Dispersible oxygen microsensors map oxygen gradients in three-dimensional cell cultures†

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Phase fluorimetry, unlike the more commonly used intensity-based measurement, is not affected by differences in light paths from culture vessels or by optical attenuation through dense 3D cell cultures and hydrogels thereby minimizing dependence on signal intensity for accurate measurements. This work describes the use of phase fluorimetry on oxygen-sensor microbeads to perform oxygen measurements in different microtissue culture environments. In one example, cell spheroids were observed to deplete oxygen from the cell-culture medium filling the bottom of conventional microwells within minutes, whereas oxygen concentrations remained close to ambient levels for several days in hanging-drop cultures. By dispersing multiple oxygen microsensors in cell-laden hydrogels, we also mapped cell-generated oxygen gradients. The spatial oxygen mapping was sufficiently precise to enable the use of computational models of oxygen diffusion and uptake to give estimates of the cellular oxygen uptake rate and the half-saturation constant. The results show the importance of integrated design and analysis of 3D cell cultures from both biomaterial and oxygen supply aspects. While this paper specifically tests spheroids and cell-laden gel cultures, the described methods should be useful for measuring pericellular oxygen concentrations in a variety of biomaterials and culture formats.

Introduction

Three-dimensional (3D) cultures that use hydrogels or specialized culture vessels are increasingly used to elicit more physiological cellular responses than are available from conventional 2D cultures. Oxygenation within these tissue cultures is an important regulator of cell function. Physiological oxygen levels in vivo range from ~14% in lung alveoli down to ~3% in some tissues, such as muscle and skin (dermal papillae). Even lower levels of oxygen exist in tissues associated with different pathologies, such as tumors, ischemia, and obesity. Re-creation of physiological oxygen levels in tissue cultures enables researchers to probe specific biological phenomena in more representative microenvironments.

3D spheroid cultures are commonly used as model systems for avascular tumors and other pathological tissues. Spheroids have a diffusion-limited supply of critical nutrients such as oxygen, resulting in marked gradients within the tissue aggregate, mirroring oxygen gradients expected in avascular tumors. Spheroids have remained popular models owing to their defined shape and structural architecture of a cell mass with a constant volume, which enables computational models to assess these structures radially, minimizing the complexity of modeling compared to radially asymmetric systems. Spheroids are formed by a broad array of methods that promote cell–cell attachment by limiting cell–substrate interaction. These methods, however, differ significantly in vessel types used and hence oxygen-transfer properties. Specific examples of spheroid cultures include low-adhesion, curved wells, agarose-coated wells, microfluidic devices, honeycomb wells, and hanging-drop cultures.
While formation of oxygen gradients within spheroids is well-appreciated, there is often less emphasis on the variations in oxygen levels within the surrounding culture medium that arise as a result of the culture method used. This paper aims to fill this gap. While a variety of oxygen sensors exist, intensity-based measurements of sensors make few, if any, of them meet the requirements for oxygen-evaluation gradient mapping of 3D cell cultures in being non-toxic to cells, non-tethered, micro-scale, and accurate. In particular, cell-compatible optical oxygen sensing microbeads with inherent variabilities associated with intensity-based optical sensing modalities. Even ratiometric imaging with an oxygen-insensitive reference dye present is challenging, as photobleaching properties and excitation/emission properties will be different between the oxygen-sensitive and oxygen-independent dyes. In contrast, measurement methods that depend on the indicator’s luminescence lifetime, such as phase fluorimetric measurements, can usually eliminate inconsistencies that arise from intensity differences, caused by dye-loading variability, photobleaching, and optical path variability inherent to 3D tissues. Using small, dispersible polydimethylsiloxane (PDMS)-based oxygen sensing microbeads with phase fluorimetry, we quantified significant dimethylsiloxane (PDMS)-based oxygen sensing microbeads with phase fluorimetry,18,19 we quantified significant differences in oxygen microenvironments between three different but commonly-used 3D culture formats. Furthermore, for the cell-laden hydrogel culture, oxygen gradient maps were used together with computer simulations to estimate the constants for the uptake rate of cellular oxygen and for the half-saturation of oxygen.

**Methods and materials**

**Microsensor fabrication and calibration**

Briefly, lab-fabricated microsensors were generated by producing PDMS microbeads using a microfluidic flow-focusing device. PDMS beads were infused with tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dichloride and then calibrated over a range of pre-mixed combinations of N2 and O2 gas spanning from 21% (room air) to 0% O2. Phase-shift measurements were taken at each oxygen interval using a custom setup similar to that already described by others. A more in-depth protocol for the fabrication and calibration of these microsensors can be found in ESI Methods and materials† with detailed information on the oxygen-sensing capability of our imaging setup.

**Cell culture**

Human embryonic kidney 293T (HEK 293T) cells (ATCC® CRL-1573™), HS-5 human bone-marrow stromal cells (ATCC® CRL-11882™), and MDA-MB-231 human breast-cancer cells (University of Michigan, Luker Lab) that stably expressed eGFP (MDA-MB-231-eGFP) were cultured with Dulbecco’s modified Eagle’s medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. The medium was refreshed every 2 to 3 days, and the cultured cells were passaged using 0.25% trypsin/EDTA (Gibco, Carlsbad, CA, USA) when sub-confluent, 70–80%. The cytotoxicity assessments were performed with HS-5 cells. The experiments with spheroid cultures were performed using HEK 293T cells. The cell-laden hydrogel work used to assess cell-generated oxygen gradients was performed using the MDA-MB 231 cells.

**Assessment of oxygen levels generated by spheroids in different culture formats**

Spheroids were generated using high-throughput hanging-drop-array plates and the culture methods recently presented. Prior to use, a hydrophilic coating was applied onto the entire surface of the hanging-drop plate by soaking the plate overnight in 0.1% Pluronic F108. The plate was then dried and sterilized by using ultraviolet light before seeding with cells. To form the hanging drops, 25 µL of a cell suspension at a concentration of 2.0 × 10^5 HEK 293T cells per mL was pipetted through the access holes on the top-side of the plate, with the tip of each pipette being inserted into the access hole to guide the sample to the bottom surface. The cell-culture medium was supplemented with A4M Methocel (Dow Chemical Co., Auburn Hills, MI, USA) at 0.024% (w/v). The spheroids formed over 24 hours. At this time microsensors were placed either into hanging drops with spheroids or into 96-well round-bottom microplate wells. The round-bottom microplate wells were then filled with 1, 5, 10, or 50 spheroids. Microsensors were also placed in media with only hanging drops and round-bottom wells as controls. The spheroids were monitored for a minimum of 18 hours in the different culture formats. A second study assessed hanging drops with 0, 1, 3, or 10 spheroids placed into hanging drops containing microsensors. These systems were monitored at various stages over the course of a single day.

**Mapping oxygen distribution in cell-patterned hydrogels**

A more complete protocol to generate a spatially-patterned, cell-laden, collagen hydrogel surrounded by a cell-free collagen hydrogel has been previously described. Briefly, spatial patterning was performed by coating PDMS onto a glass slide, which was then covered with polyacrylamide. The polyacrylamide coating was selectively oxidized using a protective mask and applying plasma oxidation (Covance MP, FemtoScience, Hwaseong-si, Gyeonggi-do, South Korea). Trypsinized MDA-MB-231 eGFP cells were mixed with the oxygen microsensors and neutralized type-I bovine collagen (BD Biosciences, San Jose, CA, USA) to create a suspension of 1.0 × 10^7 cells per mL and 2 × 10^5 beads per mL in 2 mg mL^-1 of collagen. 8 µL of this suspension were dispensed onto the adhesive surface and allowed to polymerize for 45 minutes at 37 °C. The overlaying gel consisted of a suspension of oxygen microsensors with 200 beads per mL in 2.5 mg per mL collagen. 250 µL were dispensed over the polymerized core region, and incubated at 37 °C for 1 hour to polymerize the overlaying gel. 2 mL of cell-culture media were added to each well, and the samples were
cultured at 37 °C. Oxygen levels were measured after 24 hours of culturing (steady state) and then mapped radially by using the distance from the center of the cell-laden region. The results from our system were compared with measurements using a commercially available Seahorse XF® Extracellular Flux Analyzer and Ocean Optics NeoFox. Both the oxygen-consumption rate and oxygen levels within the cells seeded in collagen gels were compared following similar protocols for processing cells and forming the collagen gel. A more in-depth description of these comparative measurement acquisition and measurements can be found in ESI Methods and materials.†

Oxygen-depletion simulations
In order to model the cell-generated oxygen gradients, finite-element analyses were conducted using COMSOL Multiphysics® v5.1 Chemical Reaction Engineering Module (COMSOL, Inc., Burlington, MA). In the model, a two-dimensional axisymmetric geometry was used to mimic the actual experimental configuration, which consisted of a region composed of a cell-free hydrogel (6 mm radius and 1.8 mm height) encapsulating the cell-laden region (0.85 mm radius and 0.1 mm height), referred to as region 1 and 2 respectively in Fig. 3. The steady-state oxygen distribution within this geometry was studied assuming two mechanisms: diffusion and Michaelis–Menten kinetics. The governing equation for such a system is

\[ 0 = D_0 \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C_{O_2}}{\partial r} \right) + \frac{\partial^2 C_{O_2}}{\partial z^2} \right] + \gamma, \]  

where \( D_0 \) is the diffusivity of oxygen and \( C_{O_2} \) is the oxygen concentration. It was assumed that the oxygen consumption activity, or reaction term \( \gamma \), only occurs in the cell-laden region (region 2) and is affected by the local oxygen concentration. This adaptive behavior of \( \gamma \) was simulated assuming Michaelis–Menten kinetics:

\[ \gamma = -V_{\text{max}} \left( \frac{C_{O_2}}{K_m + C_{O_2}} \right), \]

where \( V_{\text{max}} \) is the maximum oxygen-respiration rate, or oxygen uptake rate, and \( K_m \) is the concentration of oxygen at which the oxygen uptake rate decreases by one-half. The diffusivity of \( O_2 \) in a cell-culture medium and in a collagen gel was assumed to be similar to that in water (\( D_{O_2} = 2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \) (ref. 26)), and it was assumed to be uniform throughout the geometry. The cell-free region (region 1) is dominated only by diffusion, and the reaction term \( \gamma \) is zero. The boundaries at the air-solid interface were assumed to have a continuous influx of \( O_2 \) in order to maintain a constant concentration of \( C'_{O_2} \) as being determined by Henry's law:

\[ C'_{O_2} = p_{O_2} S \]

where \( p_{O_2} \) is the partial pressure of oxygen in air (\( p_{O_2} = 158.8 \text{ mmHg} \)) and \( S \) is the solubility of oxygen in culture media (\( S = 1.29 \times 10^{-3} \text{ mol m}^{-3} \text{ mmHg}^{-1} \) (ref. 27)). A dimensional analysis of the problem shows that the values of \( V_{\text{max}} \) and \( K_m \) scale with the assumed values of \( D_{O_2} \) and \( C'_{O_2} \), respectively, and that there are no other dimensionless groups with unknown parameters. Therefore, parametric sweeps using just these two variables were used to identify a range of values for \( V_{\text{max}} \) and \( K_m \) that resulted in a prediction that bracketed the experimental data.

Results
Oxygen monitoring in different spheroid culture platforms
The oxygen microsensors were calibrated using both intensity-based and phase-shift measurements. Phase fluorimetry showed a highly consistent response to different oxygen levels ranging from 0–21%, in contrast to intensity-based quenching (Fig. SI 2A and C†). To reduce the variation inherent in the microsensors, phase fluorimetry of our monodispersed oxygen microsensors was performed throughout this work. The micro-sensors were used to compare two different formats of spheroid cultures: hanging drops and 96-well round-bottom plates. The oxygen levels were measured after 18 hours for a single HEK 293T spheroid (5.0 × 10⁵ cells per spheroid) in these two culture formats. The oxygen levels within spheroids in the hanging drop cultures did not change within 5 days (data not shown) when compared to the media controls without cell cultures (both hanging-drop and round-bottom wells). However, the oxygen levels around the spheroids in round-bottom wells were lower (Fig. 1). Since a significant drop in oxygen was measured in the microwell, even with just a single spheroid, the platform was investigated further to explore possible effects of multiple spheroids. As seen in Fig. 1, more spheroids (e.g., one, five, ten, and fifty) resulted in lower steady-state (at 18 h) oxygen levels. Fig. 1C shows the ability to perform real-time, continuous monitoring of peri-tissue oxygen concentrations, as well as the spheroid number-dependence (1, 5, or 50 spheroids) of the rate of decrease in oxygen concentration in round-bottom wells. Fig. 1D further highlights that the oxygen levels in hanging drop cultures do not exhibit a significant change compared to media controls, even with 10 spheroids maintained in the hanging drops.

Oxygen gradient mapping and modeling of 3D hydrogel cultures
The oxygen microsensors were also dispersed into micropatterned cell-laden hydrogel constructs† (Fig. 2A). Using phase fluorimetry, we observed oxygen concentrations that varied from hypoxic levels of ~0.5% \( O_2 \) (0.00496 mol m\(^{-3}\)) within the interior of the cell-laden region to higher oxygen levels in the cell-free regions of the hydrogel (Fig. 2B). The oxygen profile was fit to a computational model (Fig. 3A) with a two-dimensional axisymmetric geometry, where the best fit gave a \( V_{\text{max}} = (1.0 \pm 0.27) \times 10^{-2} \text{ mol m}^{-3} \text{ s}^{-1} \) and \( K_m = (6.0 \pm 1.0) \times 10^{-3} \text{ mol m}^{-3} \) (i.e., 6.0 ± 1.0 µM) (Fig. 3B). Again, it is emphasized that these values are based on the assumptions that
Discussion

Oxygen is important not only as a key substrate for respiration but also as a regulatory molecule that alters gene expression and cell function. Because oxygen is only sparingly soluble in aqueous solutions, with a reduced concentration compared to that in the atmosphere, and because oxygen diffusion is relatively slow, pericellular oxygen concentrations can be significantly altered by uptake of oxygen by the cells themselves. 3D cell-culture platforms are particularly susceptible to such effects because of the high density of cells used compared to 2D cultures, as well as diffusion-limited characteristics of these cultures. The wealth of new information from custom 3D cultures, which more aptly reconstruct aspects of the cellular microenvironment milieu, has been well documented in recent years. Oxygen gradients through these 3D tissues are well recognized. On the other hand, oxygen gradients that can form in the pericellular microenvironment are not as commonly appreciated. For example, spheroid cultures in non-stick microwell cultures are commonly used interchangeably with hanging-drop cultures, as both form spheroids. In this work, we highlight the variation that can occur as a function of culture vessel-type or from the use of a gel-embedded culture format.

While a variety of oxygen sensors exist, we prepared custom, lab-fabricated oxygen sensors in a dispersible microbead format (Fig. SI 1†). A major reason is that cell-compatible optical oxygen sensing microbeads reported previously²⁸,²⁹ are easily dispersible, and could be easily incorporated into different culture formats, but have been prone to inaccuracies owing to heterogeneities in the sensors²⁹,³⁰ and to inherent variabilities associated with intensity-based optical-sensing modalities (Fig. SI 2A†). On the other hand, while phase fluorimetry-based oxygen sensors also exist commercially, they primarily are large, patch-based sensors or very small, nano-scale sensors that can be taken up by cells. By using phase fluorimetry with ~100 micron-sized sensor beads, we demonstrated tighter reproducibility and peri-cellular oxygen concentrations across the physiological range of oxygen concentrations (Fig. SI 2C†). Phase-fluorimetric measurements¹⁸,²⁰ are used to eliminate inconsistencies that arise from dye-loading variability,²⁹ photobleaching, and optical-path variability inherent to 3D tissues,³¹ enabling a more robust measurement system than intensity-based imaging.

$D_{O_2} = 2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (ref. 26) and $c_{O_2}^* = 0.205 \text{ mol m}^{-3}$, with $V_{max}$ and $K_m$ scaling with each parameter, respectively.

**Fig. 1** Oxygen levels as a function of cell mass and culture vessel. (A) Schematic illustration of the hanging-drop (left) and round-bottom (right) spheroid-culture systems. These schematics illustrate a single spheroid with an oxygen microsensor to highlight the difference in culture geometries and spheroid position with respect to the air–liquid interface. Black arrows identify the air–liquid interface at the closest point to the spheroids. (B) Microsensors were placed into 96 round-bottom wells with 0 (media control), 1, 5, 10, or 50 spheroids or into hanging drops (HD) with one spheroid or just media (control). Samples were observed over 18 hours with the stabilized oxygen values presented for 18 hours. Oxygen measurements were averaged per sample well together, regardless of microsensor position with regard to cell mass, in order to generate an oxygen level per well, error bars presented are one standard deviation, $n = 4$ well samples assessed per condition. (C) Oxygen levels monitored in a microplate well after mixing and aeration of media followed by static culture, demonstrating real-time continuous measurement capability of the microsensors. (D) Microsensors were placed into HD with 0 (media control), 1, 3, and 10 spheroids and monitored over the course of one day. Error bars presented are one standard deviation, a minimum of 3 samples were assessed per condition.
Previously described with bead-based microsensors,\textsuperscript{28,29} in addition to enhancing the reproducibility of our measurement system between batches, we identified a relatively long shelf life for the sensors (Fig. S1 2D\textsuperscript{†}), eliminating the concern for the use of the sensors over the course of the present study. This was possible because phase-fluorimetry measurements minimize the dependence on intensity, which drops over time in beads, as seen in Fig. S1 2D, SI 3, and Table SI 1.\textsuperscript{†}

Once validated, we used our microsensors to evaluate different 3D cell cultures. For cultures of the same sized spheroids, we observed higher peri-cellular oxygen levels (similar to no-cell controls) in hanging-drop cultures, whereas round-bottom-microwell cultures showed significantly lower oxygen levels at 18 hours. We attribute these differences to the distance between the spheroids and the air-liquid interface (~0 mm for the hanging drops and ~4 mm for the microwells, a schematic of the different geometries can be found in Fig. 1). Because of the less efficient oxygen-transfer rate of microwells, an increase in cell mass (number of spheroids) led to even lower steady-state oxygen levels (Fig. 1B), some of which approach hypoxic/anoxic levels.\textsuperscript{32–34} This observation was in contrast to those observed by placing multiple spheroids in a hanging drop, which did not appear to significantly affect the oxygen levels in the hanging-drop cultures. These results highlight the need to evaluate the role of micropatterned vessel-type on oxygen transfer rates, as this may have a substantial impact on cell function.

We also mapped oxygen concentrations (Fig. 2) within and around an island of cells patterned within a hydrogel. The MDA-MB-231 cells used for this portion of the study do not spontaneously form tight spheroids,\textsuperscript{22} enabling us to simulate a 2D slice of a spheroid in a 3D culture-like environment. Employing a prevalent practice for the qualification of bioreactors,\textsuperscript{35} we generated a computational model of oxygen supply, diffusion, and uptake (Fig. 3) by using the geometry of the experimental system and a range of literature values of oxygen $V_{\text{max}}$ and $K_m$ (ref. 36–42) to run a parametric sweep. We approximated the best fit for $V_{\text{max}}$ and $K_m$ to be $(1.06 \pm 0.27) \times 10^{-2}$ mol m\textsuperscript{−3} s\textsuperscript{−1} and $(6.0 \pm 1.0) \times 10^{-3}$ mol m\textsuperscript{−3} (i.e. $(6.0 \pm 1.0)$ μM), respectively of MDA-MB-231 cells in our system. The value for $V_{\text{max}}$ of $1.06 \times 10^{-2}$ mol m\textsuperscript{−3} s\textsuperscript{−1} corresponds to an...
oxygen uptake rate of \((3.00 \pm 0.10) \times 10^{-17}\) mol per cell per s which falls between previously reported values\(^{39}\) \((1.6 \times 10^{-17}\) mol per cell per s while in suspension and \(5.6 \times 10^{-17}\) mol per cell per s while in a monolayer culture). Commercially available systems were also used to determine the oxygen consumption rate (OCR) and the oxygen levels in uniform, unpatterned, cell-laden hydrogel culture formats. The Seahorse XFe Extracellular Flux Analyzer gave an OCR for MDA-MB-231 cells in a uniform collagen gel of \(271.63 \pm 2.72\) pmol min\(^{-1}\). This corresponds to an oxygen-uptake rate of \((1.30 \pm 0.013) \times 10^{-17}\) mol per cell per s (Fig. S1 f). This is very similar to our computationally produced values, given the differences in the culturing format. Oxygen levels were measured by using an Ocean Optics NeoFox within DMEM for a blank collagen gel, and cell-laden collagen gels (Fig. S1 f). When using the probe, all the measurements had to be end-point measurements within the collagen gel, since the ~700 µm probe tore through the gel as it was inserted. Additionally, the culture disruption and the relatively large size of the probes made accurate measurements difficult, and detailed mapping of oxygen gradients impossible. This highlights two benefits of using untethered microsensing probes, such as those presented in this work.

While there are a number of other papers on evaluating oxygen measurements for cell, tissue, and organismal cultures,\(^{43–48}\) this analysis is one of the few that has pursued sufficient accuracy and resolution, in terms of both oxygen levels and position of the measurements, to allow estimations of the cellular oxygen uptake rate. This method also has advantages over the more conventional Clark electrodes in that it does not consume oxygen during the measurements, and is less destructive in nature compared to Clark electrodes and other fiber-based probes.\(^{36,37,44,49}\)

Conclusions

The recent increase in the use of 3D cell cultures presents new challenges in understanding and regulating cellular oxygen microenvironments. We implemented non-toxic, dispersible, optical oxygen-sensing microbeads and used phase fluorimetry, rather than the more common fluorescence intensity-based methods, to accurately quantify pericellular oxygen concentrations. This sensing strategy was used to show that hanging-drop spheroid cultures maintain pericellular oxygen levels very similar to atmospheric oxygen levels while microspheres was provided by NSF (CMMI-0700232). Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the DTRA and SSC PACIFIC. SCLP was supported by an NSF Graduate Research Fellowship Program (DGE 1256260; ID: 2011101670) and the NIH Cellular Biotechnology Training Program (NIH GM008353). CM was supported by a Bannting postdoctoral fellowship from the Natural Sciences and Engineering Research Council of Canada. TK is thankful for the Yoshida Scholarship. The authors would also like to thank Dr Joseph M. Labuz for useful discussion and thoughtful input, Priyan Weerappuli for help with MATLAB image processing, and Usha Kadiyala from J. Scott VanEppls lab (University of Michigan) and Prof. Joe Lo lab (University of Michigan) for the commercial \(O_2\) sensor measurements.

References

6. A. Carreau, B. E. Hafny-Rabhi, A. Matejuk, C. Grillon and C. Kieda, Why is the partial oxygen pressure of human


