

# Optimization of a multi-mode detection model for measuring real-time cellular respiration and mitochondrial function using fluorophoric biosensors

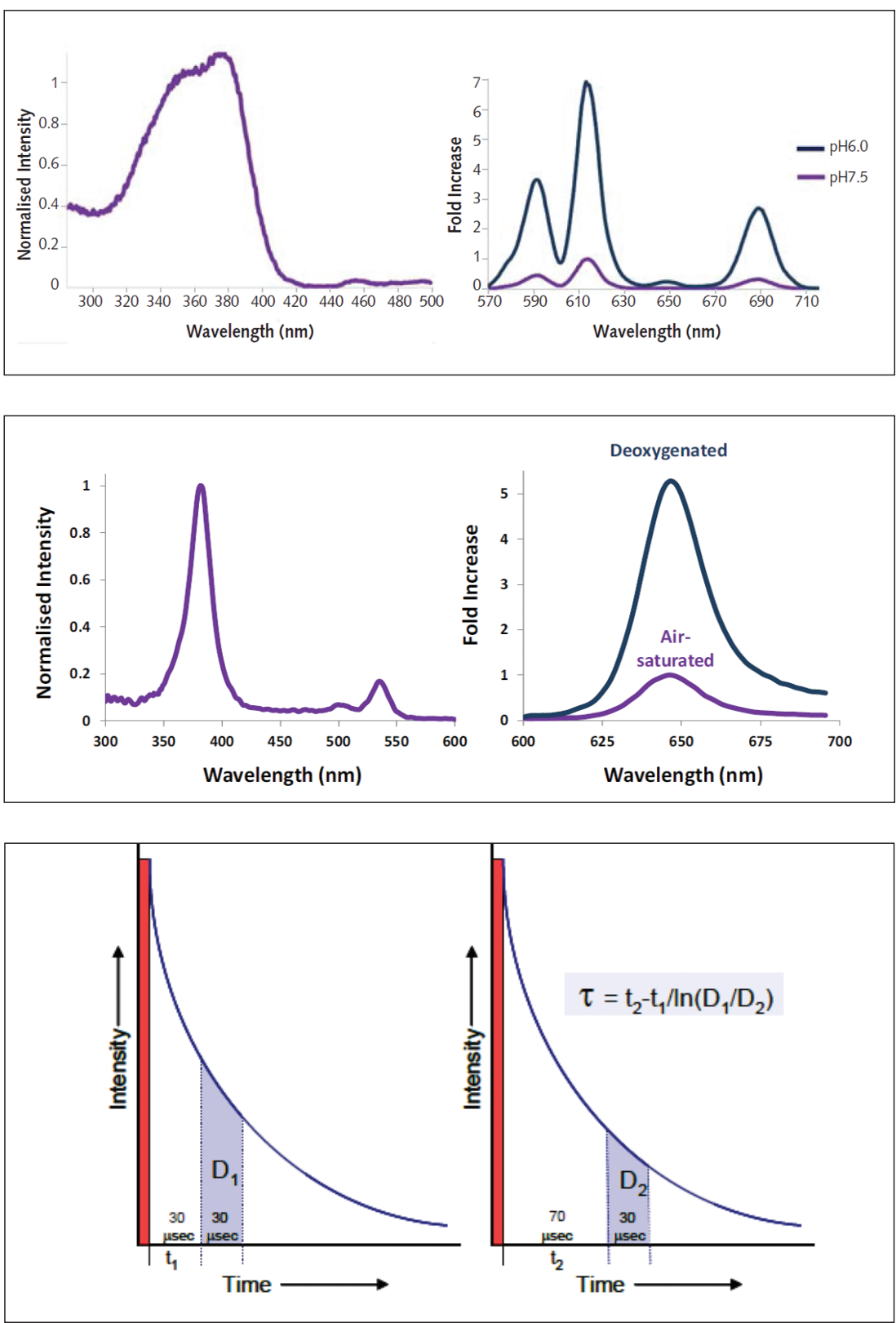


## Introduction

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 ( $\mu\text{s/hr}$ ), presenting accurate comparisons of acidification rates converted to hydrogen ion scale ( $\text{ECA}[\text{H}^+]/\text{t}$ ), and detecting intracellular oxygen levels in parallel with fluorescent imaging in live cell 2D monolayers.

## Assay Overview and Detection Principle

**The MitoXpress® Xtra – Extracellular Oxygen Consumption Assay [HS Method], pH-Xtra™ Glycolysis Assay, and MitoXpress® Intra – Intracellular O<sub>2</sub> Assay** are a family of fluorescent probes designed by Luxcel Biosciences to aide 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<sub>2</sub> or proportional to extracellular H<sup>+</sup> in the sample. O<sub>2</sub> levels, OCR, and quantification of H<sup>+</sup> levels are calculated from the changes in fluorescence signal over time.



**Figure 1.** Excitation and Emission spectra of the pH-Xtra™ Glycolysis Assay 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 620<sub>nm</sub> provides an optimal window for detecting signal change in response to pH levels. The MitoXpress® Xtra- and Intra-cellular probe show emission peaks at a normalized intensity of 380<sub>nm</sub> (center left). The inverse relationship between probe signal and O<sub>2</sub> levels is seen by a 4-fold increase of signal in deoxygenated conditions at emission peak 645<sub>nm</sub> (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<sub>2</sub> 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 Luxcel 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 Luxcel probes in standard RFU mode.

Reader, Probe	Detection Mode	Optics Position	Ex/Em, Mirror, LED, Objectives	TR-F Options (Delay, Integration)	Gain, Exposure
<b>Cytation 3</b>					
Intra	Imaging (RFU)		377-647, 1225000, 4X, 20X		10, 127, 15.6
	Lifetime (t)	Top	380/20:645/15 400nm	R1 30_30; R2 70_30	Auto, Low well @ 10000
<b>Synergy H1</b>					
Xtra	All	Top	380/20:645/15 (400nm)	R1 30_30; R2 70_30	Auto, High well @ 60000
pH-Xtra	All	Top	380/20:645/15 (400nm)	TR-F 30_100	Auto, High well @ 60000
<b>Synergy Neo</b>					
Intra	Lifetime (t)	Top, 50mw	380/20:645/15 400nm	R1 30_30; R2 70_30	Auto, Low well @ 10000
<b>Synergy 2</b>					
Xtra	RFU	Bottom	360/40:645/15		Auto, High well @ 60000

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

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## Materials and Methods

### Materials

- Luxcel Biosciences MitoXpress Xtra Oxygen Consumption Assay (HS Method) (Catalog No. MX-200); Luxcel Biosciences MitoXpress Intra – Intracellular O<sub>2</sub> Assay (Cat No. MX-300); Luxcel Biosciences pH-Xtra – Glycolysis Assay (Cat No. PH-100) • Glucose Oxidase (GOX) powder (e.g. Sigma Cat No. 49180) reconstituted in sterile water
- Respiration Buffer (1M Glucose+DMEM media to final 40mM glucose concentration) • Phosphate Buffered Saline (PBS e.g. Sigma P4417) at pH 7.25 and 6.2 adjusted w/ 0.1mM NaOH or HCl • Respiration Media (DMEM(Sigma D5030, powder), 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 (Thermo Scientific p/n 165305) • HEK293 cells stably transfected with antibiotic resistant marker (proprietary) • HepG2 cells grown and cultured from stock • Rotenone (2.5mM final) vehicle sterile water • FCCP in vehicle DMSO • AntiMycin A in vehicle DMSO • Phenformin (50μM final) in vehicle sterile water

Mito-Xtra, -Intra	1	2	3	4	5	6
A	140μL DMEM + 10μL Probe + (100μL HS Oil) [21% O <sub>2</sub> ]			140μL DMEM + 10μL Probe [21% O <sub>2</sub> ]		
B	130μL DMEM + 10μL Probe + 10μL GOX + (100μL HS Oil) [0% O <sub>2</sub> ]			130μL DMEM + 10μL Probe + 10μL GOX [0% O <sub>2</sub> ]		
C	150μL DMEM + (100μL HS Oil)			150μL DMEM		
	High Sensitivity (HS) Oil for Xtra probe only			No Oil		
pH XTRA	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 Luxcel 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.

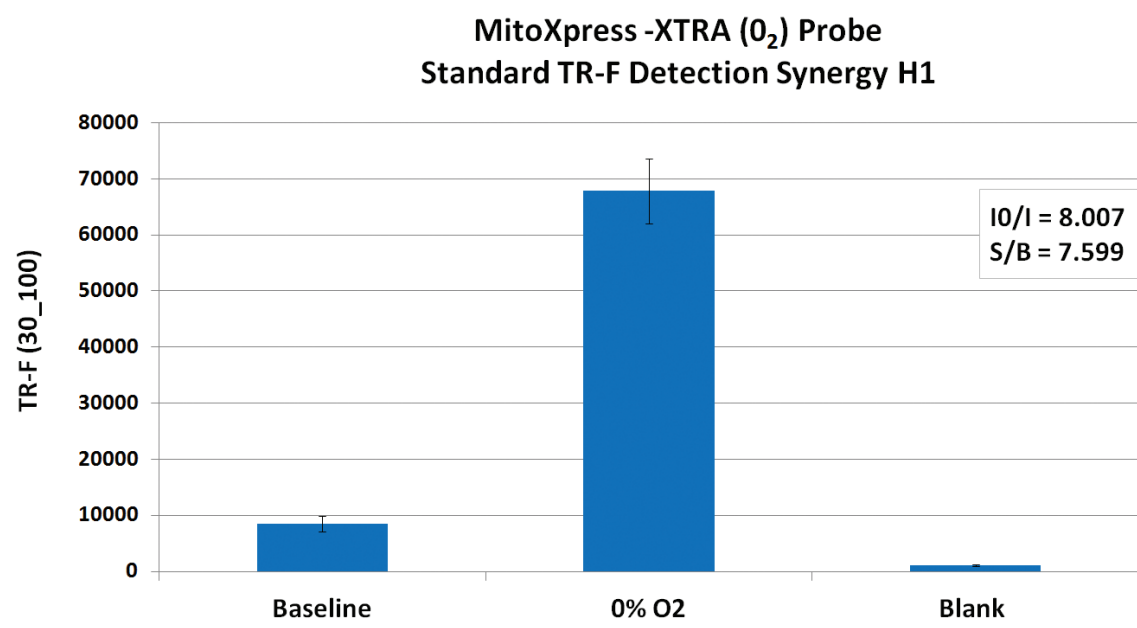
### Signal Optimization, H<sup>+</sup> Quantitation, Cellular Metabolic Analysis

**MitoXpress Xtra:** 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 (1 μM final); Phenformin (50 μM final); and FCCP (1 μM final, or as a 1:2 dilution from a start concentration of 20 μM) on Day 2 of the protocol. Blanks (150 μL media only), Signal Control (15X probe in media), and PC (10 μL 15X probe, 10 μL 15X GOX (solubilized 1mg/mL in 1mL sterile water) to 130 μL media per well) were run on each plate. Data shown by Figures 6 and 8.

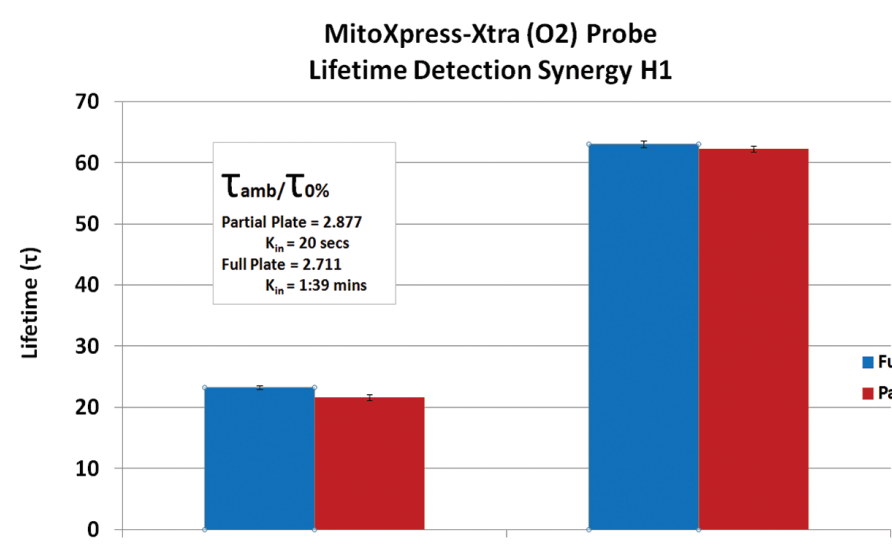
**pH-Xtra:** For signal optimization and pH scale/H<sup>+</sup> conversion - assay in volumes and locations shown in Figure 2 (bottom). Commence 90 minute kinetic read (fastest interval) on H1 at 30 °C in full and partial plate read modes using lifetime detection parameters defined by Table 1. Data shown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

**MitoXpress Intra:** For cellular metabolic analysis - cells were plated in 200 μL culture media and incubated ON at 37 °C 5% CO<sub>2</sub>. On Day 2 probe was reconstituted 1:11 in culture media. Spent cell media was aspirated and replaced with 100 μL/well of Intra Probe in media stock then incubated ON at 37 °C 5% CO<sub>2</sub>. 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 Xtra probe. 1 μL 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.

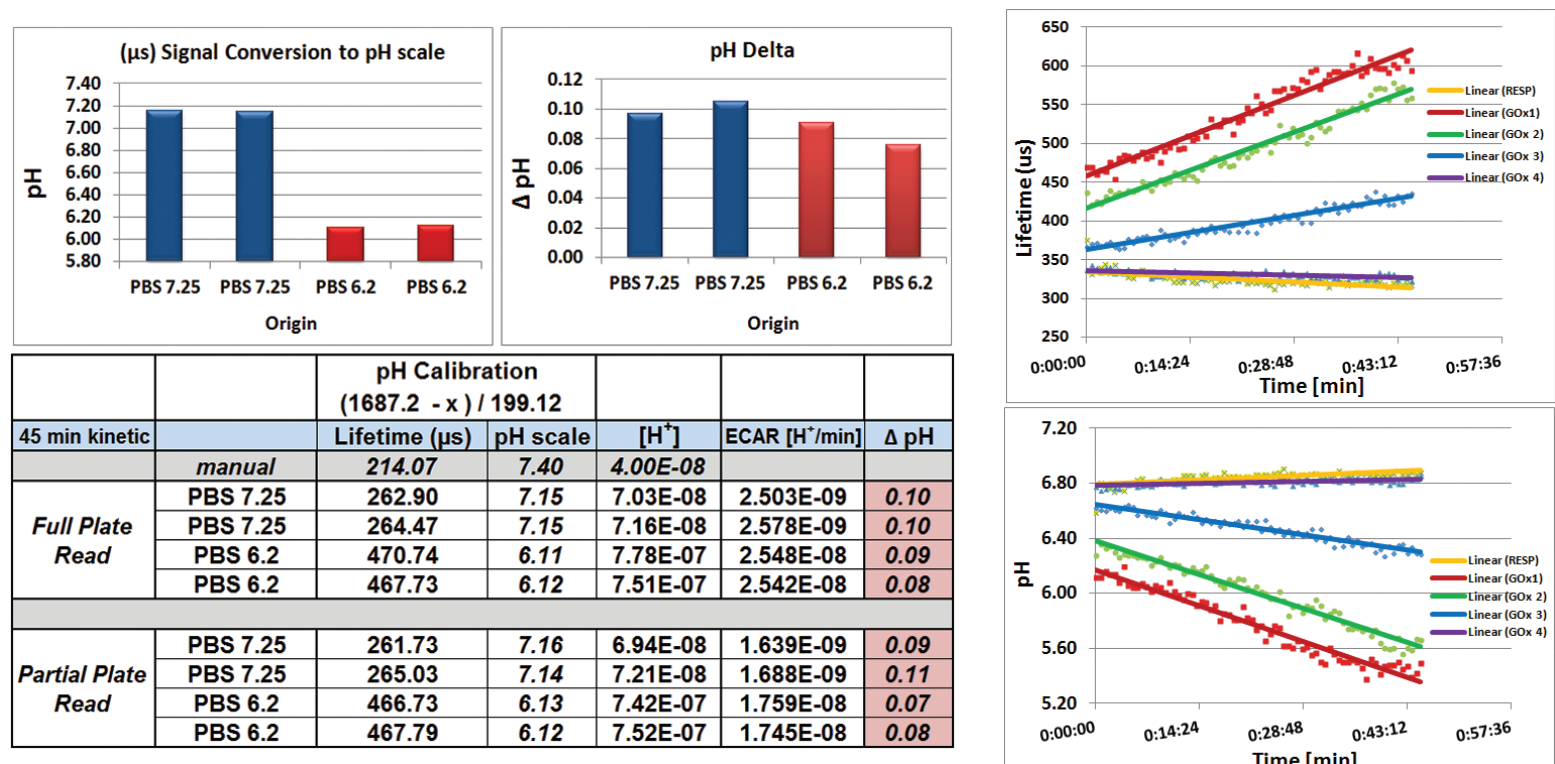
## Results



**Figure 3.** Assay window results on average signal for ambient (~19% O<sub>2</sub>) 0% O<sub>2</sub> (glucose depletion via GOx) and blank in standard TR-F mode for MitoXpress Xtra. The kinetic interval for a full plate was 50 seconds between reads over 45 mins.

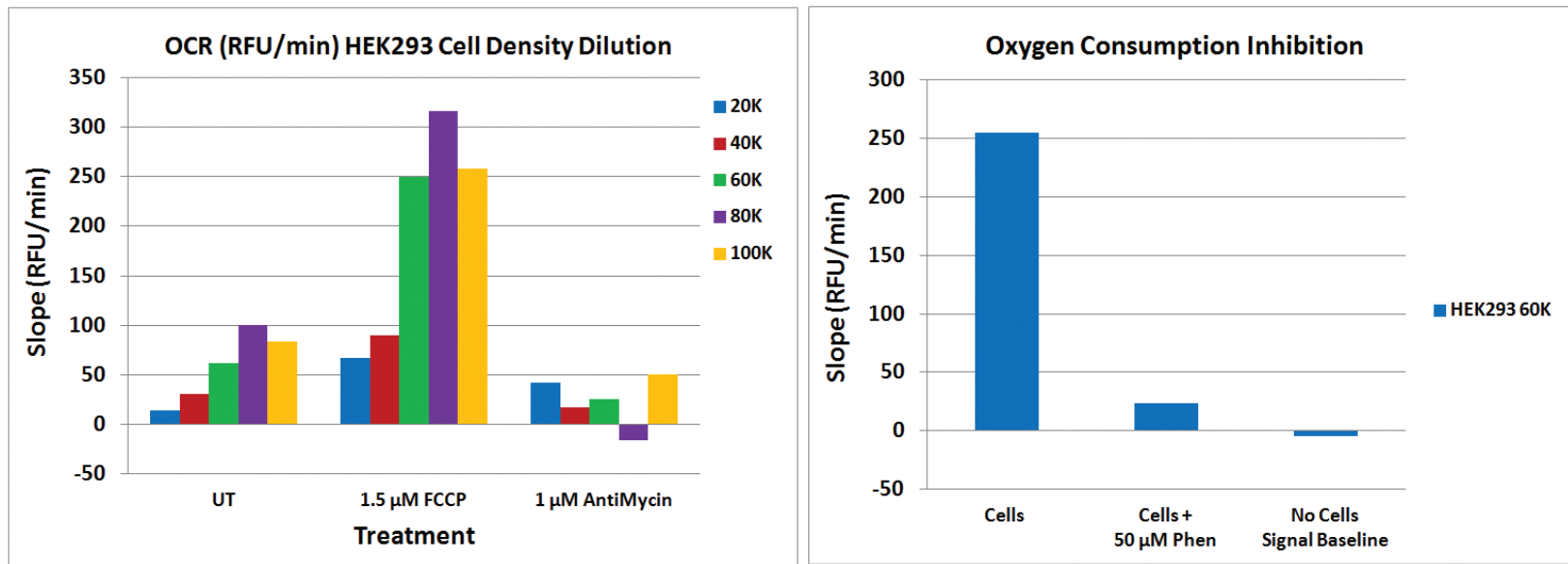


**Figure 4.** Correlation of lifetime signal stability for partial and full plate reads for MitoXpress Xtra. The full plate interval results in a 0.0274/sec change in assay window compared with 0.14385/sec in partial plate mode.

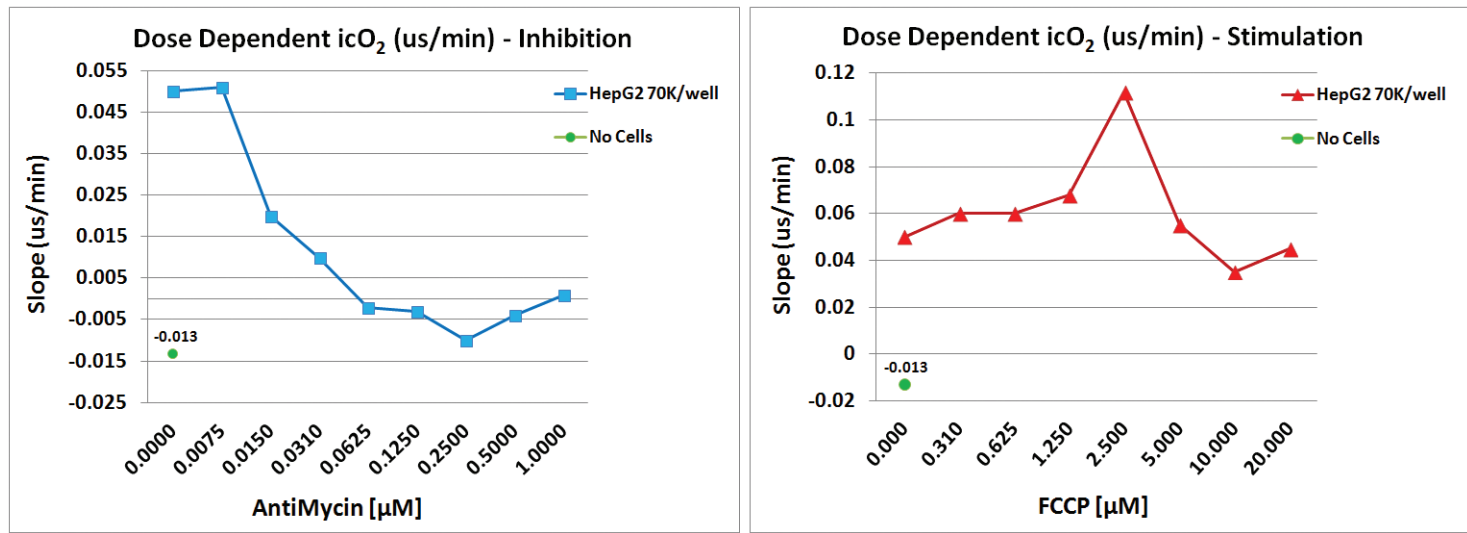


**Figure 5.** Lifetime signal reproducibility between full and partial plate reads for pH-Xtra probe on the Synergy H1 at 30 °C. (Left) Using a default conversion function, pH is calibrated from lifetime values and H<sup>+</sup> is quantified from pH. ECAR is calculated from H<sup>+</sup> on average 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).

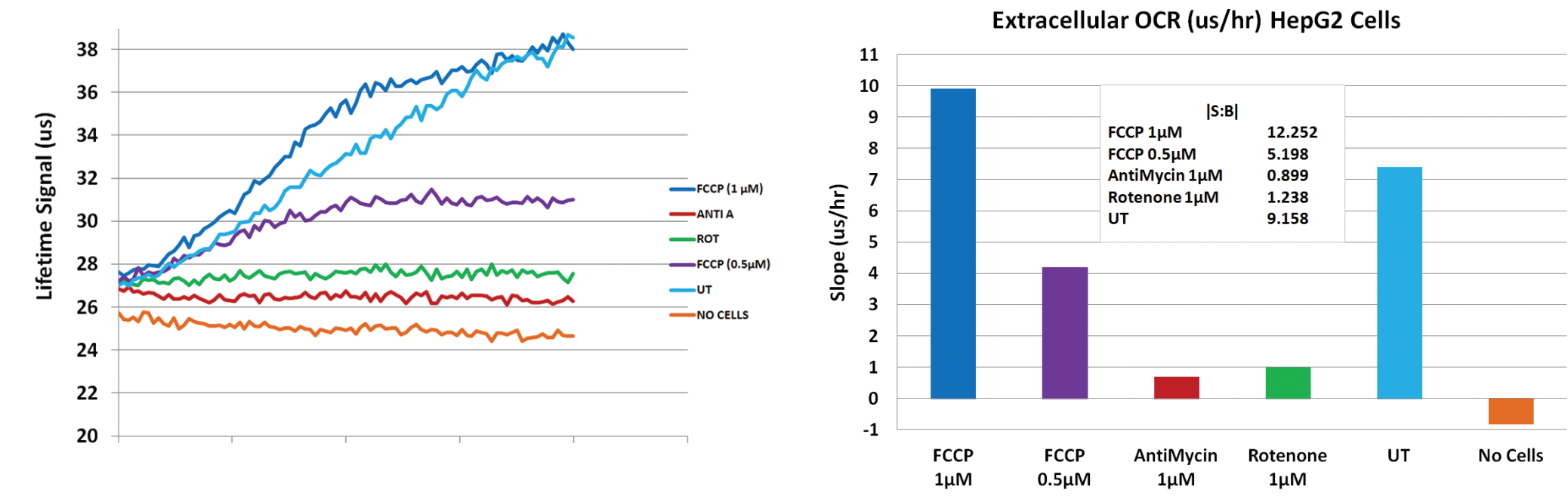
## Results Cont.



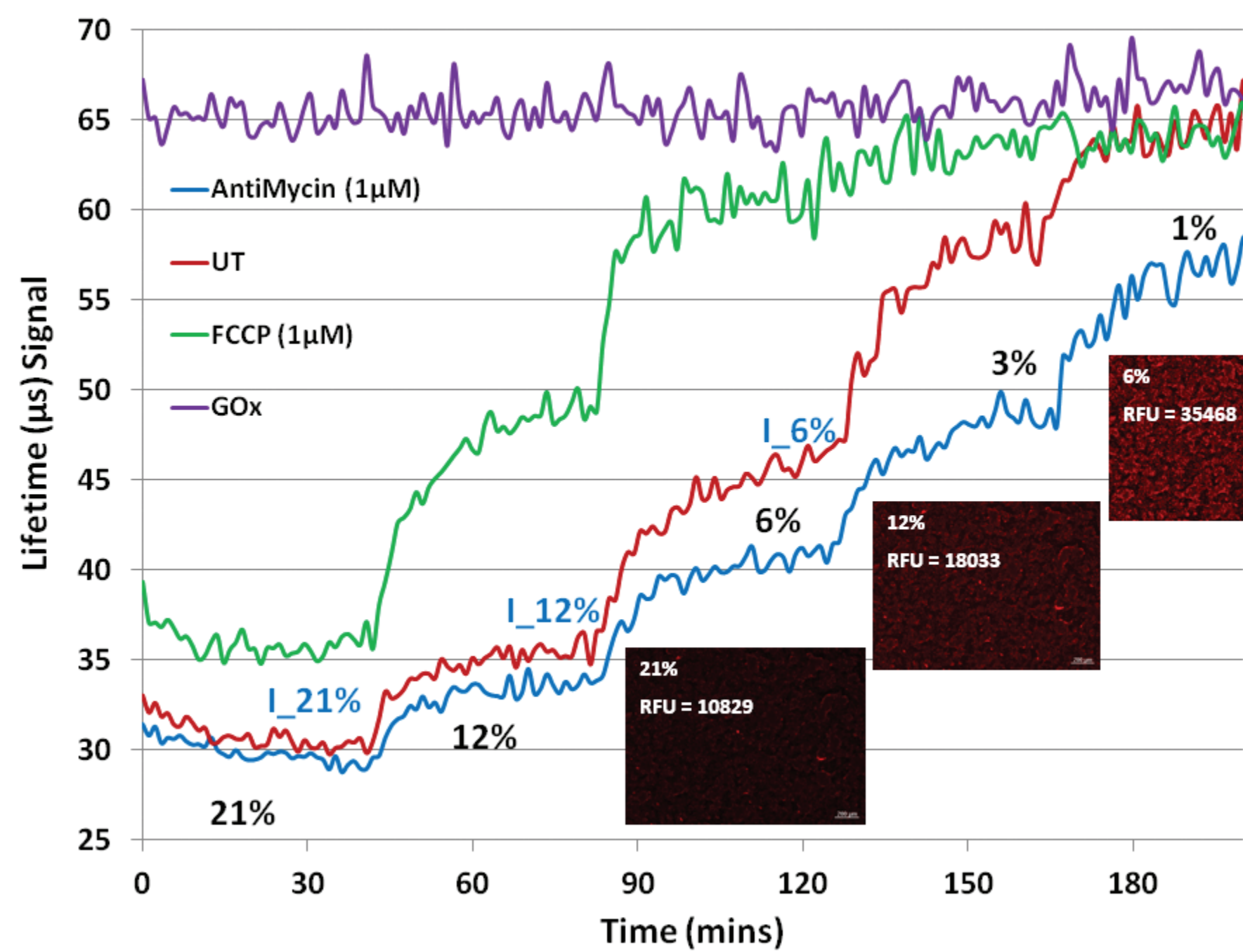
**Figure 6.** Synergy 2 RFU detection of Extracellular O<sub>2</sub> probe in HEK293 cells at 5 cell densities under 2 drug treatments compared to basal response. OCR becomes non-linear at 100K cells/well in basal and FCCP treated cells. 1μM of Antimycin inhibits oxygen rates regardless of cell density. HEK293 cells plated at 6 x 10<sup>4</sup>/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<sub>2</sub> 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<sub>2</sub> levels, where 1μM dose results in complete inhibition and 0.0075 μM 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<sup>4</sup> 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 (\_\_\_\_) of decreased oxygen levels in AntiMycin treated cells illustrates the principle detection MOA of the Luxcel probes.

## Conclusions

- Luxcel MitoXpress –Xtra, -Intra, and pH-Xtra 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 Luxcel 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 pH-Xtra probe in lifetime mode allows direct conversion of signal to pH scale and H<sup>+</sup> quantitation using a default conversion function.
- BioTek readers and Luxcel probes are compatible for analyzing extracellular oxygen consumption rates and intracellular O<sub>2</sub> levels of live cell response to drug treatment.
- The MitoXpress-Intra 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<sub>2</sub> levels in response to progressive oxygen depletion.