Cellular Energy Flux in Real Time: Optimization of a multi-mode detection model for measuring real-time cellular respiration and mitochondrial function using fluorophoric biosensors

Cytation™ 3 Imaging Reader | Mito-ID® Metabolism & Mitochondrial Function Assays

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Mito-ID® Extracellular O_2 Sensor Kit (High Sensitivity) (ENZ-51045)

Mito-ID® Extracellular pH Sensor Probe (ENZ-51048)

Mito-ID® Intracellular O_2 Sensor Probe (ENZ-51046)

INTRODUCTION

Characterization of cellular metabolism is being aided by the development of new tools designed to provide ease-of-use, higher throughput, and multiplexed data markers for analysis. One of these tools is a simple mix and measure assay compatible with a variety of cellular matrices that utilizes fluorophoric probes to measure oxygen consumption rates (OCR), extracellular acidification (ECA), and intracellular oxygen levels useful to inform on the activity of the electron transport chain (ETC) and glycolytic flux. These probes can be detected using standard fluorescence, time-resolved fluorescence, or lifetime fluorescence with reduced background and increased signal dynamic range dependent on the detection mode. Optimization of biosensor recognition in all three fluorescent modes was done in microplate format using multiple cell lines and drug compound treatments. In particular, the lifetime time-resolved fluorescent mode is highlighted for generating drug compound dose response against OCR (µs/hr), presenting accurate comparisons of acidification rates converted to hydrogen ion scale (ECA[H+]/t), and detecting intracellular oxygen levels in parallel with fluorescent imaging in live cell 2D monolayers.

ASSAY OVERVIEW AND DETECTION PRINCIPLE

The Mito-ID® Extracellular O_2 Sensor Kit (High Sensitivity), Mito-ID® Extracellular pH Sensor Probe, and Mito-ID® Intracellular O_2 Sensor Probe are a family of fluorescent probes designed to aid in the study of real-time analysis of mitochondrial function, metabolism and toxicity in a variety of biological matrices. The probes are chemically stable and inert, water-soluble, and can be multiplexed. The amount of fluorescent signal is an inverse relationship to intra- or extracellular O_2 or proportional to extracellular H^+ in the sample. O_2 levels, OCR, and quantification of H^+ levels are calculated from the changes in fluorescence signal over time.

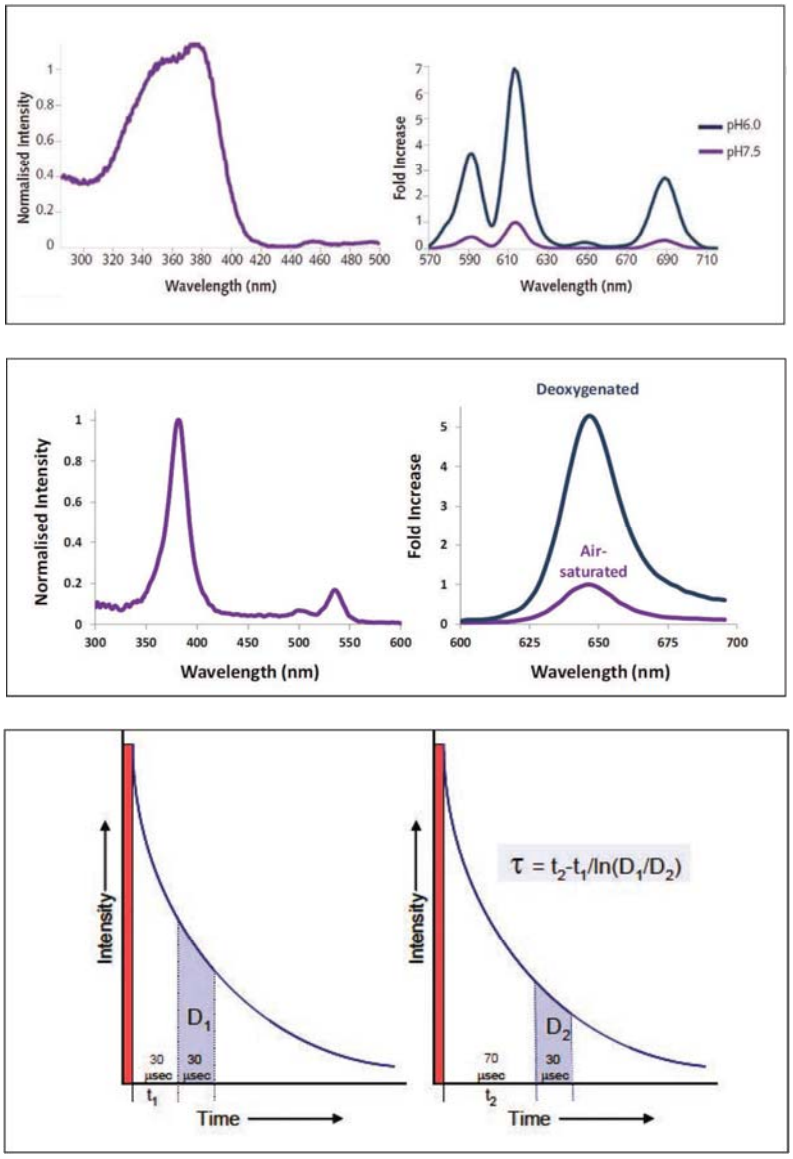


Figure 1. Excitation and Emission spectra of the Mito-ID® Extracellular pH Sensor Probe demonstrating normalized excitation (top left) and 3- to 6-fold increase in emission peak signals in response to increased acidification (top right). A 6-fold delta at 620nm provides an optimal window for detecting signal change in response to pH levels. The Mito-ID® Extracellular and Intracellular O_2 probe show emission peaks at a normalized intensity of 380nm (center left). The inverse relationship between probe signal and O_2 levels is seen by a 4-fold increase of signal in deoxygenated conditions at emission peak 645nm (center right). Principle of an optional dual time-resolved fluorescent lifetime (τ) detection mode that utilizes two reads at different times over the decay of the probe to increase stability and dynamic range in signal acquisition (bottom).

BIOTEK® INSTRUMENTATION

Cytation 3™ Cell Imaging Multi-Mode Reader with Gas Control Module combines automated digital microscopy and conventional microplate reading in one instrument. Its unique patent pending design is ideal for research and assay development applications in the field of cell biology. It was used in both imaging and filter based detection mode as shown by **Table 1** at 37°C using the O_2 gas control module.

Synergy™ H1 Hybrid Reader is a flexible monochromator-based multi-mode microplate reader that can be turned into a high-performance Hybrid System with the addition of a filter-based optical module. The filter module is a completely independent add-on that includes its own light source, and a high performance dichroic-based wavelength selection system that was used as shown by **Table 1**.

Synergy™ Neo Hybrid Reader is a patented HTS multi-mode microplate reader with multiple parallel detectors for ultra-fast measurements and a dedicated filter-based optical system for live cell assays. **Table 1** contains the optical parameters used for signal optimization and validation of Enzo probes for live cell metabolic analysis.

Synergy™ 2 Multi-Mode Reader offers performance, speed and sensitivity. Based on BioTek's popular Synergy™ HT platform, Synergy 2 has been further enhanced with improved sensitivity in Fluorescence Intensity by utilizing a dedicated optical element. **Table 1** contains the detection configuration used for validation of Enzo probes in standard RFU mode.

Reader, Probe	Detection Mode	Optics Position	Ex/Em, Mirror, LED, Objectives	TR-F Options (Delay_Integration)	Gain, Exposure
Cytation 3					
Mito-ID® Intracellular O_2	Imaging (RFU)		377:647, 1225000, 4X, 20X		10, 127, 15.6
	Lifetime (τ)	Top	380/20:645/15 400nm	τ R1 30_30: R2 70_30	Auto, Low well @ 10000
Synergy H1					
Mito-ID® Extracellular O_2	All	Top	380/20:645/15 (400nm)	τ R1 30_30: R2 70_30 TR-F 30_100	Auto, High well @ 60000
Mito-ID® Extracellular pH	All	Top	360/40: 620/10 and 380/20:620/10 (400nm)	τ R1 100_30: R2 300_30 TR-F 100_100	
Synergy Neo					
Mito-ID® Intracellular O_2	Lifetime (τ)	Top, 50mw	380/20:645/15 400nm	R1 30_30 R2 70_30	Auto, Low well @ 10000
Synergy 2					
Mito-ID® Extracellular O_2	RFU	Bottom	360/40:645/15		Auto, High well @ 60000

Table 1. Detection settings for Enzo probes on BioTek readers for results shown.

MATERIALS AND METHODS

Materials

- Mito-ID® Extracellular O_2 Sensor Kit (High Sensitivity) (ENZ-51045)
- Mito-ID® Intracellular O_2 Sensor Probe (ENZ-51046)
- Mito-ID® Extracellular pH Sensor Probe(ENZ-51048)
- Glucose Oxidase (GOX) powder reconstituted in sterile water
- Respiration Buffer (1M Glucose+DMEM media to final 40mM glucose concentration)
- Phosphate Buffered Saline at pH 7.25 and 6.2 adjusted w/ 0.1mM NaOH or HCl
- Respiration Media (DMEM, 20mM HEPES, 1mM Sodium Pyruvate, 20 mM Glucose, 10% FBS, 10% Pen-Strep)
- DMEM culture media + additives and 20mM glucose, depending on cell type(s)
- Corning COSTAR 96-well microplate (#3904)
- Nunc™ MicroWell™ 96-Well Optical-Bottom Plates
- HEK293 cells stably transfected with antibiotic resistant marker (proprietary)
- HepG2 cells grown and cultured from stock
- Rotenone (2.5nM final) vehicle sterile water
- FCCP in vehicle DMSO • Antimycin A in vehicle DMSO
- Phenformin (50µM final) in vehicle sterile water

Mito-ID Extracellular O_2, Mito-ID Intracellular O_2	1	2	3	4	5	6
A	140µL DMEM + 10µL Probe + (100µL HS Oil) [21% O_2]			140µL DMEM + 10µL Probe [21% O_2]		
B	130µL DMEM + 10µL Probe + 10µL GOX + (100µL HS Oil) [0% O_2]			130µL DMEM + 10µL Probe + 10µL GOX [0% O_2]		
C	150µL DMEM + (100µL HS Oil)			150µL DMEM		
	High Sensitivity (HS) Oil for probe only			No Oil		

Mito-ID® Extracellular pH	1	2	3	4	5	6
A	140µL PBS (pH 7.25) + 10µL Probe			140µL Resp Buffer + 10µL Probe		
B	140µL PBS (pH 7.25) + 10µL Probe			130µL Resp Bufr + 10µL Probe + 10µL GOX 1		
C	140µL PBS (pH 6.2) + 10µL Probe			130µL Resp Bufr + 10µL Probe + 10µL GOX 2		
D	140µL PBS (pH 6.2) + 10µL Probe			130µL Resp Bufr + 10µL Probe + 10µL GOX 3		
E	150µL PBS (pH 7.25) only			130µL Resp Bufr + 10µL Probe + 10µL GOX 4		
F				150µL Resp Bufr only		

Figure 2. 96-well Plate Map and reagent volumes for wet test signal optimization of Enzo fluorescent probes on BioTek Instrumentation

Method

All methods used pre-warmed plates, media, and buffers. Probes are reconstituted in 1mL sterile water and assayed at RT. Compounds are kept at -20°C and brought to RT. Partial plate analysis was done by only reading assay wells of the 96-well plate (**Figure 2**). Full plate analysis was done by reading all wells of the 96-well plate.

Signal Optimization, H+ Quantitation, Cellular Metabolic Analysis

Mito-ID® Extracellular O_2 Sensor Kit (High Sensitivity): For signal optimization - assay in volumes and locations shown in **Figure 2** (top). Commence 45 minute kinetic read (fastest interval) at 30°C using detection mode and parameters defined by **Table 1**. for Synergy H1, Synergy 2, and Cytation 3. **Figures 3 and 4** show data in kinetic average. For cellular metabolic analysis – assay following the kit insert procedure using compound treatments of Antimycin (1µM final, or as a 1:2 dilution from a start concentration of 1µM); Rotenone (1µM final); Phenformin (50µM final); and FCCP (1µM final, or as a 1:2 dilution from a start concentration of 20µM) on Day 2 of the protocol. Blanks (150µL media only), Signal Control (15X probe in media), and PC (10µL15X probe, 10µL 15X GOX (solubilized 1mg/mL in 1mL sterile water) to 130µL media per well) were run on each plate. Data shown by **Figures 6 and 8**.

Mito-ID® Extracellular pH Sensor Probe: For signal optimization and pH scale/H+ conversion - assay in volumes and locations shown in **Figure 2** (bottom). Commence 90 minute kinetic read (fastest interval) on H1 at 30°C in full and partial plate read modes using lifetime detection parameters defined by **Table 1**. Data shown by **Figure 5**.

Mito-ID® Intracellular O_2 Sensor Probe: For cellular metabolic analysis - cells were plated in 200µL culture media and 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 100µL/well of Mito-ID® Intracellular O_2 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 150µL of fresh Respiration media was added to the wells. Controls were run on each plate as described for the Xtra probe. 1µL of test compounds were then added. Commence kinetic read at 37°C using either Neo or Cytation 3 with parameters shown by **Table 1**.

Data shown by **Figures 7 and 9**.

RESULTS

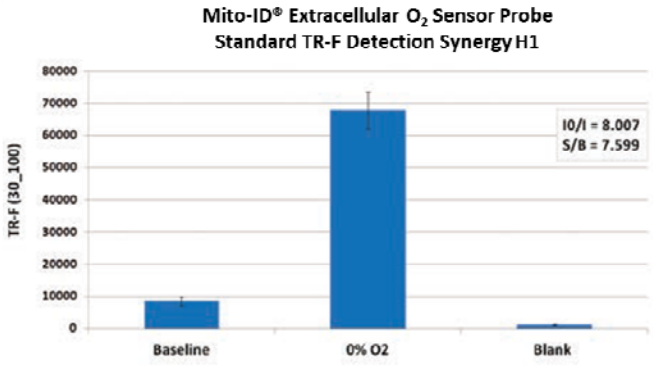


Figure 3. Assay window results on average signal for ambient (~19% O_2) 0% O_2 (glucose depletion via GOx) and blank in standard TR-F mode for Mito-ID® Extracellular O_2 Sensor Probe. The kinetic interval for a full plate was 50 seconds between reads over 45 mins.

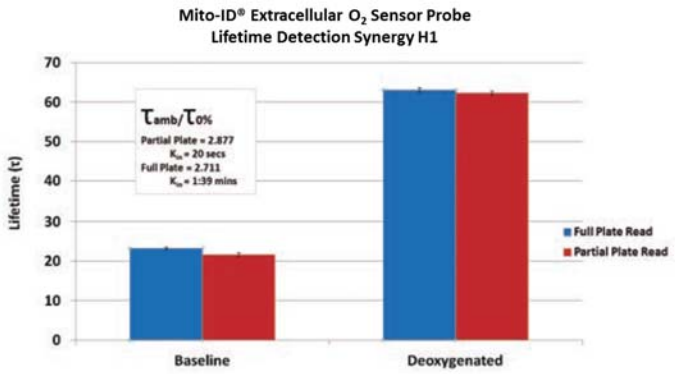
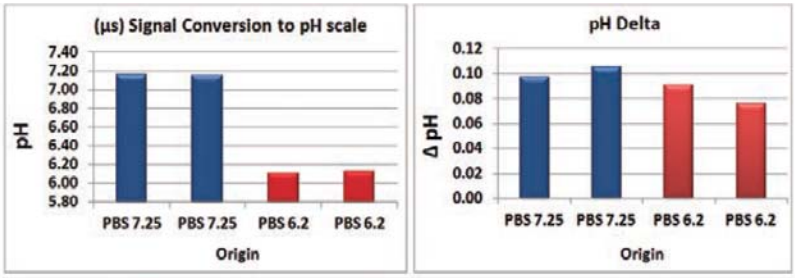


Figure 4. Correlation of lifetime signal stability for partial and full plate reads for Mito-ID® Extracellular O_2 Sensor Probe. The full plate interval results in a 0.0274/sec change in assay window compared with 0.14385/sec in partial plate mode.



		pH Calibration (1687.2 - x) / 199.12				
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*

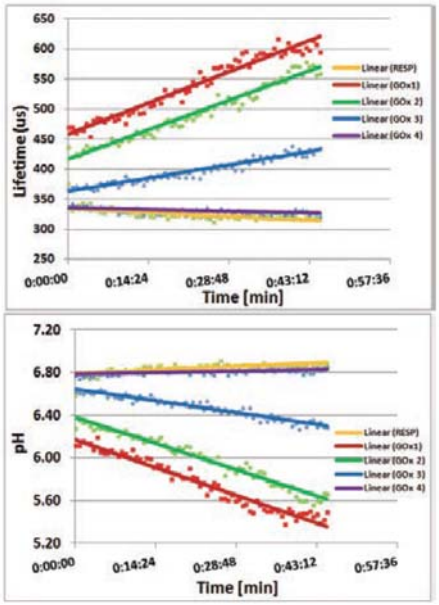


Figure 5. Lifetime signal reproducibility between full and partial plate reads for Mito-ID® Extracellular pH Sensor Probe on the Synergy H1 at 30°C. (Left) Using a default conversion function, pH is calibrated from lifetime values and H^+ is quantified from pH. ECAR is calculated from H^+ on average pH over the 45 min kinetic read. Δ pH are all <1.6% of origin although slightly higher than within error range. Delta pH can be improved by performing instrument specific pH calibration to adjust calculation variables. (Right) Lifetime profiles of GOx diluted 1:10 in respiration buffer from GOx1 (top) converted to pH scale (bottom) demonstrates the analogous relationship between probe signal and acidification (greater acidity-higher signal).

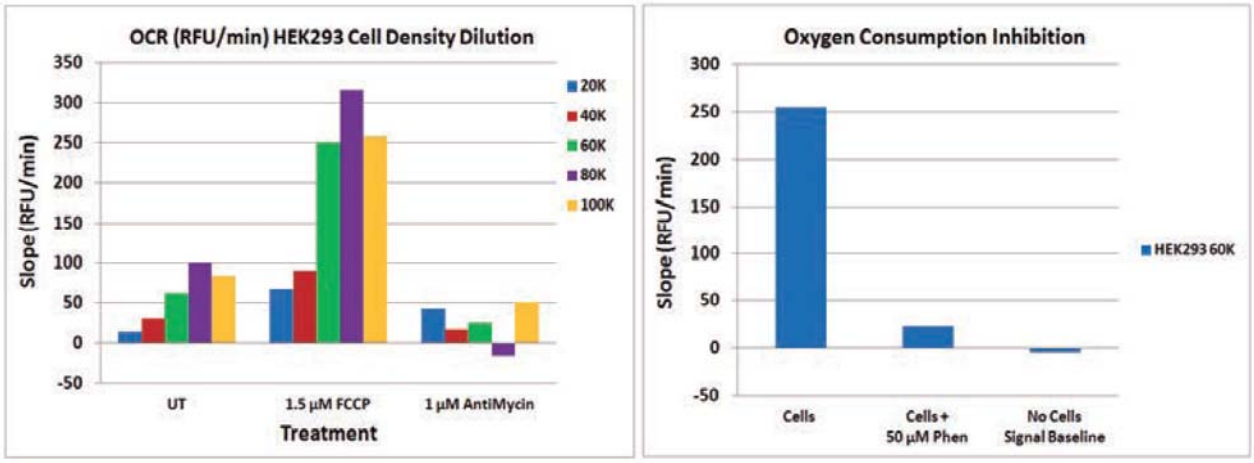


Figure 6. Synergy 2 RFU detection of Extracellular O_2 probe in HEK293 cells at 5 cell densities under 2 drug treatments compared to basal response. OCR becomes non-linear at 100K cells/well in basal and FCCP treated cells. 1µM of Antimycin inhibits oxygen rates regardless of cell density. HEK293 cells plated at 6 x 10^4/well was used to validate OCR inhibition compared to both basal cell response and signal baseline of extracellular probe (right). RFU is converted to OCR (MeanV = RFU/min) from a 40 minute kinetic read with results illustrating 10 fold rate inhibition from compound dose shown.

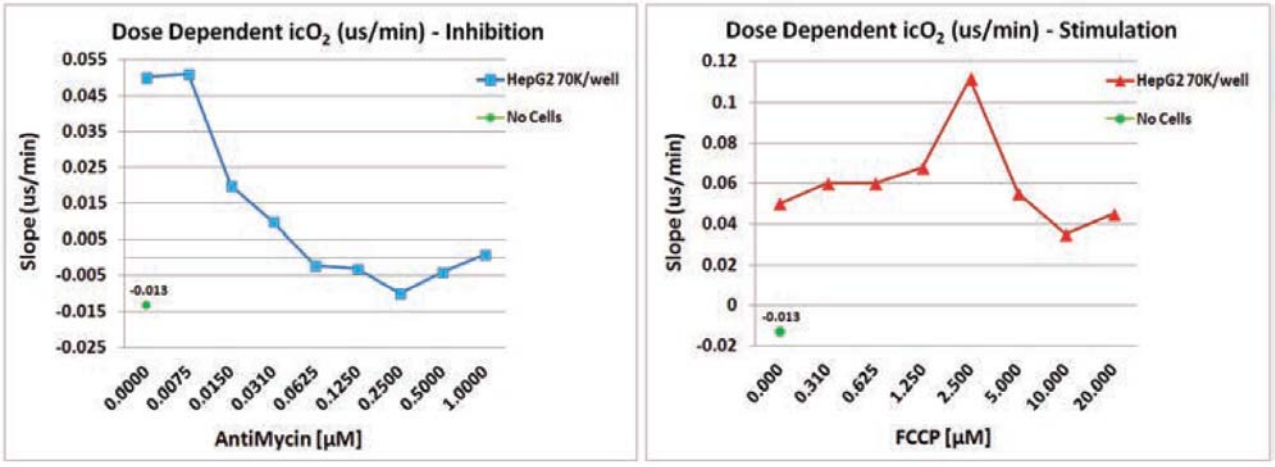


Figure 7. Response of intracellular O_2 in HepG2 cells as calculated from Neo lifetime measurement from the linear portion (9:16 -130 mins) of a 3 hour kinetic run. (Left) AntiMycin dose dependent inhibition of ETC and resulting increase in intracellular O_2 levels, where 1µM dose results in complete inhibition and 0.0075µM reflects basal cell level. (Right) FCCP stimulation of maximal respiration is indicated at 2.5µM followed by inhibition of oxygen depletion to below basal levels at higher concentrations.

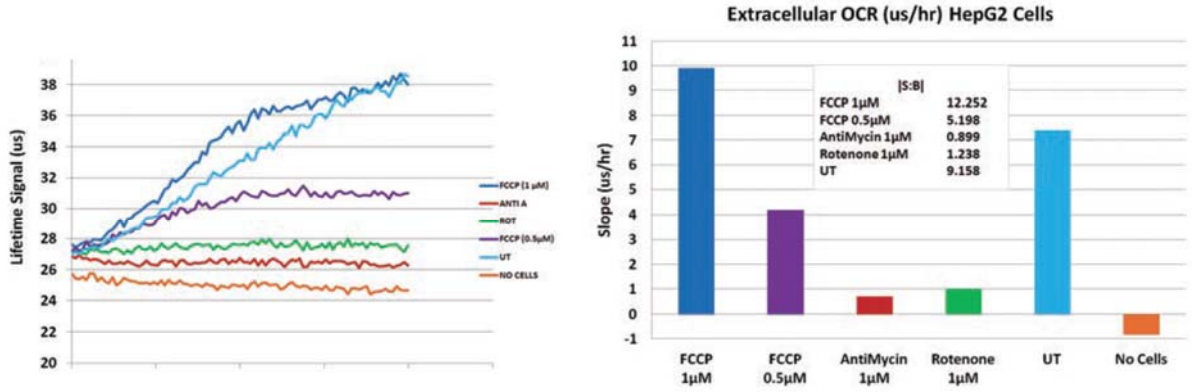


Figure 8. Lifetime detection of extracellular oxygen consumption in HepG2 cells detected on Cytation 3. Slope is calculated from the linear portion (10-80 mins) of a 2 hour kinetic run. Consistent, stable lifetime measurements are shown over the full time course at a kinetic interval of 2:19 mins (left). Cells reflect strong basal (UT) OCR and expected, well differentiated response to agonist/antagonist treatment (right).

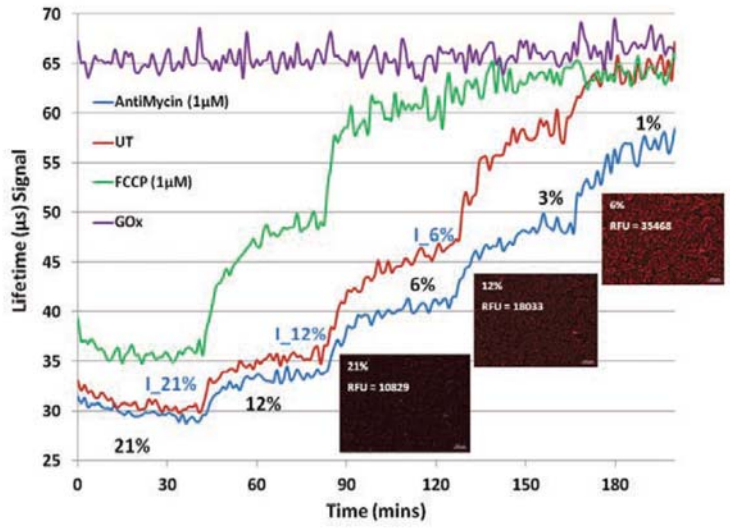


Figure 9. Intracellular response to progressively higher oxygen depletion is measured in parallel by fluorescent imaging (4X) and lifetime detection of HepG2 cells using the Cytation 3 and gas controller over a 3 hour kinetic time course. Cells were plated at 7 x 10^4 cells/well and read on Day 3. Oxygen levels were decreased at intervals shown without interrupting the read. Mean RFU values calculated from images at 3 intervals (I_n) of decreased oxygen levels in AntiMycin treated cells illustrates the principle detection MOA of the Enzo probes.

CONCLUSIONS

- Enzo's Mito-ID® Extracellular O_2 Sensor, Mito-ID® Intracellular O_2 Sensor Probe, and Mito-ID® Extracellular pH Sensor Probe probes can be detected in RFU, standard TR-F, and lifetime detection modes using a variety of BioTek readers to inform cellular metabolic analysis.
- Signal acquisition of Enzo probes using the BioTek lifetime detection algorithm is stable for kinetic interval times from 20 sec to 2:19 minutes. Standard TR-F and RFU detection modes can be done on a full plate in kinetic intervals <= 50 seconds.
- Detecting Mito-ID® Extracellular pH Sensor Probe in lifetime mode allows direct conversion of signal to pH scale and H^+ quantification using a default conversion function.
- BioTek readers and Enzo probes are compatible for analyzing extracellular oxygen consumption rates and intracellular O_2 levels of live cell response to drug treatment.
- The Mito-ID® Intracellular O_2 Sensor Probe is conducive to fluorescent imaging, facilitating increased data analysis options – demonstrated here paired with lifetime detection and a gas controller for measuring changes in intracellular O_2 levels in response to progressive oxygen depletion.

REFERENCES

1. Hynes James et al, 'A high-throughput dual parameter assay for assessing drug-induced mitochondrial dysfunction provides additional predictivity over two established mitochondrial toxicity assays', Toxicol In Vitro, 2012 Mar; 27(2):560-569.
2. Hynes James et al, 'Fluorescent pH and oxygen probes for the assessment of mitochondrial toxicity in isolated mitochondria and whole cells', Curr Proto Toxicol., 2009 May; Chapter 2:Unit 2.16.
3. Hynes James et al, 'In vitro analysis of cell metabolism using a long-decay pH-sensitive lanthanide probe and extracellular acidification assay', Analytical Biochemistry, 2009; 390:21-28.
4. Hynes James et al, 'Investigation of drug-induced mitochondrial toxicity using fluorescence-based oxygen-sensitive probes', Toxicol Sci., 2006; 92(1):186-200.
5. Martin D. Brand and David G. Nicholls, "Assessing mitochondrial dysfunction in cells", Biochem. J. (2011) 435:297–312.
6. Minh-Son To, Edoardo C. Aromataris, Joel Castro, Michael L. Roberts, Greg J. Barritt, and Grigori Y. Rychkov, "Mitochondrial uncoupler FCCP activates proton conductance but does not block store-operated Ca^{2+} current in liver cells", Archives of Biochemistry and Biophysics 495 (2010) 152–158.