Rapid method for assessing oxygen consumption rate of cells from transient-state measurements of pericellular dissolved oxygen concentration

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Abstract

Recently we described a method for estimating the oxygen consumption rate (OCR) of cells in static culture from equilibrium measurements of dissolved oxygen concentration (dO_2), using an oxygen-sensing microplate and the steady-state solution to Fick's Law (Guarino et al. 2004). Here we describe a complementary method for estimating OCR from the transient-state rate of change of measured dO_2 . Although the system is open to the atmosphere and subject to a significant lag in sensor response, the rate of change of the measured dO_2 immediately after seeding correlated directly with both cell number and steady-state OCR. This transient-state method is linear with cell number to a much higher density than is possible with the steady-state method because it derives from measurements made before diffusion limitations can be established. For a given sensor thickness, the same correlation line between the transient and non-diffusion-limited steady-state estimates of OCR was found to apply for various preparations of rat hepatocytes. The correlation slope varied predictably with sensor thickness. Thus, despite the non-idealities of this system, the initial rate measurement offers a rapid method to obtain an estimate of absolute OCR. To demonstrate the utility of this method, we purposefully treated rat hepatocytes in ways expected to change OCR. Cells deprived of oxygen by storage under several centimeters of medium showed decreases in both OCR and viability with time. Likewise, the OCR of hepatocytes exposed to the oxidative phosphorylation inhibitor rotenone decreased, whereas those exposed to the uncoupler dinoseb increased.

Introduction

Monitoring of the oxygen consumption of cells in culture is a routine practice for large-scale cell culture operations but is not common for microplate-scale cultures primarily due to the lack of convenient assays. A series of recent publications (O'Riordan et al. 2000; Papkovsky et al. 2000;

Wodnicka et al. 2000; Hynes et al. 2003; Alderman et al. 2004; Deshpande and Heinzle 2004; Guarino et al. 2004; Hutter and John 2004; Ramanathan et al. 2005) indicate that such assay technologies are becoming available, and are of interest for both detailed and high-throughput studies of cellular viability, growth kinetics, cytotoxicity, tumorigenesis, oxidative burst, and

oxidative phosphorylation, among others. Oxygen consumption rate (OCR) is also emerging as an assay of interest in the clinic, as a means of assessing Islets of Langerhans or embryos, for instance, prior to implantation (Houghton et al. 1996; Steurer et al. 1999; Sweet et al. 2002; Wilson-Fritch et al. 2004; Fraker et al. 2005; Wang et al. 2005), and also in the area of food safety. (Matos and Garland 2005).

The most classic method for computing the OCR of cells is to suspend them in a sealed, well-stirred vessel and measure dO_2 over time (Harvitt and Bonanno 1998; Kindig et al. 2003a, b; Heimbürg et al. 2005). Under such conditions, OCR is computed directly from the rate of decrease in dO_2 . Another common method is to use a flow-through chamber and to measure the decrease in dO_2 between the inlet and the outlet (Li et al. 2001; Sweet et al. 2002). Each of these setups affords a conceptually and mathematically simple and straightforward assessment of OCR, but is cumbersome in practice. They also require the use of specialized equipment, and are inherently serial and of low throughput.

By contrast, our previously published method for computing OCR from steady-state measures of pericellular dO_2 in an oxygen-sensing microplate affords parallel, high-throughput analysis of multiple wells at once. Unlike the classic method, quantitation of OCR via this microplate method is based not on the rate of change of dO_2 , but rather on the magnitude of the equilibrium dO_2 gradient, which is directly proportional to the oxygen consumption rate (OCR) of the cells at the well bottom, as described by the steady-state solution to Fick's Law (McLimans et al. 1968a, b; Metzen et al. 1995; Mamchaoui and Saumon 2000). While simple in both theory and practice, one limitation of this protocol is that it requires the culture to have achieved equilibrium, which we and others (Mamchaoui and Saumon 2000; Guarino et al. 2004) have demonstrated can take several hours. Another limitation of the equilibrium method is that its upper limit of detection is governed by the maximal oxygen transport rate (OTR_{max}) into the plate, which can be a limitation when samples of high OCR – such as primary cells or tissue slices – are being studied.

We describe herein an alternate method for computing OCR from transient (pre-steady-state) measurements of dO_2 in the same oxygen-sensing

microplates. This new method combines the advantages of both the classic and steady-state microplate methods, and is based upon the empirical observation that the initial rates of change of measured dO_2 vary linearly with both cell number and the computed equilibrium OCR. In addition to a faster estimate of OCR, this method offers a much larger upper detection limit than the steady-state microplate method, since it can be applied during the transient phase (*i.e.*, immediately following seeding) before the dO_2 reaches zero. As such, this method is far more suitable for use with high-OCR cultures, such as primary hepatocytes, high-density 3D constructs, or tissue biopsies. Because they are non-proliferative, primary and cryopreserved hepatocytes were used for the purposes of demonstrating the method.

Materials and methods

Hepatocyte isolation and culture

Cryopreserved Sprague–Dawley rat hepatocytes or human hepatocytes were obtained from BD Biosciences (Woburn, MA). Briefly, cells were rapidly thawed at 37 °C and then washed either using a 24% percoll gradient followed by a medium rinse or by gentle centrifugation through Isom's medium alone. Cells were resuspended and cultured as described below.

Primary rat hepatocytes were isolated from 150–230-g Sprague–Dawley rats by a modification of Seglen's two-step collagenase perfusion procedure (Seglen 1976). Briefly, livers were first perfused with a calcium-free solution containing 0.5 mM EGTA for ~ 10 min at a flow rate of 25 ml/min after which they were perfused with buffer containing 0.35 mM calcium chloride, 1% BSA and 75–100 units/ml collagenase (Worthington) for ~ 10 min. Hepatocytes were dispersed, washed through a percoll gradient by low-speed centrifugation, and then resuspended in either Isom's Medium (Isom and Georgoff 1984) or BD HepatoStimTM Medium supplemented with 4 mM glutamine, 0.1% (v/v) mixture of penicillin and streptomycin and 10% (v/v) FCS, unless otherwise noted. Viability was determined by Trypan Blue exclusion. Cells were then re-suspended to the proper concentration in appropriate test medium.

In general, cells were diluted to 1×10^6 cells/ml stock concentration.

In a parallel experiment, 2.5×10^6 cells were placed in 10 ml of medium in each of several 15 ml centrifuge tubes (BD, Franklin Lakes, NJ). The tubes were stored at either 4 °C or 37 °C for 0, 2, 7 and 27 h. After incubation, the cells were spun down, assessed for viability, and plated as described above. OCR measurements were taken over an extended time period after plating.

BD Oxygen Biosensor Systems were prepared by performing an initial fluorescent read (to be used for normalization purposes, as described below) and then adding 100 μ l of medium to each well. Cells were then serially diluted across the plate. In studies using test compounds, 100 μ l of cells at the designated concentration were plated directly into 100 μ l of compound at 2 $\times$ the final concentration. Test compounds rotenone and dinoseb were obtained from Sigma. Compounds were solubilized at 10 mg/ml in DMSO, adjusted to 10 μ g/ml in medium, and then serially diluted. Drug solutions were controlled for any potential background fluorescence (none was observed). The final volume per well was 200 μ l unless otherwise noted. Wells containing medium alone (no cells) or sodium sulfite (100–1000 mM) were included on each plate as ambient and anoxic controls, respectively.

Plates were stored in a standard incubator (37 °C, 5% CO₂) and manually moved between the incubator and the fluorescence plate reader only for data collection. Care was taken not to agitate the media in the wells during transport, as this would disrupt the evolving oxygen gradient. Fluorescence data were collected on a variety of commercial plate readers (PE, Cytofluor, BMG), using the bottom plate-reading configuration. Nominal wavelengths of the bandpass filters were 485 ± 20 nm for excitation and 610 ± 20 nm for emission. Data were collected at selected time points during the first few hours after seeding, and then periodically over a 24 h period.

Oxygen-sensing microplates

The BD Oxygen Biosensor System is an oxygen-sensing microplate, as described previously (Wodnicka et al. 2000; Stitt et al. 2002; Guarino

et al. 2004), in which an oxygen-sensitive fluorophore is incorporated into a silicone rubber matrix in the bottom of each well of an otherwise standard 96-well round-bottom microplate. Most of the studies herein utilized the standard Oxygen Biosensor System (OBS) from BD Biosciences (Bedford, MA), for which there is 7.6 μ l of sensor added per well. For experiments investigating the effect of sensor volume, wells coated with 4, 12, or 20 μ l of sensor were tested in addition to the standard sensor volume. In all cases, the sensor was cured for at least 3 h at 50 °C. The standard OBS does not accommodate hepatocyte attachment. To allow cells to attach, the wells of a standard OBS plate were overlaid with a thin film of collagen I, covalently coupled by EDC/NHS carbodiimide chemistry to acid groups introduced onto the silicone sensor surface via the UV/ozone method of Genzer and Efimenko (2000). All plates were sterilized by exposure to gamma (25–40 kGy) or UV irradiation (> 1000 kJ/cm²) prior to use. Dissolved oxygen concentration in the sensor was computed from the normalized fluorescence intensity, N , via the one-parameter Stern–Volmer equation, as described previously. (Guarino et al. 2004; Guarino et al. 2005)

Determination of steady-state oxygen consumption rate (OCR)

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. OCR may then be computed from the steady-state solution to Fick's Law, as given in Equation (1) (Guarino et al. 2004; Guarino et al. 2005), adapted from Mamchaoui and Saumon (2000):

$$\text{OCR} = \frac{D S L \Delta p}{H} \quad (1)$$

D is the diffusion constant of oxygen in the medium (for simplicity we assume this to be the same as in water, 3.3×10^{-5} cm² s⁻¹). S is the surface area of the medium exposed to the atmosphere (0.32 cm² for a 96 w plate). H is the diffusion path length in cm (the distance between the atmosphere and the cells, which is the depth of the medium: 0.63 cm for 200 μ l). L is a unit conversion factor (6.0×10^7 L cm³ s min⁻¹ fmol μ mol⁻¹). Δp is the

difference in oxygen partial pressure (expressed as a concentration, dO_2 , in μM) between the air/medium interface and the medium/cell interface, as given by $[O_2]_a - [O_2]$, where $[O_2]$ is the measured value at the well bottom.

Estimation of OCR from pre-steady-state measures of dO_2

The initial slope of a plot of the measured dO_2 vs. time was determined from the data points collected immediately after seeding, during the period when the decrease in measured dO_2 was essentially linear. Typically, the decrease in measured O_2 was approximately linear during the first 2 h after seeding, although the duration of the linear portion varied between experiments. Slopes determined in this fashion are referred to as “initial rates.” Using the empirically determined proportionality constant between “initial rates” and equilibrium values of OCR, the initial rates were converted to OCR values. OCR values determined in this fashion are dubbed “rapid OCR.” Oxygen utilization rates (OUR) per cell were computed by taking the slope of the linear portion of a plot of OCR against cell number. (Guarino et al. 2004).

Results and discussion

Correlation of initial rate of change of dO_2 to cell number and equilibrium OCR

The earlier paper (Guarino et al. 2004) showed hepatocyte data which indicated that it took the system several hours to reach equilibrium under our typical use conditions (200 μl per well, 37 °C), which is consistent with the low diffusivity of oxygen through water. Figure 1 replots these data as on a linear scale, with the independent variable expressed as the decrease in dO_2 from ambient. It can be seen that dO_2 decreases quasi-linearly at first, and at a rate that varies with cell number. Least-squares estimates of the initial rates of change of dO_2 during this portion of the transient phase are indeed linear with cell number, as can be seen in Figure 2. This fact alone suggests that this initial rate is proportional to OCR. This assertion is bolstered by Figure 3, which shows that the initial rate also correlates linearly with equilibrium

Figure 1. Computed decrease in sensor dO_2 as a function of time for various numbers of primary rat hepatocytes (as identified in the legend) seeded in 200 μl in a standard OBS plate. Data are re-plotted from Figure 12 in the previous paper by Guarino et al. (2004).

Figure 2. Initial rate of decrease of measured dO_2 ($\mu M h^{-1}$) during the first 1–2 h after seeding for the kinetic dO_2 traces shown in Figure 1. Least-squares fit excludes data for the highest cell density (shown as an open symbol), for which there were too few measurements to determine a reliable initial rate.

OCR (OCR computed from the data between 6 and 22 h), which has also been shown (Guarino et al. 2004) to vary linearly with cell number (for densities below those which induce oxygen starvation). These results constitute an empirical demonstration that it is possible to predict OCR from pre-equilibrium data.

To understand whether the relationship between the initial rate of change of dO_2 and the equilibrium method is specific to a given experimental set-up or is more generally applicable, experiments were performed with multiple preparations of hepatocytes to compare the rapid and equilibrium

Figure 3. Equilibrium OCR values computed from the data between 6 and 22 h in Figure 1 (Guarino et al. 2004) for each cell density as a function of the corresponding initial rate of decrease of measured dO_2 (from Figure 2). The least-squares fit to the data excludes the equilibrium OCR points corresponding to oxygen starvation (open symbols).

methods. Experiments were performed with fresh male, and cryopreserved male and female rat hepatocytes. Experiments were performed on regular BD OBS plates and plates on which the OBS surface was chemically modified to allow collagen 1 coating, which enabled the rat hepatocytes to attach to the sensor. By contrast, hepatocytes did not attach to the surface of standard OBS plates, but rather attached to one another in spheroid-like formations after 24 h.

For a given sensor volume, it was found that the same correlation slope held, provided that the cells were of general good health and reached and maintained a quasi-constant dO_2 by ~ 6 h. Figure 4 plots the measured rapid and equilibrium values of OCR for eight different lots/preparations of hepatocytes, each indicated by a different symbol (see figure legend). Although the actual measured OCR per cell varied considerably among these preparations (from 9 to 25 $\text{fmol min}^{-1} \text{ cell}^{-1}$), the data superimpose onto a single master curve, the linear (sub- O_2 -starvation) portion of which exhibits a linear slope of 4035 $\text{fmol min}^{-1} \mu\text{M}^{-1} \text{ h}$ for the commercial sensor. Thus, despite the fact that the measured dO_2 in the sensor layer lags behind that in the medium (see below), its rate of change appears to exhibit a universal correlation to OCR, thereby making it quantitatively predictive of OCR.

For the purposes of establishing the generality of the correlation between the two measurements, the OCR of the cells had to be constant over the

Figure 4. Equilibrium OCR values for each cell density tested plotted as a function of the corresponding initial rate of decrease of measured dO_2 for each of eight separate experimental conditions. The least-squares fit to the data excludes the equilibrium OCR points from the plateau (which correspond to oxygen starvation). Legend entries refer to the date of isolation for the cells (e.g., 030709) or the BDTM Gentest lot number of cells (e.g., 18). Entries showing “RTC-OBS” refer to plates chemically modified to allow coating with rat-tail collagen I. For all other test conditions, the cells were cultured on unmodified OBS plates. The results from Figures 1–3 are included (test condition 030716).

duration of the experiment. Such was the case for all data shown in Figures 1–4. Comparable trends have been observed with proliferative cell types, such as HL-60 or MCL-5 cells, when values of equilibrium OCR estimated at time zero by extrapolation (Guarino et al. 2004) are used (data not shown), and also for pancreatic islets (Fraker et al. 2005). By contrast, cell cultures that were not of good health, such as those exposed to prolonged anoxia (see below), did not achieve and maintain a constant dO_2 . In these cases, dO_2 showed an initial decrease during the transient phase, but during steady-state exhibited a monotonic increase back toward ambient, which indicated an ever-decreasing OCR. Because the assumption of constant OCR during the duration of the experiment was clearly violated, the results of these experiments were not used in establishing the correlation. Nonetheless, even in these situations there is linearity between cell number and the initial dO_2 slope, meaning the initial slope is still proportional to initial OCR.

This method allows for the near-instantaneous assessment of OCR. Any upstream treatment of the cells (such as cryo-preservation, trypsinization, high-density culture, pre-exposure to drug, or

change in medium composition) which could impact OCR per cell would thus be reflected in the measurement. Attempts to quantitate these effects separately from 'basal' OCR would require experiments with suitable control conditions.

As mentioned above, most of the results reported herein are for hepatocytes cultured in suspension, which adopt a spheroid-like morphology. Our analysis assumes this 3-dimensional morphology which does not significantly impact the dO_2 gradient and kinetics. Work is on-going to assess the degree to which this method can be extended to the study of larger 3-dimensional tissue constructs. Preliminary data suggest that qualitatively similar trends are observed, so long as the tissue thickness is small compared to the medium depth. One potential advantage of the rapid method for 3D tissues is that it should have a higher upper limit of detection because a reading can be obtained before diffusion limitations set in.

Effect of sensor thickness

The sensor layer in the OBS plates is not infinitely thin; therefore, the pO_2 in the sensor (which is what is actually measured) lags behind the pO_2 in the medium at the well bottom where the cells are located. The magnitude of this lag phenomenon varies directly with the thickness of the sensor layer, and should, in theory, extrapolate to zero at the limit of zero thickness. Custom sensor plates prepared with three different volumes of sensor (4, 12, and 20 μl) confirm this hypothesis. In all cases the initial slope of dO_2 was linear with cell number and with equilibrium OCR, with lower volumes of sensor yielding a higher rate of change of measured dO_2 for the same equilibrium OCR. Since equilibrium OCR should be independent of sensor volume (there should be no difference in the pO_2 of the sensor and the medium at the well bottom at equilibrium, by definition), this comparison is valid. As shown in Figure 5, the ratio between the initial rate and equilibrium OCR varied monotonically with sensor volume across the range tested.

Effect of oxygen deprivation on hepatocyte OCR

Storing cells under a tall column (~ 8 cm) of medium severely limits their supply of oxygen.

Figure 5. Absolute (left axis) or relative (right axis) ratio of equilibrium OCR to the initial rate of change of dO_2 as a function of sensor volume. The relative ratio is computed relative to the value predicted at zero sensor volume from the shown linear least-squares fit to the data.

Using the Fick's Law model, it can be computed that the rate of O_2 diffusion through the medium is approximately two orders of magnitude lower than the O_2 demand by the cells. As the summarized results in Figure 6 indicate, storage under oxygen-limited conditions at 37 $^{\circ}C$ was clearly detrimental to the cells, as both viability and OCR decreased immediately and were essentially immeasurable by 7 h. For the cells stored at 4 $^{\circ}C$, the effect was less drastic, with viability and OCR decreasing partially over this time. Since OCR per cell would likely be significantly lower at the colder

Figure 6. Measured OUR (diamonds) and Trypan Blue viability (squares) of hepatocytes as a function of how long they were under oxygen-limited conditions at either 37 $^{\circ}C$ (filled symbols) or 4 $^{\circ}C$ (open symbols).

Figure 7. OCR relative to no-drug controls for primary rat hepatocytes exposed to various concentrations of rotenone (filled squares) or dinoseb (open triangles).

temperature (Jorjani and Ozturk 1999) and the O_2 solubility greater, this difference was expected. The values of OUR (oxygen utilization rate, per cell) reported here are computed by converting the initial rate into an OCR via the conversion factor described above for the standard OBS, and then dividing by cell number.

Effect of rotenone and dinoseb on hepatocyte OCR

As another demonstration of the utility of this new rapid method for estimating OCR, the OCR of primary rat hepatocytes was determined immediately after plating in various concentrations of rotenone and dinoseb. The results are plotted in Figure 7, where it can be seen that OCR increased with dose for dinoseb, an uncoupler of oxidative phosphorylation, and decreased with dose for rotenone, an inhibitor of mitochondrial respiration. At very high dose of dinoseb, there was a downturn in OCR due to toxicity to the cells (Palmeira et al. 1994a, b).

Final words

The rapid OCR analysis explicitly excludes the impact of diffusion on the measured dO_2 . In theory there should be no contribution from diffusion to a true initial rate, since there will be no diffusion before there is a dO_2 gradient to drive it. Moreover, since the time constant for diffusion in this system is several hours ($L^2 D^{-1} \sim 3.3$ h) (Stewart

2003), the contribution will be small, even when data as late as 2 h are used to compute the initial slope.

It should be stated explicitly that the initial rate of change of the measured dO_2 is not identical to OCR. However, the fact that data from numerous 'healthy' cell preparations fall onto the same master correlation line (Figure 4) confirms that it is reproducibly predictive of OCR. For the standard sensor formulation, the proportionality constant between both was $4035 \text{ fmol min}^{-1} \mu\text{M}^{-1} \text{ h}$. This constant can thus be used to convert initial rate measurements to OCR, assuming our equilibrium OCR model (Guarino et al. 2004) is correct. Work assessing the validity each of the assumptions implicit in the equilibrium model, and comparing OCR values to those measured via more traditional means, is underway.

There have been previous reports of estimating OCR from the rate of change of dO_2 in a microplate. Hynes (Hynes et al. 2003) showed that the initial rate of change of fluorescence of a soluble oxygen probe in a static culture of lymphoblasts was proportional to cell number. Though no attempt was made to account for the nonlinear relationship between fluorescence lifetime and dO_2 , the empirical fit was decent, presumably because of the pseudo-linearity of fluorescence with dO_2 near ambient. A recent paper by Deshpande and Heinzle (2004) describes a method for computing OCR from dO_2 measured using an oxygen-sensing microplate. This method employed a shaken microplate, an empirically determined

mass transfer coefficient, and an assumption of uniformity of dO_2 throughout the well. Computed OCRs varied linearly with cell number, and the data indicate that the measured dO_2 decreased at a rate proportional to cell number, though the authors do not point this out. Per-cell OCR estimates resulting from this method, however, seem unusually large, which suggests that the mixing was inadequate to prevent a dO_2 gradient.

The new method described herein involves the rate of change of dO_2 in the sensor at the well bottom, which was shown to decrease at a rate proportional to both cell number and OCR computed from the steady-state Fick's Law model. While no theoretical basis is offered for why this trend should exist, it was empirically shown to exist universally for a range of cell preparations and across the measurable range of OCR. The rate of change of dO_2 for a given OCR increased as the thickness of the sensor layer was decreased, consistent with expectation. Based on the observed high degree of correlation, we are confident that the rate of change of measured dO_2 affords a number directly proportional to OCR.

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