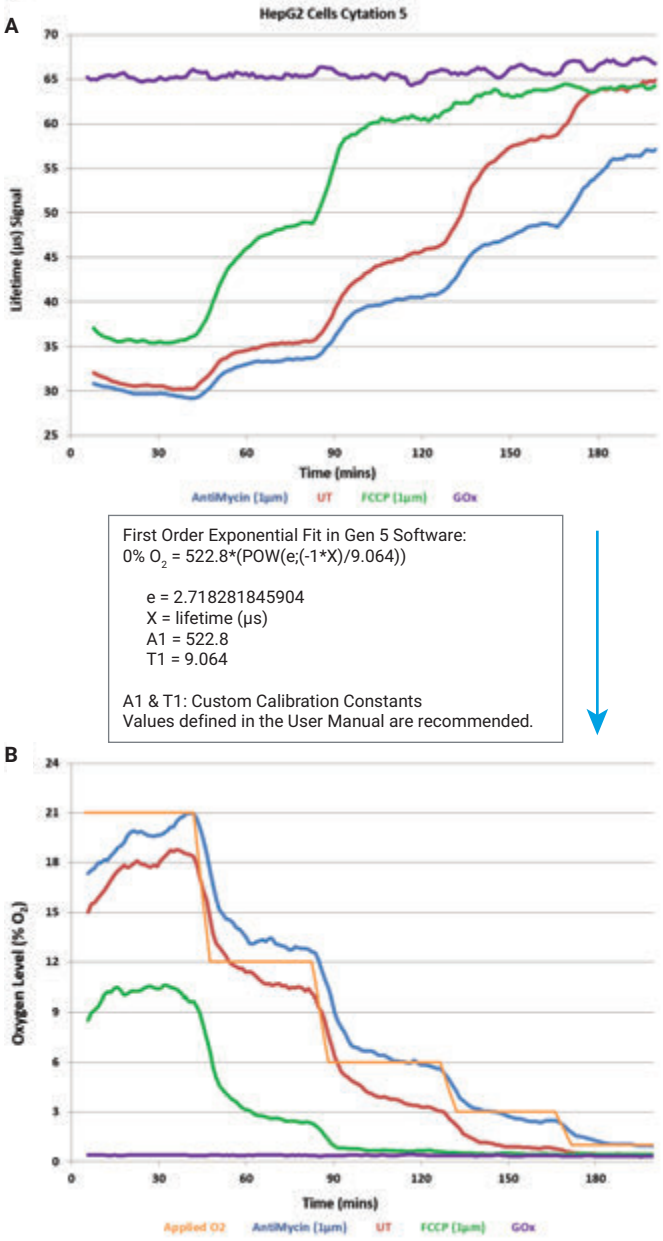


Figure 9. (A) Lifetime detection of intracellular oxygenation of HepG2 cells in response to decreasing levels of applied O_2 using a gas controller peripheral with Agilent BioTek Cytation 5 over a 3-hour kinetic time course. Cells were plated at 7×10^4 cells/well and read on Day 3. Oxygen levels were decreased at 40 to 50-minute intervals. (B) Lifetime conversion to Intracellular O_2 (%O_2) is done using a first order exponential fit within Agilent BioTek Gen5 software. A set of customized calibration constants is used for the A1 and T1 values.

Conclusion

A variety of Agilent BioTek readers are compatible with the Agilent MitoXpress Xtra Oxygen Consumption Assay, MitoXpress Intra Intracellular Oxygen Assay, and MitoXpress pH Glycolysis assay. They read in either standard TRF or lifetime mode, enabling diverse cellular metabolic studies. Signal acquisition of the Agilent probes using the Agilent BioTek lifetime detection algorithm is stable for kinetic interval times from 20 seconds to 2:30 minutes, although it is recommended to run all assays using the same interval time. Detecting the pH probe in lifetime mode allows direct conversion of signal to pH scale and H^+ quantification for ECAR analysis using a default conversion function. Agilent BioTek readers and Agilent probes are compatible for analyzing extracellular O_2 consumption rates and quantifying intracellular O_2 levels in treated and untreated whole cells using the dual-read TRF measurement mode and subsequent conversion to lifetime values. Intracellular O_2 concentration ($\%O_2$) can be directly calculated using a first order exponential fit in Agilent BioTek Gen5 software.

References

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