Chapter 8
Monitoring of Extracellular and Intracellular O_2 on a Time-resolved Fluorescence Plate Reader

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Abstract

Oxygen is an essential molecule in maintaining healthy cellular homeostasis and metabolism and is fundamental in both health and disease in eukaryotic organisms. The ability for the mitochondria to generate ATP *via* oxidative phosphorylation is totally dependent on oxygen. As oxygen is a key molecule, the ability to study and quantify its usage is a powerful tool to investigate the dynamic relationship between cellular bioenergetics and phenotype. The capability to accurately measure oxygen within a cell population has made great advances in the last 30 years, ranging from the traditional electrodes to modern intracellular oxygen sensing probes using time-resolved fluorescence plate readers. In this review we discuss the advances made in the field of cellular oxygen sensing, with emphasis on medium to high throughput cell-based assays.

8.1 Introduction

Oxygen is a life-sustaining molecule with a crucial role in cell homeostasis and metabolism, integral to both health and disease in eukaryotic organisms. Approximately 90% of the oxygen we consume contributes to the process of mitochondrial oxidative phosphorylation (OxPhos).[1] Oxygen is required by normal differentiated cells for the efficient oxidative metabolism of substrates including glucose and fatty acids. Mitochondrial oxidation converts substrates into carbon dioxide and water yielding the high energy substrate ATP. The remaining 10% of oxygen is used for enzymatic reactions *e.g.* oxidation and hydroxylation. A residual proportion of oxygen contributes to the production of reactive oxygen species (ROS) and this can vary in disease states and when the respiratory chain compromised.[1] Therefore, oxygen has an important role to play in maintaining mammalian cell function providing the necessary ATP for cellular work, assisting in enzymatic reactions and generating ROS, which plays a role in a number signaling pathways.[2] The capability to study and quantify the effects of oxygen utilisation and the level of oxygenation in relation to mitochondrial function is extremely important for understanding the dynamic relationship between cellular bioenergetics and the effect it can have on cell phenotype.

8.2 Measuring Oxygen

8.2.1 Clark Electrodes

Today, there are numerous methods available that allow us to measure oxygen consumption and respiration *in vitro*. Clark type electrodes were, for many years, the workhorses in oxygen assessment and measuring mitochondrial oxidative metabolism. Clark type electrodes use amperometric oxygen sensors based on the principle that when oxygen is dissolved in a solution and placed in the sample chamber oxygen can be detected and monitored by polarography.[3] The instrumental setup consists of a positively charged anode (silver) and a negatively charged cathode (platinum) immersed in a saturated potassium chloride solution.[4] An oxygen permeable Teflon membrane surrounds the probe (Figure 8.1A). Upon the application of the electric current oxygen contained in the test solution of interest will diffuse through the Teflon membrane towards the cathode and get reduced, resulting in the production of hydrogen peroxide. The H_2O_2 subsequently oxidises the silver on the anode producing an electrical current. Oxygen tension is proportional to the measured current generated by the electrode (Figure 8.1C). There are advantages of using such a system: (1) it can be used on intact cells as well as permeabilised cells and isolated mitochondria; (2) ease of addition of drug/inhibitor/substrate combinations for monitoring electron transport chain (ETC) complex activity.[5] However, some of the shortcomings of this technique are: (1) it can be time consuming to assemble the apparatus, thus making it unsuitable for high throughput applications; (2) the addition of inhibitors/drugs will require the sample chamber to be thoroughly cleaned to remove any residual compounds from interfering with subsequent measurements; (3) large volumes are required which would need a higher number of

cells; (4) the electrode is known to introduce interferences as it consumes oxygen.[6]

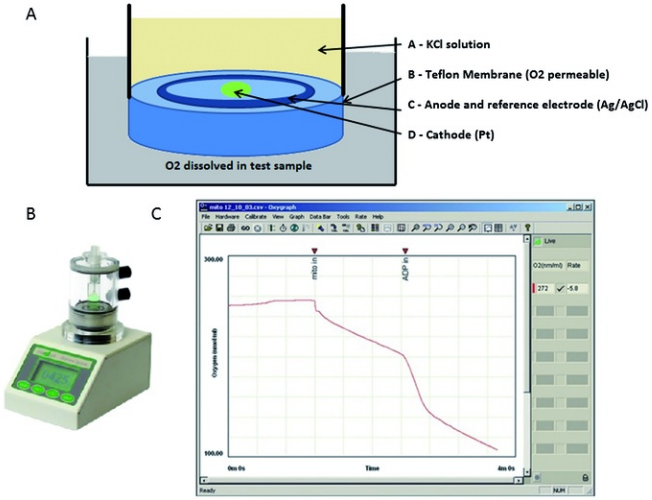

Fig. 1 A typical Clark electrode setup showing the design of the measuring probe (A), a reading chamber (B) and a representative Oxygraph data output (C).

8.2.2 Phosphorescent Porphyrin-based Probes

Porphyrin-based sensors, first developed in the 1980s, provide the basis of current plate based oxygen sensing systems.[7] These phosphorescence-based sensors are oxygen-sensitive and subject to quenching by high oxygen concentrations. In this chapter we will describe two plate-based approaches, the Seahorse Bioscience XF Analyser (Agilent) and Luxcel MitoXpress assay which uses porphyrin-based oxygen-sensing probes.[3] The key difference between the two systems is that the Seahorse XF Analyser requires a specialised instrument to read the specialised plates while the Luxcel MitoXpress® system is compatible with many standard fluorescence plate readers and microplates.

8.2.2.1 Seahorse XF Analyser

The Seahorse Bioscience XF24 Extracellular Flux Analyser uses oxygen-sensitive fluorophores to determine the oxygen consumption rate (OCR) of intact cells (Figure 8.2). It couples this with a measurement of media acidification giving information on extracellular acidification rate (ECAR), linked to glycolytic flux. The solid-state sensors (oxygen and proton) sit on the ends of plastic pistons that make up the XF sensor cartridge. This cartridge then fits over the assay plate (24- and 96-well plate formats are available for higher throughput analyses). When the sensor cartridge is in the reading position thus measuring, the oxygen and proton sensors are approximately 5 millimetres above the cells. Such a distance would require several hours to detect a significant change in oxygen or proton concentrations. In addition, the well would need to be sealed to prevent back diffusion of atmospheric oxygen from dissolving into the media as is the case with the MitoXpress®-Xtra assay, discussed later. To avoid the need for a sealed system and speed up measurement times, the sensors are lowered to within 200 µm of the cells. This close proximity creates a better sealed micro-chamber of either 3 or 7 µL, in the 96- and 24-well plate formats respectively, where back diffusion interference is minimal.[3] After reading, the cartridge is raised to its original position and the medium re-equilibrates. Subsequent measurements can be made every few minutes for the duration of the assay.[8] Some of the major advantages of this system include:

(1) Much smaller volumes of sample and lower numbers of cells are required compared with Clark type electrode measurements.

(2) OCR as a standalone measurement will give an indication of mitochondrial function in intact cells allowing the impact of cellular metabolism (*i.e.* substrate flux and metabolic pathways) on mitochondrial function to be determined. This information cannot be obtained from studies of isolated mitochondria. OCR can also be paired with acidification rate (ECAR) to provide parallel information on glycolysis within the same wells.

(3) Has a higher throughput multi-well plate format allowing for either 24 or 96 wells to be measured per run. This enables a variety of treatments with multiple replicates to be measured simultaneously.

(4) Enhanced sensitivity due to the creation of a microchamber.

(5) The presence of four injection ports allows for compound/substrate addition.

(6) The assays are considered non-destructive so the cells can be used for subsequent downstream analyses such as ATP or viability tests, which can serve to normalise the Seahorse XF data.

(7) With the addition of mitochondrial respiratory modulators such as oligomycin, FCCP and antimycin A, an insight into the maximal rate of mitochondrial respiration and spare reserve capacity is obtained, Figure 8.2C.

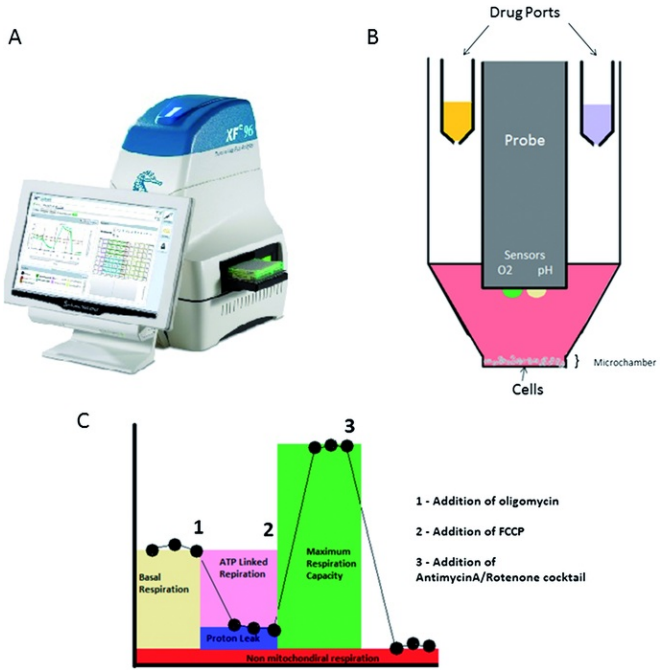

Fig. 2 The Seahorse XF Flux Analyser (A) is a dedicated stand-alone instrument used for the measuring and assessment of mitochondrial oxidative metabolism and extracellular acidification due to glycolysis. The oxygen and pH sensors are mounted on a moving probe (B) adjacent to injection ports for substrate/drug additions. The resulting data output resembles the graph shown in (C) giving information about various aspects of cellular respiration upon the addition of drugs at time point 1 (oligomycin), 2 (FCCP) and 3 (antimycin A/rotenone).

Notwithstanding the many benefits of this system over older methods there are still a number of drawbacks including the expense of the instrument and the associated consumables (sensor cartridges and plates). Furthermore, the addition of mitochondrial inhibitors and uncouplers to the cells may affect cellular function and impede downstream assays thus limiting the potential for a multiplexed analytical approach. Overall, the Seahorse XF assays offer users an easy to measure platform. The real-time kinetic information generated is far superior to more single read endpoint type assays. The facility to inject compounds that perturb the mitochondria gives a more rounded evaluation of the cellular bioenergetics of the cell being measured. However, the cost of the instrument and its very specialised applicability means it may not be achievable for many end users.

8.2.3 Oxygen Sensing Plate Based Assays Using Standard Tissue Culture Plates

A complementary system to the Seahorse Bioscience XF and one that also uses an oxygen sensitive fluorophore is MitoXpress developed by Luxcel Biosciences. Similar to the Seahorse XF, MitoXpress assays are based on a phosphorescent probe being quenched by high oxygen concentrations in the surrounding cellular environment.[9] Both systems facilitate high throughput research using whole cells or isolated mitochondria making them preferable in a drug discovery setting. However, there are some significant differences between the two approaches. As mentioned previously, the Seahorse XF is a standalone instrument, using bespoke plates and pre-prepared probe assay kits. In contrast, the MitoXpress kit can be used on standard black tissue culture plates with clear bottom and the assay can be performed on a standard fluorescence plate reader. Readers with time-resolved fluorescence mode (TRF) provide enhanced performance and ones that incorporate an atmospheric control unit (ACU) have a large advantage over the Seahorse XF allowing for tighter regulation of oxygen and carbon dioxide. The data outputs for the two systems are quite different. The Seahorse XF approach does not present the raw data but provides an output showing oxygen consumption rates following the sequential modulators of mitochondrial function (Figure 8.2C). These are then used as an output of mitochondrial fitness providing information on different aspects of mitochondrial function. The MitoXpress assays provide an output linked to the software available on the plate reader. Raw data is available and can be used to calculate rates of oxygen consumption with the benefit of observing mitochondrial respiration over several hours. Plate reader manufacturers *i.e.* BMG Labtech now provide specific software modifications which allow for a rapid analysis of raw data. The MitoXpress assay can be used in the same way as the Seahorse XF system to identify aspects of mitochondrial fitness with the modulator compounds added during the assay set up.

8.2.3.1 MitoXpress® Oxygen Probes

The Luxcel Biosciences MitoXpress probe system uses a phosphorescent Pt-porphyrin based oxygen-sensitive probe to measure oxygen consumption rates. Oxygen reversibly quenches the probe phosphorescence so depletion of oxygen in the media due to the activity of respiring cells will result in an increase in measured phosphorescence intensity and lifetime signals.[10] The probe can be measured on any standard fluorescence capable plate reader although ones with TRF capability are preferred. The instrumental set up consists of dual delay time resolved measurements (Exc. 340–400 nm/Em. 640–660 nm, with delay times of 30 and 70 µs and the subsequent conversion of RFU values to phosphorescence lifetime values using the following equation: Lifetime = $(t_2 - t_1)/\mathrm{Ln}(D_1/D_2)$ where t is delay time and D is measured TRF intensity value). From this equation, a lifetime curve is generated where time (x-axis) and lifetime (y-axis) are plotted (Figure 8.3). The OCR is then determined from the slope of the linear portion of the lifetime curve. Lifetime is defined as the time in which a phosphorescent O_2-sensitive molecule exists in an excited state before returning to the ground state. Lifetime values are an important means of normalising the data from these probes, minimising and often eliminating variables like concentration of fluorophore added or variations in the intensity of excitation and allowing simple calculation of oxygen concentration.

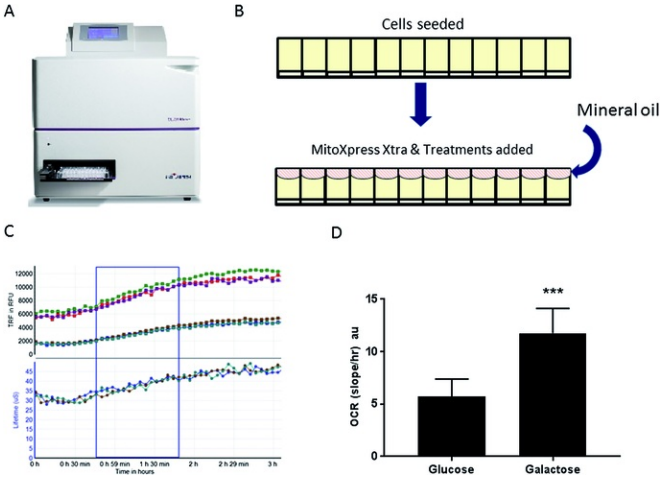

Fig. 3 The MitoXpress oxygen sensing probe can be used on standard plate readers, pictured (A) is a BMG Labtech CLARIOstar reader with an ACU. Assays are set up in standard black clear bottom culture plates (B). The conversion of TRF to lifetime (C) is required for data utilisation where the blue box is placed on the linear portion of the curve to determine slope (µs/h), which corresponds to oxygen consumption rate (OCR). (D) Example data of cells incubated for 72 hours in glucose/glucose free (galactose) medium.

8.2.3.2 MitoXpress-Xtra

Due to some shared features with the Seahorse XF, MitoXpress-Xtra comes with many of the same advantages including ease of use, small sample volumes and having a high sample throughput. Both systems measure oxygen levels in the culture media. Some additional advantages of the MitoXpress-Xtra system include:

(1) Relatively inexpensive consumables allow for smaller experiments to be set up where only part of the plate needs to be used.

(2) MitoXpress will work on any fluorescence plate reader ruling out the need for expensive dedicated equipment;

(3) Depending on the plate reader used there can be a greater variety of multi-well plate assay formats, from 6- to 384-well plates;

(4) MitoXpress-Xtra has a multiplexing capability with other assays run in the same well or in adjacent wells on the same microplate;

Despite needing smaller sample volumes than a Clark electrode measurement the MitoXpress-Xtra system does not create as small a measuring volume as the Seahorse XF. Typical volumes are in the range of 150 µL thus requiring up to 120 minutes in order to achieve measurable changes in lifetime values. The system is therefore sealed using a mineral oil, making it more difficult to perform some downstream assays, in addition to MitoXpress-Xtra assay. If multiplexing with other assays they must be conducted before the oil is added or simultaneously with oxygen measurements. Simultaneous measurement of time resolved fluorescence with other fluorescence applications is possible with many readers, including the BMG Labtech CLARIOstar system using MARS software. Injections to the wells can be made if the reader is equipped with injectors and any additions to be made must be at the start before mineral oil is added. The measured OCR rates are influenced by (A) the metabolic activities of the cells being examined but also (B) the number of cells present in the well. Any deviation in the cell numbers can lead to compromised reproducibility. For MitoXpress-Xtra assays it is useful to run parallel standard reader based quantification techniques including Hoescht 33342 and Crystal violet (DNA) or BCA (protein), to normalise the rate of oxygen consumption

to cell number/protein content. Cell number cannot be determined on the cells used in the OCR measurement, as the oil used to seal these samples does not allow for the recovery of the cells for further analysis. Due to the larger well volume cells with slower rates of oxygen consumption, which often need to be assayed in suspension, are easily measured using the MitoXpress-Xtra assay. These include human peripheral blood mononuclear cells (PBMC). A typical experiment is shown in Figure 8.4: for human control PBMCs 500 000 cells are required to be added to each well. As can be observed oxygen consumption rates in these cells are low in non-activated cells with a considerable amount of oxygen consumption due to mitochondrial respiration (total respiration rate assessed by antimycin A treatment). When stimulated with the phorbal ester PMA, PMBCs demonstrate a large increase in oxygen consumption which is unaffected by co-incubation with mitochondrial inhibitors (Figure 8.4). This suggests that the burst of oxygen consumption is not derived from mitochondrial respiration and is most likely due to the NADPH oxidase, a known response to a pathogen with cells generating high levels of superoxide. As the MitoXpress-Xtra assay can be followed for several hours, an interesting difference is observed between PMA stimulation with and without mitochondrial inhibitors. The cells treated with PMA alone maintain a higher rate of oxygen consumption than those treated with inhibitors suggesting that mitochondrial function is important post PMA stimulation in maintaining cellular function.

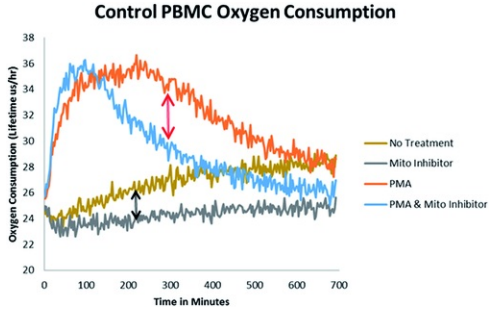

Fig. 4 Lifetime profiles of PBMC cells untreated (yellow), treated with mitochondrial inhibitors antimycinA and rotenone (grey), PMA (orange) or PMA + mitochondrial inhibitors (blue). The black and red arrows indicate the mitochondrial contribution of the PBMC to the depicted profiles.

In the majority of eukaryotic cells, ATP is produced by oxidative phosphorylation in the mitochondrial respiratory chain. The integrity of the mitochondrial membrane potential is critical for maintaining the physiological function of the respiratory chain to generate ATP. Therefore, the ability to investigate dual parameters of cellular health such as mitochondrial function and cell viability is of great value as it saves time, material and cost. Parallel assays in other wells on the plate could be used in conjunction with MitoXpress-Xtra giving further information about cell death, *e.g.* CellTox Green (Promega) and mitochondrial membrane potential (TMRM or JC-1, Cayman Chemicals). For instance, using the oxygen sensing MitoXpress Xtra probe in conjunction with the mitochondrial membrane dye JC-1 enable to measure the bioenergetic state of the cells by monitoring both the mitochondrial membrane potential and the oxygen consumption in response to stimuli.

8.3 Measurement of Intracellular Oxygen

The importance of oxygen availability cannot be overstated. There are many physiological and indeed pathophysiological requirements for oxygen especially in cancer and the adaptive response to hypoxia. Cells and tissues are exposed to different levels of oxygen. Most tissues have a partial pressure of oxygen (pO_2) of 20–40 mm Hg but a large tumor can be severely hypoxic or anoxic with pO_2 levels <0.1 mm Hg.[11,12] Hypoxia is observed in most solid tumors with up 60% exposed to <1% oxygen.[13] Hypoxia and HIF signaling are important in the metabolic reprogramming of cancer cells so measuring *in situ* hypoxia and oxygenation is essential in studies looking to target hypoxic cancer cells which tend to be resistant to current drug therapies. Cells vary in their energy needs and depending on their location within the body can be exposed to varying oxygen tensions. Epithelial cells lining the bronchi are exposed to atmospheric oxygen levels while chondrocytes are exposed to low oxygen.[14] Most standard tissue culture experiments are carried out in an atmosphere of 20% oxygen, which is higher than found in well oxygenated parts of the body and considerably higher than levels found in lower oxygenated regions. Because methods have not been available to accurately measure and control intracellular oxygen levels, the studies of impact of oxygen on cell metabolism *in vitro* beyond hypoxia (<1% oxygen) have not been carried out properly. It is conceivable that oxygen levels between 1–20% could have an impact on cell function. Oxygen levels have been shown to have a significant impact on metabolism, differentiation and development[15-17] and senescence.[18,19] With ROS heavily involved in signalling[20,21] levels of oxygen in the environment could have a significant impact on a variety of cellular processes. As oxygen levels fluctuate *in vivo* having a different impact on cells in an organ depending upon their proximity to blood vessels, the ability to control and determine intracellular levels during *in vitro* experiments is of vital importance. Oxygen concentrations and consumption rates can be difficult to monitor as there is constant diffusion between the cells and the extracellular environment and between the atmosphere and cell culture media solution.[22] As described earlier techniques to measure oxygen based on phosphorescence quenching are well established[23] but mainly as an extracellular means of assessing oxygen levels and consumption rates.[10] However, monitoring this analyte within a cell has

remained problematic for years. Previous attempts to quantify intracellular oxygen have included using probes that needed to be microinjected,[24] electroporation or induce endocytosis.[25] Complex, time consuming and invasive such methods have hindered advances in this area.

8.3.1 MitoXpress-Intra Probe

In an effort to address some of these issues a new product recently added to the Luxcel Biosciences portfolio is the MitoXpress®-Intra probe. MitoXpress-Intra is potentially an extremely valuable *in vitro* tool to investigate the level of intracellular oxygenation. The MitoXpress®-Intra probe consists of cationic hydrogel nanoparticles loaded with an oxygen-sensitive PtTFPP (Pt(II)-5,10,15,20-tetrakis-(2,3,4,5,6-pentafluorophenyl)-porphyrin) dye (Figure 8.5). It works on the same principle as the MitoXpress-Xtra, discussed earlier, whereby molecular oxygen present in the cells is able to quench the emission of the probe reversibly. However, the difference lies in its cell-penetrating properties that enable the real-time quantification of oxygen within the cell monolayer using plate reader and microscopy-based techniques.[26] Cells are cultured to 80–90% confluence in appropriate culture media and left to adhere. Media is then replaced with 150 µL fresh culture media containing 10 µg mL^{-1} of probe. The particles are internalised *via* endocytosis[26] and the probe remains in the cells and does not leak back over time. However, upon cell division it will become less concentrated. Maximum probe uptake generally occurs in 6–16 hours. The small particle size (<50 nm) allows high penetration with minimum damage/toxicity to the cells.[22]

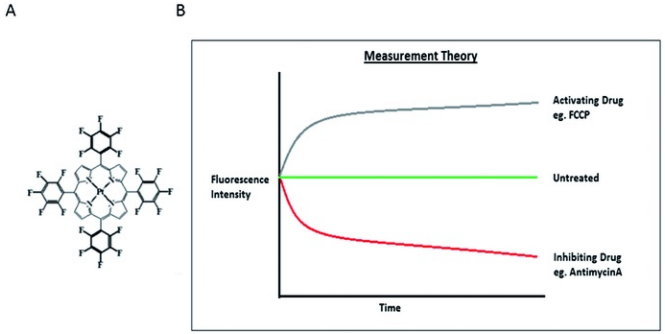

Fig. 5 The structure of the MitoXpress-Intra probe (A) and the theory behind the drug response observed when using it (B). If a treatment increases oxidative phosphorylation it will cause an increase in fluorescent signal and conversely a decrease in signal will be seen if a treatment results in inhibition. (A) reprinted with permission from A. Fercher, S. M. Borisov, A. V. Zhdanov, I. Klimant and D. B. Papkovsky, *ACS Nano*, 2011, **5**, 5499–5508. Copyright (2011) American Chemical Society.

Unlike previous extracellular oxygen sensing probes, which acted in sealed environments, the Seahorse XF (plunging probe) and MitoXpress-Xtra (oil), the wells are left unsealed so steady-state is reached between oxygen consumption by the cells and back diffusion into the well from the environment. Cells will reach a “resting steady-state” characterised by a flat lifetime signal, which correlates with the intracellular oxygen level. When cells are stimulated to consume more oxygen *e.g.* with uncouplers like FCCP or inhibited from using their mitochondria *e.g.* with antimycin A treatment, changes in phosphorescence lifetime signal will be observed. Reduced ETC activity results in a decrease in probe signal (*i.e.* high cellular O_2) as a result of reduced oxygen consumption by the cells. Conversely, an increase in probe signal will be seen where there is a reduction in cellular oxygen due to stimulated respiration (*i.e.* increased ETC activity). This is depicted in Figure 8.5.

To facilitate quantification of intracellular oxygen levels a calibration curve for MitoXpress-Intra, which converts measured lifetime values into the percentage oxygen within the cell monolayer, is provided by the vendor. To verify this calibration, cells loaded with MitoXpress-Intra probe are treated with a cocktail of 1 µM rotenone and 10 µM antimycin A. These compounds inhibit cellular respiration thereby increasing intracellular oxygen levels within cells to environmental oxygen levels in the plate reader chamber. The BMG Labtech CLARIOstar plate reader comes equipped with an atmospheric control unit that allows the user to control the temperature, oxygen (range from 19 to 0.1%) and carbon dioxide levels in the microplate chamber. To construct a calibration curve the antimycin A and rotenone- treated cells are placed in the reader at 37 °C with 5% CO_2. When the assay is started the oxygen level should be set to atmospheric ~19% and allowed to run until the fluorescence equilibrates to a flat line. Once this equilibrium is reached the oxygen levels can be dropped in a stepwise manner to 12, 8, 4, 2, 1 and 0.1% allowing for a steady state to be reached at each concentration before reducing the oxygen levels. At this point, glucose oxidase can be injected into the wells thereby chemically deoxygenating them and allowing a 0% oxygen concentration to be achieved. Once the lifetime kinetic trace has been produced (Figure 8.6A), a calibration curve of lifetime *versus* % inspired oxygen is plotted (Figure 8.6B). To this plot a mono-exponential decay fit is applied giving the model fit equation (Figure 8.6B).

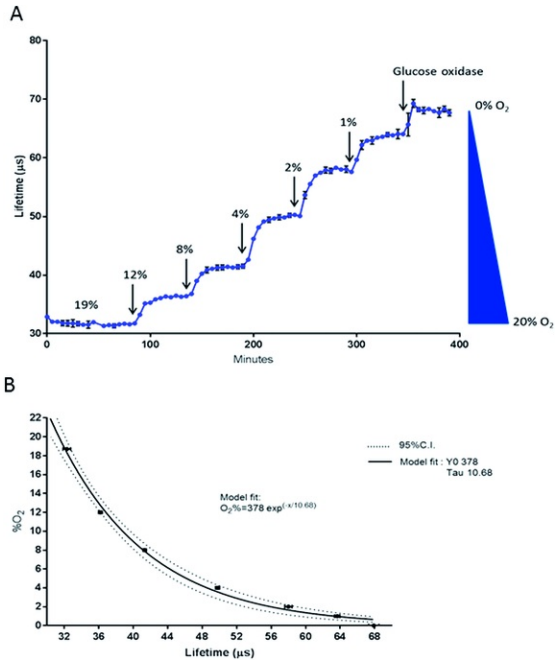

Fig. 6 Kinetic trace of the intracellular probe showing the response to decreasing atmospheric oxygen concentrations (A). Starting atmospheric oxygen concentration was 19%, once the signal had equilibrated the oxygen was dropped to 12%, 8%, 4%, 2%, 1%. Complete de-oxygenation was achieved by the addition of glucose oxidase. (B) Intracellular probe calibration curve of Lifetime *versus* % inspired Oxygen with a monoexponential fit curve.

Once the model fit has been calculated, this equation allows future determinations of the intracellular oxygen levels within a cell monolayer by inputting the lifetime value into the equation, where % O_2 = 378 $\exp^{(-x/10.68)}$ and x is the measured lifetime value.

The calibration curve is only generated once and can be used to determine the oxygen concentration for that cell line in all subsequent assays. In practice, the calibration curve should work for all cell lines as long as the inhibition of respiration by the respiratory chain inhibitors is complete. The utilisation of MitoXpress-Intra will be of great benefit in future studies by allowing more accurate determination of oxygen concentrations within a cell population. The impact of metabolic stimulation and drug effects can now be easily examined and compared under controlled oxygen concentration, more closely simulating their physiological environment. This probe enables us to further our understanding of intracellular oxygen gradients, which will be vital in studies investigating metabolic disorders and mitochondrial function.

8.3.2 Application of the MitoXpress-Intra Probe

MitoXpress-Intra has proven to be a useful tool in the area of cancer metabolism and the Warburg effect. Potter *et al.* employed the method to examine the metabolism of two established cancer cell lines, RD (rhabdomyosarcoma) and U87MG (glioblastoma multiforme) under different applied oxygen concentrations with the results highlighting some interesting findings.[27] RD cancer cells cultured on standard DMEM with high glucose (25 mM) do not rely solely on glycolysis for ATP production. In fact, they displayed high rates of oxygen consumption on high glucose media consistent with a cell with an aerobic phenotype. When ambient oxygen is set to 18% oxygen one might assume that the cells are at 18% as it has been reported that the rate of oxygen diffusion into the cells is faster than the oxygen consumption rate.[28] However, when the intracellular oxygen levels were assessed the aerobically proficient RD cells, at ambient oxygen concentrations of 18, 10 and 5%, had intracellular levels of 13.67, 6.15 and 2.5% respectively. In contrast, U87MG cells, which are well known for their glycolytic metabolism, do not deplete the intracellular oxygen and as such they reflect the applied oxygen concentration. In the study, U87MG cells displayed oxygen levels of 17.9, 9.65 and 4.6% when incubated at 18, 10 and 5% oxygen. Overall this study highlighted that in aerobically competent cells intracellular oxygen levels can be significantly lower than what you might anticipate and this may have far reaching implications especially in the field of hypoxia with the applied oxygen not necessarily reflecting intracellular levels. Further studies have now expanded this work demonstrating that cells with high rates of oxygen consumption including INS-1 and HEPG2 cells have intracellular oxygen levels below 1% when incubated in an atmosphere of 2% O_2.[29] Not all cell types respond to hypoxia in the same way. Some cells can survive in anoxia while others will die. Chi *et al.* found significant heterogeneity in the hypoxic responses between a variety of cell types.[14] Three mechanisms underpinning the difference in hypoxia response in tumours have been proposed including (i) variations in oxygen tension in the tumours; (ii) cell type-specific differences in the

extent/threshold of the response to hypoxia; or (iii) genetic changes that result in incorrect activation of the hypoxia response (pseudohypoxia).[14] While the hypoxic response in some tumours is caused by mechanisms (ii) and (iii) such as loss of von Hippel Lindau (VHL) gene in renal clear cell carcinomas the hypoxia response in most tumours is thought to lie in the variations in oxygen tension. Accurately measuring the transient changes in oxygen tension is therefore crucial for hypoxia studies and MitoXpress-Intra could prove to be a vital tool.

Standard 2D cell culture conditions are ubiquitous in cell biology and it is well documented that these models do not accurately portray the complexity of *in vivo* environments. 3D spheroids provide heterogeneous microenvironments that more accurately reflect tissue, including cell-to-cell interactions, cell–matrix interactions as well as gradients of oxygen, nutrients, waste products and drugs. MitoXpress®-Intra in 3D models has the potential to further our understanding of individual oxygen concentration gradients in 3D systems and is an important area to develop. Oxygen gradients will exist in the structure that create heterogeneous sub-populations of quiescent, hypoxic and anoxic cells each of which will have distinct metabolic profiles.[30] Measuring oxygen consumption externally has proven to be useful but has its limitations, which the MitoXpress-Intra can overcome. It can assess drug effects on 3D structures in a real time and non-invasive manner. Traditional methods of assessing spheroid "health" are time-consuming and involve spheroid dissociation or fixing and staining prior to sectioning and immunohistochemistry. Using the intracellular probe potentially allows the assessment of spheroid health in minutes with the ability to assess the effects of a drug screen in real time.

8.4 Future Applications of Plate-based Oxygen Monitoring Systems

Molecular oxygen has an important role in the ETC and is therefore a valuable marker of mitochondrial function and cellular metabolism crucial to many disease research areas. Standard cell culture protocols use hyperoxic conditions of 21% (160 mm Hg). However, apart from alveolar cells, which experience 110 mm Hg (14.5%) not many cells in the body are actually ever exposed to such high levels of oxygen under normal physiological conditions. In the circulation dissolved oxygen can be anywhere between 5–13% (40–100 mm Hg). Cells and tissues that are not in close proximity to blood vessels display considerably lower oxygen concentrations depending on: (1) the distance from the nearest blood vessel, and (2) the metabolism (oxygen consumption) of a particular cell type. Cellular levels of oxygen have been reported to lie between 1.3–2.5% while in the mitochondria oxygen is often less than 1%.[31] Using more appropriate, biologically relevant conditions is important as cells are likely to behave differently according to changes in their environment which may also impact on the efficacy of potential drug treatments being explored as therapeutic agents.[32,33]

As researchers we need to be more mindful of our *in vitro* cell conditions by considering substrate concentrations (*e.g.* glucose, glutamine, fatty acids and lactate) in addition to oxygen levels. As we are starting to observe with glucose, the conditions we work in can have a significant impact on cell metabolism[34] and the impact pharmacological agents.[35] Developing models which allow better control of environmental conditions maintaining substrates such as glucose at tissue levels (1–5 mM) in long-term experiments are essential if we are to make the relevant conclusions from our *in vitro* culture systems. We need to change our thinking from our current belief that leaving cells, in what is essentially glucose syrup, for many days will produce meaningful data. This is especially relevant in data produced from an experiment, which explores, for example, the long-term effects of hypoxia. Cells grown in non-physiological conditions will have significantly altered metabolic programs.[36]

One example of potential problems caused as a consequence of cell culture conditions relates to the Warburg Effect. For decades the dominating belief was that the Warburg Effect (also commonly known as aerobic glycolysis) was a primary feature of cancer. In 2011 it was even assigned as a hallmark of the disease.[37] However, in more recent times studies are emerging that lay challenge to this. Due to advances in the technologies that allow the study of cellular metabolism using metabolic tracers *in vivo* and a new-found realisation of the importance of controlling and accounting for non-physiologic conditions of *in vitro* testing cancer cells are proving to be a diverse mix of bioenergetic profiles which have a significant degree of plasticity depending upon environmental conditions. For instance, the well-documented Crabtree effect results in a reduction in respiration when glucose levels are elevated.[38] Glucose concentration can have a major impact on the bioenergetics of a cell and depending on the level of mitochondrial activity, cells may respond differently to xenobiotics designed to target the mitochondria. Marroquin *et al.* demonstrated that glycolytic HepG2 cells were resistant to some well-known mitochondrial toxicants like rotenone and antimycin A when cultured under standard (high glucose) conditions. However, when glucose media was replaced with media containing galactose cells derived ATP from oxidative metabolism and sensitivity to the mitochondrial toxicants was restored.[35] Using this approach, mild to moderate impairment of mitochondrial respiration can be identified; impairments that may have been masked when cultured in high glucose.[39] This highlights the potential importance of controlling cell culture conditions to better reflect the conditions in actual cells/tumours in order to gain a deeper insight into cellular bioenergetics. Oxygen can have similar effects to glucose as shown in recent study by Huang *et al.* using the PI3K inhibitor LY294002.[40] The drug displayed a differential effect on cell lines at 20% and 1% oxygen with significantly reduced levels of killing under low oxygen conditions. In experiments carried out in 25 mM glucose the two cell lines RD (high OxPhos) and U87MG (low OxPhos, high glycolysis) also showed different drug effects with the U87MG cells adopting a glycolytic phenotype being much more sensitive to PI3K inhibition. As U87MG cells switch to a more OxPhos phenotype under physiological glucose conditions (1 mM)[34] it is very likely the drug response to the PI3K inhibitor will be significantly different in this cell line under physiological glucose conditions.

Moving into the future being mindful of and adopting more relevant cell culture practices is imperative. There is an array of technology available that allows for in-depth investigation of cellular metabolism

but their use and relevance are reliant on adopting more physiological *in vitro* culturing protocols. The plasticity of cells to adapt their metabolism based on substrate supply/availability and the inaccurate assumption that intracellular oxygen can be inferred from applied oxygen concentrations cannot be disregarded. To do so may lead to inaccurate results and flawed conclusions, which only serve to weaken our knowledge and understanding.

References

1 J. W. Baynes, *Oxygen and Life*, MOSBY Elsevier, 2009, ch. 37

2 X. Xu. , Z. Li, X. Leng, M. He, J. Wang, S. Cheng and H. Wu, Roles of reactive oxygen species in cell signalling pathways and immune responses to viral infections, , *Archives in Virology,*2017, **163**, 603-610.

3 M. Koopman, H. Michels, B. M. Dancy, R. Kamble, L. Mouchiroud, J. Auwerx, E. A. Nollen and R. H. Houtkooper, *Nat. Protoc.,* 2016, **11,** 1798–1816.

4 C. Diepart, J. Verrax, P. B. Calderon, O. Feron, B. F. Jordan and B. Gallez, *Anal. Biochem.,* 2010, **396,** 250–256.

5 J. Zhang, E. Nuebel, D. R. Wisidagama, K. Setoguchi, J. S. Hong, C. M. Van Horn, S. S. Imam, L. Vergnes, C. S. Malone, C. M. Koehler and M. A. Teitell, *Nat. Protoc.,* 2012, **7,** 1068–1085.

6 O. S. Wolfbeis, *BioEssays,* 2015, **37,** 921–928.

7 W. L. Rumsey, J. M. Vanderkooi and D. F. Wilson, *Science,* 1988, **241,** 1649–1651.

8 Chomicz. S. , D. Ferrick and W. Min, *Genet. Eng. Biotechnol. News,* 2007, **27**, **19.**

9 Y. Will, J. Hynes, V. I. Ogurtsov and D. B. Papkovsky, *Nat. Protoc.,* 2006, **1,** 2563–2572.

10 J. Hynes, E. Natoli Jr and Y. Will, *Curr. Protoc. Toxicol.,* 2009.ch. 2, Unit 2 16

11 J. Aragones, P. Fraisl, M. Baes and P. Carmeliet, *Cell Metab.,* 2009, **9,** 11–22.

12 G. L. Semenza, *Science,* 2007, **318,** 62–64.

13 P. Vaupel, M. Hockel and A. Mayer, *Antioxid. Redox Signaling,* 2007, **9,** 1221–1235.

14 J. T. Chi, Z. Wang, D. S. Nuyten, E. H. Rodriguez, M. E. Schaner, A. Salim, Y. Wang, G. B. Kristensen, A. Helland, A. L. Borresen-Dale, A. Giaccia, M. T. Longaker, T. Hastie, G. P. Yang, M. J. van de Vijver and P. O. Brown, *PLoS Med.,* 2006, **3,** e47.

15 M. Takahashi, *J. Reprod. Dev.,* 2012, **58,** 1–9.

16 N. Haque, M. T. Rahman, N. H. Abu Kasim and A. M. Alabsi, *Sci. World J.,* 2013, **2013,** 632972.

17 K. Fynes, R. Tostoes, L. Ruban, B. Weil, C. Mason and F. S. Veraitch, *Stem Cells Dev.,* 2014, **23,** 1910–1922.

18 S. Parrinello, E. Samper, A. Krtolica, J. Goldstein, S. Melov and J. Campisi, *Nat. Cell Biol.,* 2003, **5,** 741–747.

19 D. H. Betts, S. D. Perrault and W. A. King, *Biogerontology,* 2008, **9,** 19–31.

20 M. Benhar, *Biochim. Biophys. Acta,* 2015, **1850,** 2476–2484.

21 D. P. Jones and H. Sies, *Antioxid. Redox Signaling,* 2015, **23,** 734–746.

22 A. Fercher, S. M. Borisov, A. V. Zhdanov, I. Klimant and D. B. Papkovsky, *ACS Nano,* 2011, **5,** 5499–5508.

23 D. B. Papkovsky, *Methods Enzymol.,* 2004, **381,** 715–735.

24 E. Schmalzlin, J. T. van Dongen, I. Klimant, B. Marmodee, M. Steup, J. Fisahn, P. Geigenberger and H. G. Lohmannsroben, *Biophys. J.,* 2005, **89,** 1339–1345.

25 T. C. O'Riordan, K. Fitzgerald, G. V. Ponomarev, J. Mackrill, J. Hynes, C. Taylor and D. B. Papkovsky, *Am. J. Physiol.: Regul., Integr. Comp. Physiol.,* 2007, **292,** R1613–R1620.

26 A. V. Kondrashina, D. B. Papkovsky and R. I. Dmitriev, *Analyst,* 2013, **138,** 4915–4921.

27 M. Potter, L. Badder, Y. Hoade, I. G. Johnston and K. J. Morten, *Adv. Exp. Med. Biol.,* 2016, **876,** 257–263.

28 I. Papandreou, T. Goliasova and N. C. Denko, *Int. J. Cancer,* 2011, **128,** 1001–1008.

29 K. J. Morten, M. Potter, L. Badder, P. Sivathondan and R. Dragovic, *et al.*, *Wellcome Open Res.,* 2017, **2, 14.**

30 R. I. Dmitriev, A. V. Zhdanov, Y. M. Nolan and D. B. Papkovsky, *Biomaterials,* 2013, **34,** 9307–9317.

31 A. Carreau, B. El Hafny-Rahbi, A. Matejuk, C. Grillon and C. Kieda, *J. Cell. Mol. Med.,* 2011, **15,** 1239–1253.

32 J. A. Marwick, D. A. Dorward, C. D. Lucas, K. O. Jones, T. A. Sheldrake, S. Fox, C. Ward, J. Murray, M. Brittan, N. Hirani, R. Duffin, I. Dransfield, C. Haslett and A. G. Rossi, *J. Leukocyte Biol.,* 2013, **94,** 1285–1292.

33 A. G. Vij, K. Kishore and J. Dey, *Indian J. Med. Res.,* 2012, **135,** 211–216.

34 M. Potter, E. Newport and K. J. Morten, *Biochem. Soc. Trans.,* 2016, **44,** 1499–1505.

35 L. D. Marroquin, J. Hynes, J. A. Dykens, J. D. Jamieson and Y. Will, *Toxicol. Sci.,* 2007, **97,** 539–547.

36 G. Gstraunthaler, T. Seppi and W. Pfaller, *Cell. Physiol. Biochem.,* 1999, **9,** 150–172.

37 D. Hanahan and R. A. Weinberg, *Cell,* 2011, **144,** 646–674.

38 H. G. Crabtree, *Biochem. J.,* 1929, **23,** 536–545.

39 C. Aguer, D. Gambarotta, R. J. Mailloux, C. Moffat, R. Dent, R. McPherson and M. E. Harper, *PLoS One,* 2011, **6,** e28536.

40 X. Huang, M. Potter, B. Pilgrim, R. Ardkhean, M. Kabeshov, T. Claridge, M. Wiseman, K. J. Morten, T. Donohoe and H. Townley, *Int. J. Cancer Ther. Oncol.,* 2017, **5, 1.**

Queries and Answers

Query: Figure 2 appears to be of low resolution and therefore appears rather blurred. Would you like to resupply the image at higher resolution (ideally >600 dpi in TIF format)?
Answer: We have tried to change this figure but we are unable to improve the quality. We could potentially get these figures from Agilent but this will take longer than a week

Query: Figure 3 appears to be of low resolution and therefore appears rather blurred. Would you like to resupply the image at higher resolution (ideally >600 dpi in TIF format)?
Answer: We can't produce a high resolution image than this. The detail looks fine on the proofs.

Query: The size of the text appears to be small in the artwork of Figure 3. Please resupply the artwork (preferably as a TIF file at 600 dots per inch) with larger fonts if possible.
Answer: The scale on c) are as they come off the MARS software. The font sizes can not be changed

Query: Figure 5 appears to be of low resolution and therefore appears rather blurred. Would you like to resupply the image at higher resolution (ideally >600 dpi in TIF format)?

Answer: We have tried to improve this but failed to improve the quality. With more time we could potentially reproduce the chemical structure by asking Luxcel for help with this

Query: Figure 6 appears to be of low resolution and therefore appears rather blurred. Would you like to resupply the image at higher resolution (ideally >600 dpi in TIF format)?
Answer: Unable to change the quality will take longer than a week to do this. The high resolution image looks fine to me

Query: Ref. 2: Please provide the last name for the 1st author.
Answer: added

Query: Ref. 2: Please provide the journal title and page (or article) number(s).
Answer: Added

Query: Ref. 8: Please provide the last name for the 1st author.
Answer: Should be S Chomicz

Query: Ref. 8, 29 and 40: Please provide the page (or article) number(s).
Answer: Done