

Monitoring the Oxygen Consumption Rates of Cells in Culture

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Introduction

Research in tissue engineering, cell therapy and drug discovery all involve studies of cellular response to biochemical molecules and drugs. These studies involve following relevant cellular parameters such as intracellular calcium, pH or membrane potential, and correlating the response of the parameters to cellular activity. Historically, oxygen consumption could not easily be included among the monitored parameters because there were no convenient oxygen consumption assays. While there have been numerous literature reports studying the oxygen consumption of cells¹⁻¹², none of these has been in a format readily amenable to automated, high-throughput usage.

The doubling time of a culture is a measure of its rate of proliferation, and is a fundamental indication of the compatibility between the cell and its culture environment. Whether the goal is to optimize the expansion of proliferative cells or to optimize conditions for retaining differentiation, the doubling time offers an indication of the cell's state on the proliferation/differentiation spectrum. Traditional methods to assess doubling time require either actual counting of cells or indirect assessments of cellular content by chemical means. The former set of methods are direct and straightforward, but are intrusive and labor-intensive, and require easy access to the cells. While the latter set does not require immediate access to the cells, they are disruptive in that they require the addition of reagents to the cells, and are frequently terminal or irreversible in nature. For the culture of cells in three dimensions, however, these methods are unattractive, as it is often difficult to visualize cells *in situ* or undesirable to perform an invasive or destructive assay, particularly if eventual implantation is a goal.

The BD™ Oxygen Biosensor System (BD™ OBS) is a new cell culture technology that enables researchers to repeatedly and non-invasively monitor the rate of oxygen consumption by cells. The BD OBS incorporates an oxygen-sensitive luminophore into a silicone rubber matrix at the bottom of the wells in a standard BD Falcon™ microplate. When monitored on a standard fluorescence plate reader, the intensity and lifetime of the emitted luminescence varies inversely with oxygen concentration, in a manner consistent with the quenching mechanisms described by the Stern-Volmer theory. The measured fluorescence intensity thus offers direct insight into the oxygen concentration in the well.¹³⁻¹⁴ Under static culture conditions, applying the laws of Fickian diffusion to the system allows equilibrium oxygen concentration to be converted to an equilibrium oxygen consumption rate (OCR). In addition, because the BD OBS can be read repeatedly and in real-time, following the rate of change of oxygen concentration and OCR is facile.

Recently BD Biosciences reported a simple method using the BD OBS to determine the doubling time of bacterial cultures.¹⁵ Here we report an alternate protocol based upon the rate of change of oxygen concentration, suitable for use with mammalian cells in culture. The advantage of the new method is that explicit knowledge of the kinetics of oxygen consumption affords a more informative “handle” on the status of the culture. To demonstrate this method, the results of several studies, including the proliferation of HL60 cells, the impact of glucose concentration on the doubling time of U937 cells cultured in suspension, and the doubling time of osteoblasts cultured on various three dimensional (3D) biodegradable scaffolds are reported. The ability to read the BD OBS repeatedly greatly simplifies the collection of such information.

Background

The emerging field of tissue engineering frequently entails the culture of cells in 3D, under conditions intended to more clearly resemble those found in the physiological environment. As tissue engineering pushes past the boundaries of traditional two dimensional (2D) cell culture, new challenges emerge for assessing the state of the cells. Among the challenges associated with the culture of cells in 3D are the ability to monitor cell viability in real time, oxygenation/necrosis of “interior cells” in 3D constructs and an assessment of the phenotypic or differentiation state of the cells.

The explosion of knowledge in the biology of stem and other progenitor cells means the notion of cell therapy is increasingly becoming a reality. One goal of autologous cell therapy efforts is the harvesting of a small number of progenitor cells from the patient, expanding these *ex vivo*, differentiating them into the tissue phenotype of interest, and then reimplanting them. Accomplishing this goal requires an understanding of the relationship between culture conditions and phenotype, and identifying conditions that optimize expansion by minimizing doubling time.

Under static culture conditions, Fick's law can be applied to determine the overall OCR from the equilibrium oxygen concentration. If cell number is also known, one can then determine the oxygen utilization rate (OUR) per cell. The kinetics of oxygen concentration/consumption offer much insight into the state of cells in culture, including the doubling time, the proliferative state, the relative viability or the dependence of proliferation upon pericellular oxygen concentration. Brief descriptions of several such parameters are given in *Table 1*.

Table 1: Insight into cell or tissue status that can be obtained from the kinetics of oxygen concentration/consumption:

The Proliferative State

During exponential growth when oxygen supply is not limiting, the cell number will increase exponentially. Assuming a constant per-cell OUR⁹, it is fair to deduce from an exponentially increasing OCR that cells are proliferating exponentially, especially if there is no other compelling circumstance that could explain the oxygen consumption. Any deviation from an exponential increase in OCR thus suggests a state other than exponential proliferation.

The Doubling Time

If OUR is constant during the proliferation state, OCR will correlate directly to cell number. The amount of time required for one to double will be the same for the other. The doubling time can thus be obtained from the slope of a semilog plot of OCR versus time.

Relative Viability

For cells in the proliferative state, the OCR is directly proportional to cell number. This is the rationale for the correspondence between the BD OBS signal and cell number demonstrated empirically in an earlier report¹⁴ as the basis for a cytotoxicity assay.

Cell Number

If cells are known to be proliferating exponentially and the seed density is known, it is possible to estimate cell number from the doubling time.

OUR Per Cell

If cell number is known, it is possible to normalize OCR to cell number and obtain OUR.

The Limiting Oxygen Concentration Below Which Proliferation Is Not Supported

Various cell types exhibit different needs for oxygen, and it is a straightforward to ascertain such behavior via the BD OBS.

Impact of Media Additives on Relative Metabolic State

Exposure of hepatic cells to a known P450 substrate¹⁶, for example, or immune cells to an inducer of oxidative burst¹⁶ should both induce a measurable change in oxygen consumption.

Applications

Proliferation of MC3T3 Osteoblasts on Various Scaffolds

In this first example, consider the ability of various culture substrates to support osteoblast proliferation. Numerous 3D scaffold materials were evaluated, including collagen, polylactide and foamed polystyrene. Cells were also cultured in suspension without a substrate. Cells seeded onto unwashed chitosan/hyaluronate scaffold perished due to lethally low pH and served as a negative control. All wells were seeded with 50,000 osteoblasts and followed for eight days on a BD™ OBS microplate.

Figure 1 shows a kinetic plot of the equilibrium oxygen concentration, as calculated from Equation 4 (see Theory, page 10), for these various conditions. Slopes of the linear portions of the curves (through either day three or day six) reveal the doubling times, which are summarized in Table 2.

Impact of Glucose Concentration on Doubling Time of U937

As a second example, consider the impact of media composition on growth rate. U937 cells were seeded via serial dilution onto a BD OBS microplate and cultured in suspension. One set of wells was cultured in GibCo RPMI media at 2 g/L glucose, while the other set was cultured in ATCC RPMI media at 4 g/L glucose. Fluorescence was followed kinetically for three days.

Figure 2 shows the calculated OCR values over time for the various seed densities. It can be seen in both media that the lower seed densities proliferated exponentially at a fixed doubling time, as evidenced by the parallel nature of the linear portions of the curves. Comparison of the two sets of curves reveals that the cells grown in high glucose doubled more quickly and reached maximum density more quickly. The slopes of the linear regions of the proliferation curves indicate doubling times of 16 hours for the high-glucose media and 21 hours for the low-glucose media. The wells seeded at high density in the high-glucose media also exhibit a downturn in OCR after reaching maximum OCR, suggesting a decrease in either cell number or in the OUR per cell.

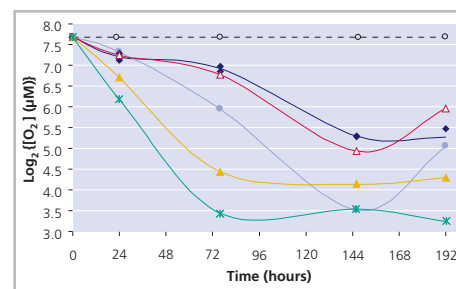


Figure 1: Calculated oxygen concentration as a function of time for MC3T3 osteoblasts seeded on various substrates and allowed to proliferate in a BD OBS. All conditions were seeded with 50,000 cells per well. Substrates tested included (○) a chitosan/hyaluronic acid mixture which was highly acidic and on which cells perished; (×) collagen, (▲) collagen/alginate, (●) polylactide, (△) polystyrene foam, (◆) no substrate.


Table 2: Doubling times in hours determined for osteoblasts cultured on various scaffolds.

Collagen	18
Collagen / alginate	23
Poly lactide	34
Polystyrene foam	55
No substrate	65

HL60 Proliferation

As a final example, HL60 cells were seeded at various densities in a BD OBS microplate and were allowed to proliferate in suspension as previously reported.¹⁴ Applying Equation 4 (see Theory, page 10) to such data, it is simple to demonstrate the relationship between cell number (as determined by hemacytometer) and equilibrium oxygen concentration, as shown in Figure 3A. It can be seen that good correlation exists for cell densities below $\sim 3.5 \times 10^5$ cells/well ($\sim 1.8 \times 10^6$ cells/mL), but that above that threshold the measured $[O_2]$ has plateaued at ~ 50 μ M. Further manipulation of the data via Equation 1 (see Theory, page 10) allows us to plot the OCR versus the cell number (Figure 3B), where again the same plateauing type relationship is observed. From the slope of the linear portion of the curve, we can compute the OUR as ~ 0.4 fmol/min/cell during exponential proliferation, a value similar to those reported in the literature for several cell lines. *Continued*

Summary

Knowing the oxygen consumption characteristics of cells in culture without invasively interrogating them minimizes the number of destructive samples needing to be taken, which would be of particular interest both during the research phase and into the clinical phase. Whether the goal is to demonstrate that cells are continuing to proliferate or to show that a differentiated tissue is remaining viable, the rate of oxygen consumption by the tissue can provide significant insight. Oxygen consumption in and of itself cannot, of course, conclusively identify the phenotypic state of the cells, but if enough else is known about the biology of the cells in question under the given set of culture conditions, it can be strongly indicative. The ability to non-invasively assess this parameter in real time offers a degree of confidence not readily matched by other techniques. 

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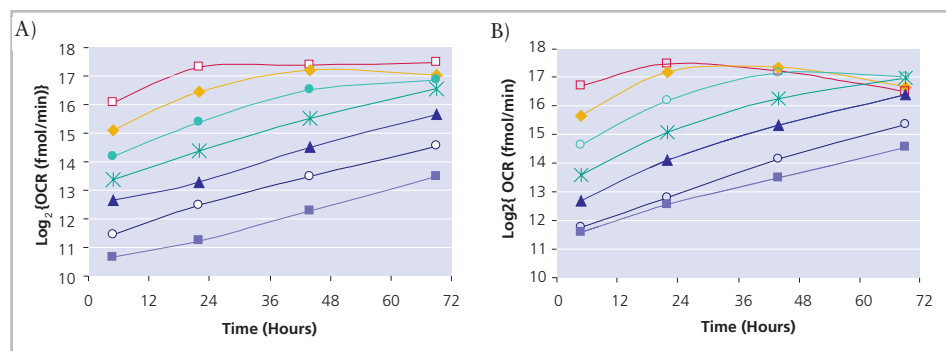


Figure 2: OCR proliferation plots over time for U937 cells seeded via serial dilution onto a BD OBS microplate in (A) GibCo RPMI media at 2 g/L glucose, or (B) in ATCC RPMI media at 4 g/L glucose. Note the parallel nature of the curves during the exponential growth phase. The slope of these linear regions indicates doubling times of (A) 21 hours and (B) 16 hours. Cells were seeded in a two-fold dilution series, with initial densities ranging from (A) 2.0×10^5 (\square) to 3.2×10^3 (\blacksquare) cells per well, or (B) 2.2×10^5 (\square) to 3.4×10^3 (\blacksquare) cells per well.

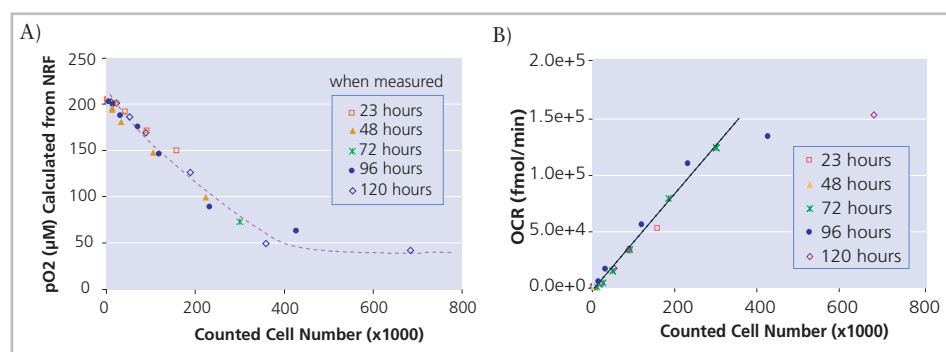


Figure 3: (A) Calculated oxygen concentration as a function of the counted number of HL60 cells. Oxygen concentration values are calculated via Equation 4 from the normalized fluorescence values and the counted cell number. (B) Calculated OCR as a function of the counted number of HL60 cells. Oxygen consumption values are calculated from the oxygen concentrations in (A) via Equation 1. The legend indicates the number of hours into the experiment when the fluorescence and counting measurements were made. N_{max} for this experiment was 5.5.

Theory

Cell Life Cycle. The analysis described herein is based upon a traditional notion of population doubling, as depicted in **Figure 1**. This model assumes that cells seeded under conditions which allow it will proliferate exponentially (stage A) until nutrient supply or mass transfer

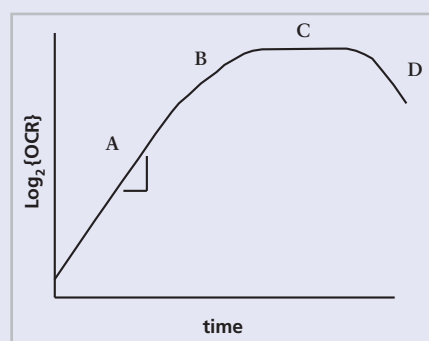


Figure 1: Schematic depiction of the oxygen consumption rate by a proliferating cell mass over time.

limitations make further exponential growth impossible. The rate of proliferation will thus decrease, as evidenced by the decrease in slope (stage B). Eventually further proliferation will become impossible, and OCR will plateau (stage C). Ultimately some nutrient other than oxygen will become exhausted and/or a toxic accumulation of metabolic waste will arise, and cells will begin to die (stage D).

While evidence indicates OUR is not constant throughout the life cycle, recent studies suggest that OUR does, in fact, remain constant during exponential growth.⁹ Thus OCR is directly proportional to cell number during exponential growth, and the rate of change of OCR will be the rate of change of cell number. A plot of the base-2 logarithm of OCR versus time is thus the doubling rate of the cells (or the reciprocal of the doubling time).

Continued

Theory *(continued)*

Stage C represents the upper limit of OCR for the culture, and by extension the lower limit of pericellular oxygen concentration. Several reports indicate that the minimum oxygen concentration which supports proliferation varies among cell types^{3,4}, with some able to proliferate into essentially anoxic conditions, while others proliferate only until the pericellular oxygen concentration drops to a much larger value. There appears to be some correlation between this tolerance for low oxygen and the distance *in vivo* of the cells or their primary parent cells from the oxygen supply.

Oxygen Diffusion. Several researchers^{4,5,11,17} have used the Fickian diffusion theory to model the oxygen gradient and pericellular oxygen concentration of static monolayer cell culture. This model uses a simple adaptation of the steady-state solution to Fick's law to relate the OCR at the well bottom, the oxygen concentration gradient in the well and the rate of oxygen diffusion. At equilibrium, when the only source of oxygen consumption is the cell mass at the bottom of the well, the rate of oxygen diffusion will equal the rate of oxygen consumption:

$$[1] \quad \text{OCR} = \frac{D S l \Delta p}{h}$$

where D is the diffusion constant of oxygen in the media (for simplicity we assume this to be the same as in water, $3.3 \times 10^{-5} \text{ cm}^2/\text{s}$), S is the surface area (in cm^2) of the media exposed to the atmosphere, h is the diffusion path length in cm (the distance between the atmosphere and the cells), l is a units conversion factor, and Δp is the difference in oxygen concentration (or partial pressure) between the air/media interface and the media/cell interface.⁵ **Figure 2** schematically depicts the geometry involved.

To express OCR as femtomoles of oxygen consumed per minute, l takes the value 6.0×10^7 . The oxygen concentration at the atmosphere interface will be governed by Henry's law. Assuming again for simplicity that the media is water and the atmosphere is standard air, the oxygen concentration at the surface will be $205 \mu\text{M}$ at 37°C . In $5\% \text{ CO}_2$, this would drop to $195 \mu\text{M}$. It can be seen from **Equation 1** that the

rate of oxygen diffusion is directly proportional to the difference between the oxygen concentration at the surface of the media and that at the cells Δp . Cells cannot consume oxygen more quickly than it can reach them by diffusion, and the upper limit of diffusional oxygen transfer can occur only when the local oxygen concentration at the cells is zero. As indicated above, however, not all cells can survive under anoxic condition, so the practical limit may, in fact, be lower.

Plugging in numbers typical of culture in a 96-well plate ($S=0.31 \text{ cm}^2$, $h=0.65 \text{ cm}$; i.e., $200 \mu\text{L}$), it can be seen that the maximum possible oxygen transport rate, (OTR_{max}) (which will occur at the maximum Δp), will be on the order of $2.0 \times 10^5 \text{ fmol}/\text{min}$. It is readily apparent from **Equation 1** that OTR_{max} varies inversely with the media depth, all else equal. This model applies only to static culture where the cells are at the bottom of the well. For cultures shaken to improve oxygen transfer, a different model would apply, but the same types of conclusions could be drawn.

BD™ OBS Signal Basics. Conversion of BD OBS signal to oxygen concentration is governed by Stern-Volmer theory¹³. In its simplest form, the Stern-Volmer equation relates oxygen concentration to the ratio of fluorescence intensity at zero oxygen concentration (I_0) to the intensity at the oxygen concentration of interest (I), as follows^{7,18}:

K_{SV} is the Stern-Volmer constant for the

$$[2] \quad I_0/I = 1 + K_{\text{SV}} [O_2]$$

fluorophore system in question. In practice, K_{SV} is a function of the fluorescence plate reader and must be determined empirically. For convenience sake, we normalize fluorescence intensity not to the zero-oxygen condition but to the fluorescence at ambient oxygen conditions (I_A), since this is a condition that can easily be generated via a simple negative control and because it is a more intuitive reference point in practice. With these substitutions, we can define the normalized relative fluorescence (N) as follows:

where we have defined the dynamic

$$[3] \quad N = I/I_A = N_{\text{max}} / (1 + K_{\text{SV}}[O_2])$$

range, N_{max} , as the ratio I_0 / I_A . This way, N will increase from 1.0 as oxygen consumption increases ($[O_2]$ decreases). N_{max} represents the largest possible value of N , which will occur at zero oxygen concentration. Solving **Equation 3** for $[O_2]$ yields the oxygen concentration at the well bottom:

$$[4] \quad [O_2] = (N_{\text{max}} / N - 1) / K_{\text{SV}}$$

K_{SV} may be determined empirically from the dynamic range as follows:

$$[5] \quad K_{\text{SV}} = (N_{\text{max}} - 1) / [O_2]_a$$

where $[O_2]_a$ is the ambient oxygen concentration, which we take as the solubility of oxygen in water, given above as $195 \mu\text{M}$ at 37°C .

The experimental values of $[O_2]$ obtained in this fashion can thus be incorporated into **Equation 1** to calculate OCR. 🌐BD

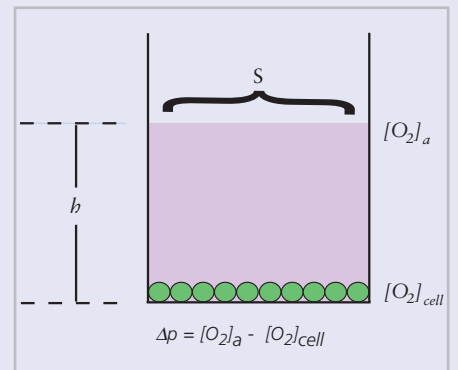


Figure 2: Schematic of the geometry relevant to the diffusion of oxygen to the cells in a microplate. The pink area represents the media, of depth h , and the green ovals represent cells at the bottom of the well. The BD™ OBS adds to this picture a layer of oxygen-sensing film directly below the cells, and therefore a reading of the oxygen concentration experienced by the cells. While this model was developed to describe the situation in a microplate, it can be generalized to any culture geometry.

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