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ab197245 Intracellular Oxygen Concentration Assay

For the measurement of intracellular oxygen concentration in cell lines, primary cells, 3D cultures and spheroids.

This product is for research use only and is not intended for diagnostic use.

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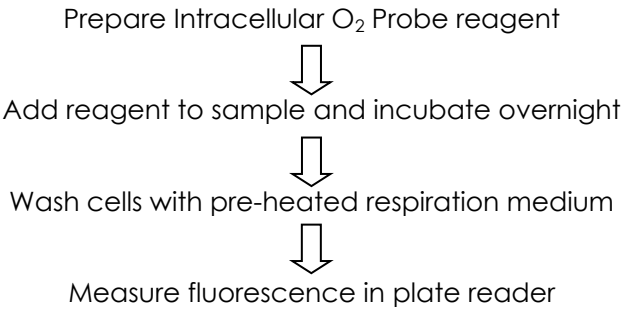
1. Overview

Intracellular Oxygen Concentration Assay (ab197245) is a flexible fluorescent approach to analyze the oxygenation levels at the cell monolayer using plate-based time-resolved fluorometry. This assay is based on the ability of oxygen to quench the phosphorescent emission of an O_2-sensitive probe. The probe signal increases with a reduction in intracellular oxygen and deceases with an increase in intracellular oxygen. The measured signal (Ex/Em = 380/650 nm) is then proportional to the intracellular oxygen concentration allowing real-time monitoring of intracellular oxygen concentration in conventional cell culture models (2D), as well as a wide range of 3D systems.

Oxygen availability significantly influences cell physiology, signal transduction and cellular response to drug treatment. However, in the majority of i*n vitro* studies, cells are cultured at ambient oxygen even though it represents a hyperoxic state for most types. Assuming that cells experience this ambient condition also ignores the significant oxygen gradient that can exist between the atmosphere and respiring cells. This assay provides a unique tool to monitor the oxygen concentration that cells in culture are actually experiencing. Additional, where cell physiology should be monitored under defined O_2 conditions, this assay is an ideal tool to identify the appropriate environmental O_2 to achieve the desired cellular O_2 concentration.
The flexible plate reader format, allows multiparametric or multiplex combination with a range of other reagents and it is suitable for HTP automation. For example, this assay can be used in combination with Glycolysis Assay [Extracellular acidification] (ab197244), allowing a detailed analysis of the interplay between O_2 availability and concomitant alterations in the balance between aerobic and glycolytic ATP production.

2. Protocol Summary



3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at 4°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing component has not been reconstituted.

Aliquot components in working volumes before storing at the recommended temperature.

Δ Note: Reconstituted reagent is stable for 3 weeks.

5. Limitations

– Assay kit intended for research use only. Not for use in diagnostic procedures.

6. Materials Supplied

Item	Quantity		Storage Condition (Before prep)	Storage Condition (After prep)
	96 tests	4 x 96 tests		
Intracellular O_2 probe	1 vial	4 x 1 vial	4°C	4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Atmospheric control module or workstation to control CO_2/O_2 environment
- Microplate reader capable of measuring fluorescence, with suitable filter and plate temperature control – see Instrument and Measurement Settings section for suitable plate readers
- MilliQ water or other type of double distilled water (ddH_2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Sterile 96 well plate (black wall with clear flat bottom), or standard clear plates for cell culture

  For cells:
- Cell culture medium
- HEPES-based DMEM buffer (for long term measurements): dissolve DMEM powder in 900 mL sterile ddH_2O. Add 20 mM HEPES, 1 mM sodium pyruvate, 20 mM glucose, 10% FBS, Pen-Strep (if required). Filter sterilize prior to use.

8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.
- Refer to Instrument and Measurement Settings table (Section 11; Table 1) for recommended settings for your plate reader.
- While compatible with all plate types, black border clear bottom plates give optimal signal-to-noise ratios.
- For first time users, we recommend performing a Signal Optimization Step (see Section 12).

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Intracellular O_2 probe:

Prepare a stock solution of the Intracellular O_2 probe by adding 1 mL of ddH_2O to the vial. Mix by gently aspirating 3 – 4 times.

Recommended working dilution = 1/10 (15 µL per 150 µL of sample). Warm to measurement temperature (usually 37°C) before use.

Do not freeze. Reconstituted reagent is suitable for 3 weeks.

10. Sample Preparation

General Sample Information:

- Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.
- Prepare test compounds for sample treatment as desired.

10.1 Adherent cells:

10.1.1. Seed cells in a 96-well plate at a density of 3 - 8 x 10^4 cells/well in 200 µL culture medium.

10.1.2. Incubate overnight in a CO_2 incubator at 37°C.

Δ Note: Typical cells are plated at a density to achieve full confluence. Lower plating concentrations should be used with long culture times unless cells are terminally differentiated.

10.2 Cells in 3D cultures and spheroids:

10.2.1. Prepare the chosen 3D construct solution in advance. Scaffold systems such as Matrigel®, RAFT™, microtissues, Alvetex® and Mimetix® can be used in this assay. Cells are generally plated at a higher density than 2D cultures.

11. Plate Reader Set-Up

11.1 Measurement Parameters

The Intracellular O_2 Concentration Reagent is a chemically stable and inert, nanoparticulate oxygen-sensing fluorophore.

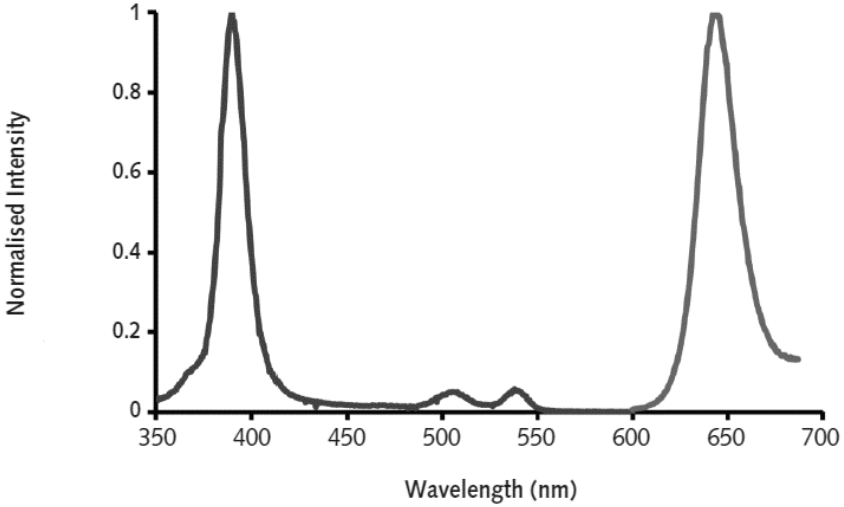


Figure 1. Excitation and Emission spectra of Intracellular O_2 probe, showing normalized excitation (Ex 360-400nm; Peak 380nm) and emission (Em 630-670nm; Peak 650nm).

	Peak Maxima (nm)	Peak (nm)
Excitation*	380	360 – 400
Emission	650	630 – 670

*Excitation at 532 ± 7.5 nm is also possible

- This assay is measured with prompt or time-resolved fluorescence (TR-F) readers, monochromator or filter-based. Optimal wavelengths are λ = 380 nm for excitation (λ = 532 nm can also be used) and λ = 650 nm for emission.
- The O_2 probe response is temperature dependent, so good temperature control of the plate during the measurement is important.

11.2 Fluorescence measurements

Outlined below are two fluorescence modalities that can be used with this assay, depending on the plate reader type and instrument setup.

11.2.1. Standard: TR-F Measurement

Using time-resolved fluorescence (TR-F) will increase performance levels. TR-F measurement reduces non-specific background and increases sensitivity.
Optimal delay time is 30 µs and measurement window (integration time) is 100 µs.

Δ Note: Intracellular O_2 probe should return Signal to Blank ratio (S:B) ≥ 8; value ≥ 10 are typical. Better S:B is achieved with filter-based optics.

11.2.2. Advanced: Dual-Read TR-F (Lifetime calculation)

Optimal performance is achieved using dual-read TR-F (Figure 2) where by two intensity measurements are taken sequentially. In combination with the Lifetime calculation, this ratiometric approach monitors the rate of the fluorescence decay of the probe, providing a more robust measurement of oxygen concentration.

Optimal dual-delay and gate (integration) times:

- Integration window 1: 30 µs delay (D1), 30 µs measurement time (W1)
- Integration window 2: 70 µs delay (D2), 30 µs measurement time (W2)

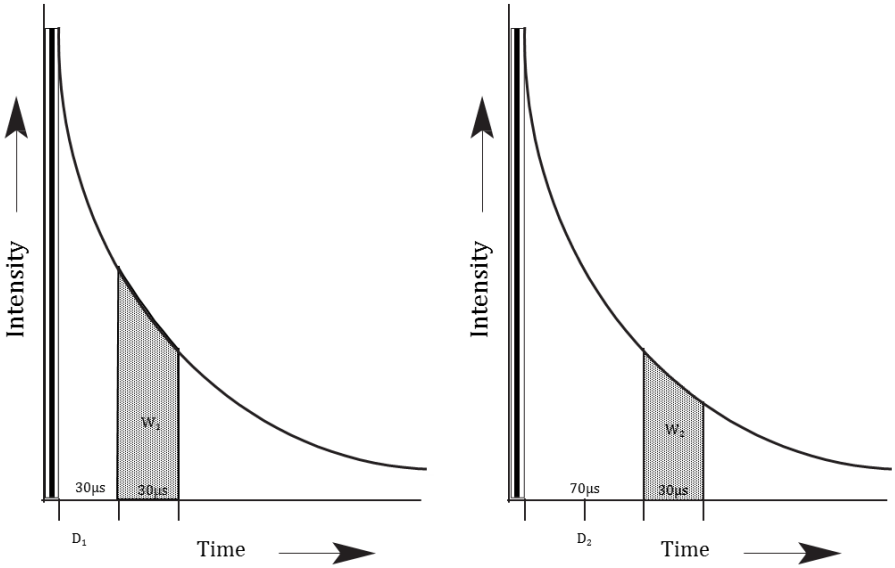


Figure 2. Illustrating dual read TR-F measurement.

See Instrument and Measurement Setting table below for instrument-specific setting and filters. Choose filter based optical configuration where available.

Table 1. Recommended Instrument and Measurement Settings

Instrument	Optical Configuration	Intg1 (D_1/W_1) Intg2 (D_2/W_2)	Optimum Mode	Ex (nm) Em (nm)
BioTek: Cytation 3 / 5	Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 380 ± 20nm Em 645 ± 15nm
BioTek: Synergy H1 / Neo / 2	Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 380 ± 20nm Em 645 ± 15nm
BMG Labtech: CLARIOstar	Filter-based Bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50nm (TR-EX) Em 665 ± 50nm or Em 645± 10nm *With LP-TR Dichroic*
BMG Labtech: FLUOstar Omega / POLARstar Omega	Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50nm (TR-EXL) Em *655* ± 25nm (BP-655)
Perkin Elmer: VICTOR series/ X4 / X5	Filter-based Top read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 40nm (D340) Em 642 ± 10nm (D642)

Instrument	Optical Configuration	Intg1 (D_1/W_1) Intg2 (D_2/W_2)	Optimum Mode	Ex (nm) Em (nm)
Tecan: Infinite M1000Pro / F200Pro	Monochromator/ Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 380 ± 20nm Em *650* ±20nm or Em 670±40nm
Tecan: Spark 10M / 20M	Filter-base / Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 380 ± 20nm Em *650* ±20nm or Em 670±40nm
BioTek: Synergy HTx / Mx	Monochromator/ Filter-based Top or bottom read	30 / 100µs n/a	TR-F	Ex 380±20nm Em 650±20nm
BMG Labtech: PHERAstar FS/ FSX	Filter-based Top or bottom read	40 / 100µs n/a	TR-F	Ex 337 nm (HTRF Module) Em 665 nm (HTRF Module)
Tecan: Infinite M200Pro / Saffire / Genios Pro	Monochromator/ Filter-based Top or bottom read	30 / 100µs n/a	TR-F	Ex 380±20nm Em 650±20nm
Perkin Elmer: EnVision / Enspire	Monochromator/ Filter-based Top read	40 / 100µs n/a	TR-F	Ex 340 ±40nm (D340) Em 642± 10nm (D642)

12. Signal Optimization

- This step is recommended for first time users.
- Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature.

12.1 Prepare 8 replicate wells of a 96-well plate, by adding 150 µL pre-warmed culture medium to each well (A1-A4, B1-B4).

12.2 Add 2.5 µL reconstituted Intracellular O_2 probe to 4 of the replicate wells (A1-A4) and 2.5 µL ddH_2O, PBS or media to the remaining replicates wells (B1-B4).

12.3 Read plate immediately in a fluorescence plate reader over 30 minutes (read every 2-3 minutes).

12.4 Examine Signal Control well (A1-A4) and Blank Control well (B1-B4) readings (linear phase) and calculate Signal to Blank (S:B) ratio.

Δ Note: For dual read TR-F, calculate S:B for each measurement window. Intra O_2 probe should return a S:B ≥ 8.

	1	2	3	4
A	Media + Intra O_2 probe	Media + Intra O_2 probe	Media + Intra O_2 probe	Media + Intra O_2 probe
B	Media	Media	Media	Media

13. Assay Procedure

- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature (typically 37°C).
- Plating density and basal metabolic rate will determine the steady-state oxygen concentration at the cell monolayer.
- Example of typical compounds that can be used as assay controls are shown in the table below.

Typical control	Treatment	Effect
Negative Biological Control: Antimycin A (Complex III inhibitor)	add 1 µL of 150 µM stock solution in DMSO	Increase in iO_2 (removes influence of OCR on intracellular O_2)
Positive Biological Control: FCCP (ETC uncoupler)	add to a final concentration of 2 µM (serial dilution should run for each new cell type to ensure optimum concentration)	Decrease in iO_2 (increases OCR by uncoupling respiration)
Positive Signal Control: Glucose oxidase	add 10 µL of 1 mg/mL stock solution in ddH_2O	Decrease in iO_2 (removes oxygen from the culture medium by reducing it with glucose)

13.1 Plate loading:

13.1.1. Adherent cells: remove culture media from all assay wells and replace with 150 µL of fresh culture media.
Cells in 3D cultures and spheroids: ready to use as prepared in Step 10.2.

13.1.2. Blank controls (we suggest using wells H11 and H12): add 150 µL fresh culture media.

13.1.3. Optional biological controls (positive and negative): as described in Sample Preparation section.

13.2 Assay set up:

13.2.1. Dilute reconstituted Intra O_2 probe 1:10 in culture media. Prepare enough volume to load the required number of wells (150 µL/well), then warm to measurement temperature (typically, 37°C).

13.2.2. Remove culture media from all assay wells and replace with 100 µL of pre-warmed Intra O_2 probe stock (Step 13.2.1).

Δ Note: Do not allow cells to dry out during aspiration.

13.2.3. Return plate to incubator and culture overnight (typically > 14 hours).

13.2.4. Warm 25 mL of culture media to measurement temperature (typically 37°C). For longer term measurements (>2 h), we recommend using HEPES buffer (see Materials Required, Not Provided).

Δ Note: A HEPES-based DMEM measurement buffer allows longer term measurement (> 2h) outside a CO_2 incubator as it maintains pH for a prolonged period without applied CO_2. This is not necessary if using plate reader models equipped with an atmospheric control module where 5% CO_2 can be maintained within the measurement chamber.

13.2.5. Put the plate on a plate block heater. Wash cells by aspirating spent media and adding 100 µl of pre-warmed measurement buffer using a multichannel or repeater pipette.

Δ Note: Take care not to dislodge cells from the base of the wells.

13.2.6. Repeat wash step.

13.2.7. Add 150 µL of pre-warmed measurement buffer to each test well, controls (if using) and designated blank wells. Blank wells are required for the proper blank correction of the measured fluorescence signal.

13.3 Measurement:

13.3.1. Insert the prepared plate into a fluorescence plate reader pre-set to the measurement temperature (typically 37°C) and commence kinetic reading.

13.3.2. Measure baseline signal for a minimum of 20 minutes to ensure sample temperature equilibration.

13.3.3. OPTIONAL: if adding test compound – for manual compound addition, pause reading, eject the plate from the reader and quickly add test compound to each well. Re-insert the plate into the plate reader and recommence the kinetic measurement. If available, plate reader injectors can also be used. After responses are observed and oxygen levels have stabilized, further compound additions can be made.

13.3.4. OPTIONAL: if modulating oxygen environment – to assess the impact of altered ambient oxygen concentration using an atmospheric control module, after baseline signal has stabilized, alter ambient oxygen concentration as per manufacturer's instructions.

14. Calculations

14.1 Plot the Blank control well-corrected Intra O_2 probe Intensity or Lifetime values versus Time (min).

14.2 Lifetime (T) values are calculated using the intensity data from Dual-Read TR-F measurements as follows:

$$\tau = \frac{40}{ln(W1/W2)}$$

Where τ represents emission lifetime and W1 and W2 represents signals measured at window 1 and window 2 respectively. Lifetime values are in µs units (range ~29 – 68 µs), and should only be calculated from samples containing Intra O_2 probe. Lifetime values should not be calculated from blank wells.

Converting into Oxygen Scale – converting from Lifetime data

Lifetime data is favored as the basis for generating data in O_2 scale. Lifetime values as calculated above can be converted in oxygen scale using the following default analytical function:

$$O2\% = 659.3\ x\ Exp\frac{-\tau}{8.475}$$

Δ Note: this function is specific to measurements at 37°C.

Converting into Oxygen Scale – converting from Intensity data

Intensity values can be converted into oxygen scale by including a positive control (glucose oxidase addition) and negative control (antimycin addition). These controls (see page 15) should be generated on each plate. Oxygen concentration ($[O_2]t$) is then calculated using the following equation:

$$[O2]t = \frac{[O2]a\ x\ Ia\ x\ (I0 - It)}{It\ x\ (I0 - Ia)}$$

Where:

$[O_2]a$ = oxygen concentration in air-saturated conditions (typically ~20.9%).

I0 = intra O_2 probe signal measured in deoxygenated conditions.

Ia = intra O_2 probe signal measured in air-saturated conditions.

It = experimental intensity values generated during measurement.

Δ Note: if test plate has equilibrated containing 5% CO_2 and 95% humidity, ambient O_2 = ~ 18.6%.

15. Typical Data

Data provided **for demonstration purposes only**.

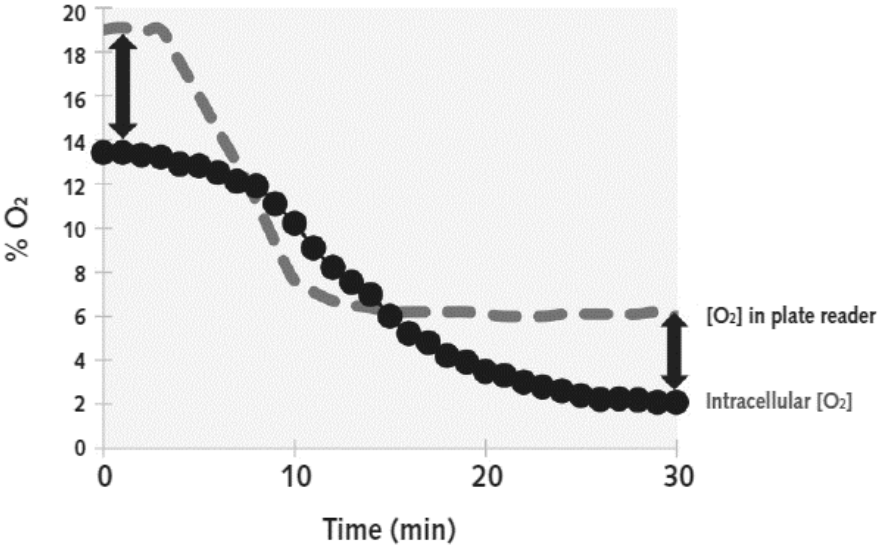


Figure 3. HEK293T cell oxygenation. HEK293T cells were cultured in 2D and measured at ambient oxygen. Intracellular O_2 levels were ~ 14%. Reducing instrument O_2 to 6% caused cellular oxygenation to drop to ~2%. Assay performed using a CLARIOstart equipped with an ACU module (BMG Labtech).

16. Additional Assays/Data

16.1 Monitoring cell respiration

A default calibration function has been generated to facilitate the conversion of Intra O_2 probe Lifetime values into O_2 scale. This conversion is described in the Calculations section and works well across multiple instruments and cell types.

If required, a bespoke calibration function specific to a particular cell type, measurement temperature or measurement conditions can be generated. This requires access to a plate reader with an atmospheric control. Calibrations are temperature specific and should be conducted at the desired measurement temperature.

Cells are loaded with Intra O_2 probe as described in the protocol and, prior to measure, antimycin is added to remove the influence of cell respiration on intracellular O_2. Measurement begins at atmospheric O_2 (room air) and, using instrument atmospheric control, O2 is reduced in a stepwise manner (typically ~20, 15, 10, 7.5, 5, 2.5, 1% CO_2). Zero values are generated using glucose oxidase (Figure 4).

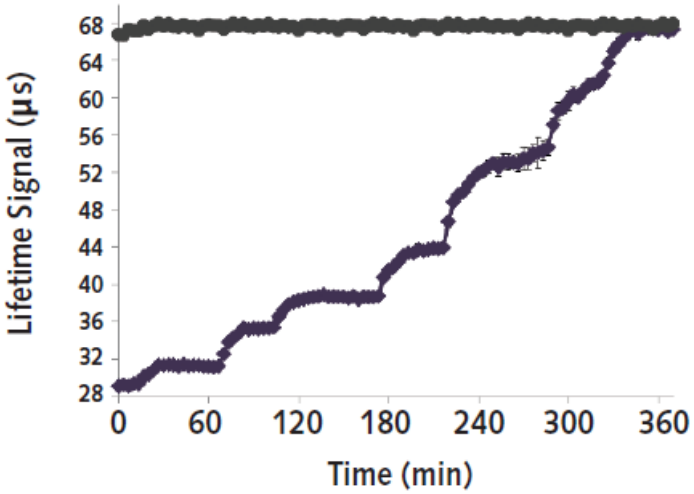


Figure 4. Sample Calibration Data. Intra O_2 probe Lifetime profiles measured at decreasing [O_2] with parallel glucose oxidase treatment to achieve 0% O_2.

Lifetime values are then plotted against applied [O_2] and a first order exponential fit applied to generate a calibration function (Figure 5).

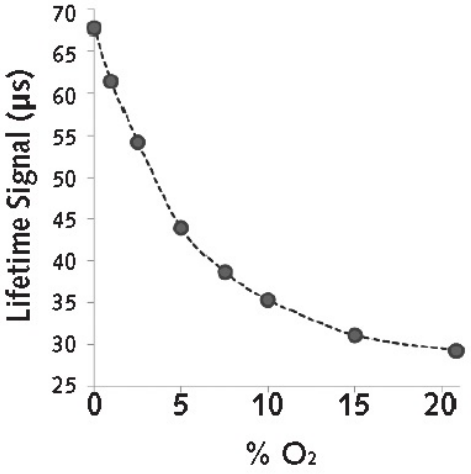


Figure 5. Relationship between probe lifetime (τ) and applied [O_2]. Applying a first order exponential fit generates a calibration function of O_2% = A1 x Exp(-τ / t1). Example: O_2% = 659.3 x Exp(-τ / 8.475).

16.2 Impact of cell metabolism on oxygenation

Cellular oxygenation can be significantly affected by alterations in cell metabolism and these biological processes can be investigated in detail using this assay. In the example presented in Figure 6, iPS derived cardiomyocytes measured at ambient O_2 (~21%) experienced an intracellular O_2 of 14% under resting conditions. If the contribution of cellular respiration is removed through the addition of antimycin (an electron transport chain (ETC) inhibitor), intracellular O_2 slowly returns to ambient levels as the cells cease to consume O_2. If cardiomyocyte beat rate is increased through addition of isoproterenol (a β-adrenoreceptor agonist), the resulting increase in ATP demand and O_2 consumption further reduces intracellular O_2, with values of ~6% observed for >15 min. This experiment illustrates the significant O_2 depletion that cells can experience despite being measured at ambient O_2.

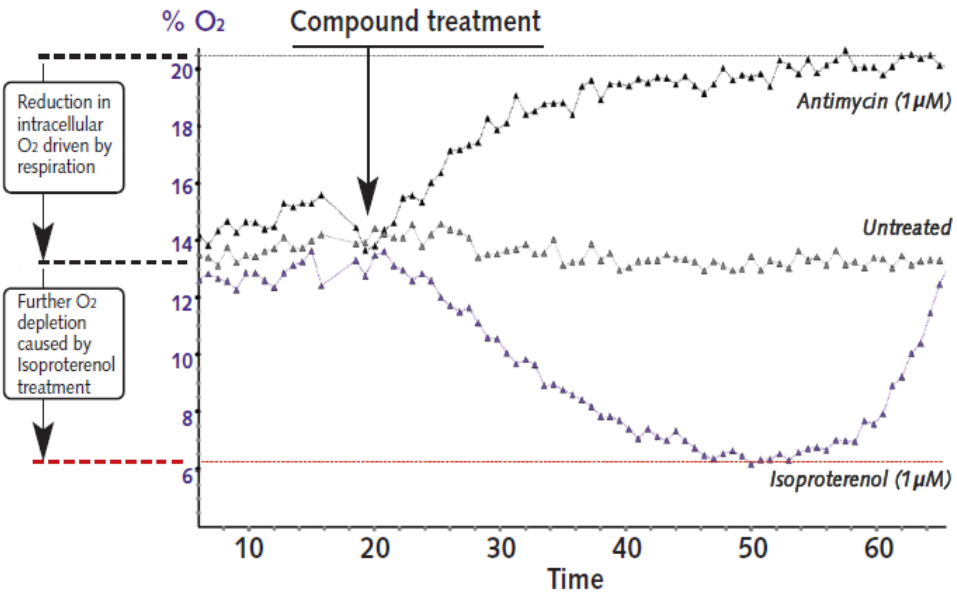


Figure 6. Measuring the impact of cell metabolism on iPS-derived cardiomyocyte oxygenation. During measurement, cells are treated with antimycin (ETC inhibitor) and isoproterenol (β-adrenoreceptor agonist).

17. Notes

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)