Implementing oxygen control in chip-based cell and tissue culture systems

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Oxygen is essential in the energy metabolism of cells, as well as being an important regulatory parameter influencing cell differentiation and function. Interest in precise oxygen control for in vitro cultures of tissues and cells continues to grow, especially with the emergence of the organ-on-a-chip and the desire to emulate in vivo conditions. This was recently discussed in this journal in a Critical Review by Brennan et al. (Lab Chip (2014). DOI: 10.1039/C4LC00853G). Microfluidics can be used to introduce flow to facilitate nutrient supply to and waste removal from in vitro culture systems. Well-defined oxygen gradients can also be established. However, cells can quickly alter the oxygen balance in their vicinity. In this Tutorial Review, we expand on the Brennan paper to focus on the implementation of oxygen analysis in these systems to achieve continuous monitoring. Both electrochemical and optical approaches for the integration of oxygen monitoring in microfluidic tissue and cell culture systems will be discussed. Differences in oxygen requirements from one organ to the next are a challenging problem, as oxygen delivery is limited by its uptake into medium. Hence, we discuss the factors determining oxygen concentrations in solutions and consider the possible use of artificial oxygen carriers to increase dissolved oxygen concentrations. The selection of device material for applications requiring precise oxygen control is discussed in detail, focusing on oxygen permeability. Lastly, a variety of devices is presented, showing the diversity of approaches that can be employed to control and monitor oxygen concentrations in in vitro experiments.

1 Introduction

In the in vitro study of cells and tissues, control of culture conditions is of paramount importance. In order to achieve results that are as predictive of the in vivo situation as possible, a multitude of parameters can be monitored and...
excellent review recently published by Brennan of monitoring oxygen concentrations when incubating tissue practical research in this area, and to stress the importance and tissue culture. It aims to provide a solid foundation for the measurement of oxygen in microfluidic devices for cell and tissue culture. It aims to provide a solid foundation for practical research in this area, and to stress the importance of monitoring oxygen concentrations when incubating tissue or cells. As such, this review serves as a complement to the excellent review recently published by Brennan et al.\(^1\)

In this introduction, the role of oxygen in the functioning of stem cells, cancer stem cells and organs is explained, as well as the theory describing its solubility and the potential use of artificial oxygen carriers to improve the transport of oxygen. The second section gives an overview of relevant examples of oxygen measurements, based on electrochemical and optical detection. The theory underlying these techniques is also discussed. The third section provides information on the oxygen permeability of widely used polymers for microfluidic culture devices. Furthermore, it reflects on the integration of both optical and electrochemical detection of oxygen into microfluidic devices for control of oxygen gradients during cell and tissue culture.

### 1.1 Oxygen in cells and tissues

Oxygen is the most abundant element in the human body in terms of mass,\(^4\) and as molecular oxygen (O\(_2\)) it is essential for various metabolic processes. Mitochondrial oxidative phosphorylation is central to the energy metabolism of most cells and provides over 95\% of the adenosine triphosphate (ATP) in the body. This process accounts for over 95\% of the O\(_2\) used in the human body, where it functions as the primary electron acceptor.\(^5\) Furthermore, oxygen is incorporated into many compounds made in the body, has a regulatory function in many signaling pathways and is a substrate for over 60 mammalian oxidase enzymes.\(^6,7\) Since both O\(_2\) excess and deficiency will result in cell damage or death, the body has various systems to maintain O\(_2\) levels in a precise range. This monitoring takes place not only in well-understood, specialized cells in the carotid body\(^‡\) that influence respiration, but in all nucleated cells. Hypoxic cells, for instance, will exhibit marked differences in gene transcription.\(^8\)

One of the signaling pathways in which O\(_2\) concentration plays a very important role affects the differentiation of stem cells. In the early days of tissue and cell culture, much attention was paid to nutrients and growth factors in the in vitro systems that were used for cell incubation, as well as the buffer systems used. Atmospheric O\(_2\) concentration (21\%) was assumed to be adequate for incubation purposes.\(^9,10\) However, following from direct measurements of O\(_2\) concentrations in developing embryos and tissues that were known to harbor stem cells, it became clear that hypoxia is necessary for maintaining an undifferentiated state in several stem cell populations.\(^10\) Stem cells are typically quiescent, using only a limited amount of O\(_2\) from a hypoxic environment. This is indicated by their high expression of hypoxia-inducible factor (HIF), HIF-1\(_\alpha\), a key regulator of the intracellular response to hypoxia.\(^11\) Hypoxia stabilizes HIFs, which in turn increase the expression of important stem cell markers.\(^10\) Comparable to oxygen-dependent formation of highly energetic phosphates such as ATP, stem cells will differentiate when exposed to O\(_2\) concentrations higher than 1–5\%.\(^9\) This property is used by stem cell researchers, along with the differing reaction of stem cells to signaling molecules such as growth factors and cytokines at varying oxygen levels, to direct differentiation into functional tissues.

A similar phenomenon is seen in cancer stem cells.\(^11\) Again, HIFs activate the transcription of genes, which in this

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\(^{‡}\) A cluster of chemoreceptors and supporting cells located along the carotid artery, which runs along both sides of the throat.

\(^{§}\) Oxygen percentage is the dissolved amount of oxygen relative to the amount that would be dissolved were the sample to be saturated. For example, the oxygen percentage of an aqueous solution in equilibrium with a gaseous phase containing 21\% O\(_2\) will be 21\%.
case play a role in angiogenesis (HIF-1 promotes release of vascular endothelial growth factor), genetic instability, immune evasion, metabolic reprogramming, invasion and metastasis, radiation resistance, and maintenance of the undifferentiated tumor properties. Although not all tumors exhibit this behavior, it is prevalent enough that a variety of pharmaceutical compounds meant to disrupt these hypoxia-dependent, tumor-regulating processes are being developed and tested.

In healthy tissue, O₂ concentrations can vary greatly from one type of tissue to the next. Concentrations of 3.6 to 6.4% in bone marrow, 12 to 14% in brain tissue and 3.3 to 12% in the liver have been reported. This appears to be mostly based on their respective metabolic activities, with more active organs showing lower O₂ concentrations. However, gradients of oxygen concentration in the organs themselves exist as well. For example, oxygen levels in the rat brain can vary to levels as low as 0.5–1% in some areas. The kidney, heart, and liver are other examples of organs exhibiting internal oxygen gradients. The liver is a particularly well-studied example of an organ in which these oxygen gradients play a strong regulatory role. Besides being an essential electron acceptor in energy metabolism as in other organs, oxygen is also a key regulator for metabolic zonation, and a necessary reactant for detoxifying xenobiotic metabolism in the liver. Hepatocytes are uniform at the histological level; however, they are heterogeneous at the level of morphometry and histochemistry. A distinction can be made between the region around the portal vein and hepatic arteriole (the periportal zone), and the region around the central vein (the perivenous zone), as shown in Fig. 1. The blood flows through the liver from the periportal to the perivenous zone through many sinusoids, microscopic capillaries that are lined by hepatocytes. Fig. 1 shows a schematic representation of one such sinusoid, where oxygen-rich blood from the hepatic artery (HA) is mixed with nutrient-rich blood from the portal vein (PV). The metabolic activity of the hepatocytes results in a gradient of oxygen along the sinusoid, with a difference of about 50% between the regions. This translates to an asymmetric distribution of enzymes regulating the various metabolic functions of the hepatocytes. This phenomenon is not only observed in hepatocytes, but also in almost all other cell types in the liver. Examples like this point out the widely varying oxygen conditions that should ideally be emulated in in vitro systems, if in vivo conditions are being strived for.

### 1.2 Organ-on-a-chip

The organ-on-a-chip is an important result of the recent drive in the life science and drug development communities towards more in vivo-like in vitro systems meant to recreate organ function ex vivo. The in vivo cellular niche is engineered by leveraging microtechnologies to fabricate small-volume devices in which cell cultures are perfused and small features can be actuated mechanically to implement organ-like function. This is the latest in a series of developments which has seen a transition from very simple, two-dimensional cell monolayer cultures to more complex three-dimensional cell culture models and associated in vitro systems. Mainly used for research in in vitro toxicology and pharmacokinetics, and increasingly in regenerative medicine, organ-on-a-chip systems differ from conventional cell cultures in many ways. Instead of growing cells in 2D monolayers submerged in static medium in a well plate, organ-on-a-chip systems incorporate means of active and controlled delivery of nutrients to cells and removal of metabolic waste products from cells, most often by perfusion. It has been noted that perfusion can enhance the response of even the simplest single-cell-type monolayer models to be more in vivo-like. However, many of the organs-on-chips reported in the literature benefit also from advances made in 3D cell culture, in which cell–cell and cell-extracellular matrix interactions are vastly improved when compared to 2D cultures. Close resemblance of the in vivo situation of the organs is further pursued through the co-culturing of different cell types. An often-named end goal of organ-on-a-chip research is the creation of interconnected organ-systems, a human-on-a-chip. Such a system should allow for sensing of metabolically important parameters, chemical signaling between the organ compartments, fluid manipulation and microscopic investigation.

A distinction between “bottom-up” and “top-down” approaches for organs-on-a-chip can be made. In most
systems, (induced) stem cells or cell lines are seeded on a substrate to grow and form tissue-like constructs – the “bottom-up” approach. This approach allows for a high degree of freedom for experimentation with different cell-type configurations in the device. The “top-down” approach is less common. Here, tissue samples (for example precision-cut tissue slices) are obtained from the organs of mammals or humans, and incubated in a device. An advantage of this approach is the faster acquisition of tissue material for actual experiments, and the preserved configuration of the different cell types in the original extra-cellular matrix. However, in both approaches, quantitative monitoring of critical parameters such as oxygen in minute quantities is essential in order to assess cell behavior and status inside the system, and thus exert greater control over the “organ” in question. In this review, many examples will be provided of ways to monitor and control oxygen concentrations in organ-on-a-chip and other relevant devices, both systemically and locally.

1.3 Oxygen solubility

The blood transfers inhaled oxygen from the lungs to the rest of the body. The concentration of dissolved oxygen depends on the temperature and partial pressure of oxygen in the gaseous phase; this is described by Henry's law (eqn (1)):

\[
k_H = \frac{c_s}{p_g}
\]

Here, \(c_s\) is the concentration of a species (oxygen in this case) in the aqueous phase and \(p_g\) is the partial pressure of that species in the gas phase at a certain temperature \(T\). The temperature dependence of Henry's constant, \(k_H\) – which is, because of this dependence, also referred to as Henry's law coefficient – is described in eqn (2).

\[
k_H(T) = k_{H0} \exp\left(\frac{-A_{H0}H}{R} \left(1 - \frac{1}{T_T}\right)\right) = \frac{c_s}{p_g}
\]

Here, \(k_{H0}(T)\) is Henry's law coefficient as a function of temperature \(T\), \(k_{H0}\) is Henry's law coefficient at \(T = 298.15\) K (= 1.3 \times 10^{-3} \text{ M atm}^{-1} \text{ for O}_2)\), and \(\frac{A_{H0}H}{R}\) is the enthalpy of the dissolution process divided by the gas constant (=1500 K for O2; the solubility of oxygen increases with decreasing temperature). Henry's law coefficient at a temperature of 310.15 K (37 °C) is therefore 1.07 \times 10^{-3} \text{ M atm}^{-1}. Using Henry's law (eqn (1)), the dissolved oxygen concentration of a saturated aqueous solution at a given partial pressure can be calculated. Since air consists of 21% oxygen, the partial pressure at sea level is 0.21 atm or 21.28 kPa; often, the dissolved oxygen concentration of a saturated aqueous solution in equilibrium with air is also referred to as 21%. However, the actual dissolved oxygen concentration in a saturated aqueous phase at 21% partial pressure and 37 °C is 225 μM, or 7.02 mg L^{-1} at 37 °C. The solubility of oxygen is thus low in aqueous solutions, including cell medium and blood plasma. It should be noted that there are multiple common units used to express dissolved oxygen concentrations. An overview of these is given in Table 1, along with conversions of several O₂ percentages in aqueous solutions at 37 °C to different units.

The evolution of oxygen-carrying molecules in blood was necessary to make the transport of oxygen from the lungs to other tissues more efficient. In humans and other vertebrates, a metalloprotein, hemoglobin, transports inhaled oxygen (O₂) throughout the body. It binds oxygen to one of its four heme groups, a porphyrin ring with an iron atom in the center. More than 98% of the oxygen in the blood is transported by red blood cells (RBCs), which are the transport vehicles for hemoglobin. In microfluidic systems, the use of this kind of oxygen carrier is more complicated, and in fact has not been reported. Oxygen carriers are absent in the culture media used, and oxygen supply to cells or tissue is wholly dependent on diffusion, which is far less efficient. The use of oxygen carriers has been investigated, though not in the context of microfluidics, as elaborated below.

1.4 Artificial oxygen carriers

The oxygen-carrying capability of blood is essential for a living organism, and a loss of this capability, for instance due to an excessive loss of blood, can be fatal. Nowadays, blood transfusions are a routine medical procedure. However, the donor–recipient compatibility, the large demand for whole blood and blood products, short shelf-life (35–42 days, depending on the product and national regulations) and the possibility of spreading blood-borne diseases are important reasons to investigate possible alternatives. Some blood functions, like coagulation, and properties like osmotic pressure can be provided by means that do not require blood. However, there is no artificial product as of yet that can provide all the functions of blood, and thus be used as a substitute in humans or other mammals. There has, however, been a substantial amount of research in the area of artificial blood products, which will be described briefly below.

The capability of blood to efficiently transport oxygen using red blood cells and the possibility to realize this function differently have been intensively studied for many years. Molecules that can bind oxygen and transport it, thus mimicking hemoglobin, are referred to as artificial oxygen carriers. There are two main ways in which these artificial oxygen carriers function. The first employs perfluorocarbons (PFCs), which have the ability to dissolve large amounts of

| Table 1 | An overview of some common units used to express dissolved oxygen concentration, and their respective converted values in aqueous solutions at 37 °C for several oxygen partial pressures. |
|-------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Temp 37 °C** | **% O₂** | **μmol L⁻¹** | **mg L⁻¹ (ppm)** | **hPa** | **Torr ≈ mmHg** |
| 5 | 10 | 21 | 50 | 100 |
| 53.5 | 107.0 | 224.7 | 535.0 | 1070.0 |
| 1.7 | 3.3 | 7.0 | 16.7 | 33.4 |
| 50.9 | 101.9 | 213.9 | 509.4 | 1018.8 |
| 38.2 | 76.4 | 160.5 | 382.1 | 764.3 |
oxygen (about 10–20 times more than pure water\(^{34}\)). The second approach utilizes free hemoglobin (not transported by RBCs).\(^{33}\)

### 1.4.1 Perfluorocarbons

PFCs are hydrocarbons in which all the hydrogen atoms have been substituted with fluorine. They are chemically inert, odorless and colorless, but they are not soluble in water or lipids.\(^{33,35}\) Liquid perfluorocarbons can be used as artificial oxygen carriers, but they need to be emulsified first. In order to do this, different detergents have been tried. The two most commonly used detergents are based on poloxamers (like Pluronic-F68) or on natural phospholipids (like lecithin). The poloxamers are chemically stable and do not undergo oxidation under physiological conditions, in contrast to phospholipids. However, phospholipids are preferred, as fewer adverse effects have been observed in patients, and a higher degree of control over the purity of the detergent can be achieved. To prevent the oxidation of phospholipids, a small amount (0.1–0.2% w/v) of \(\alpha\)-tocopherol can be added.\(^{36}\)

Since perfluorocarbon emulsions are intended for use in conjunction with living organisms, tissues or cells, it is also important to ensure that they have the right osmotic pressure and pH. Ideally, the final emulsion should have an osmolarity and a pH similar to those found in blood (ca. 300 mOsM dm\(^{-3}\) and pH 7.4, respectively).\(^{37}\)

The main challenge with an aqueous emulsion of PFCs is its instability and the resulting growth of droplet size, also known as Ostwald ripening.\(^{38}\) The larger droplets tend to merge with smaller ones due to molecular diffusion.\(^{39}\) The size of the droplets directly influences the surface across which oxygen transport between the aqueous and PFC phases occurs. The typical droplet size of PFC-based artificial oxygen carriers is in the range of tens to hundreds of nanometers,\(^{37}\) while the average diameter of human red blood cells is about 7.2 µm.\(^{40}\) Although PFC emulsions have a much lower oxygen solubility than hemoglobin-based oxygen carrier solutions (Fig. 2),\(^{41}\) they show superior oxygen delivery to tissue when compared to RBCs.\(^{42}\) This is partly due to the much larger oxygen exchange area, but also to the difference in the oxygen binding mechanism. Oxygen solubility in PFC is a physical phenomenon based on oxygen distribution according to Henry’s law (eqn (1)), while oxygen binding to hemoglobin is based on ligand–acceptor chemistry for cooperative binding described by the Hill equation (eqn (3), see also Fig. 2).

\[
S_a = \frac{p^*}{p^* + p_{50}}
\] (3)

Here, \(S_a\) is the dissolved oxygen content, \(p\) the oxygen partial pressure, \(p_{50}\) the oxygen partial pressure when half of the binding sites are occupied and \(n\) the Hill coefficient (for hemoglobin \(n = 2–4\), typically 2.8).

However, the potential for long-term treatment of, for example, anemia with PFCs is rather low, due to Ostwald ripening, the volatile nature of PFCs and their relatively fast clearance from the blood. Moreover, if the PFC droplets grow beyond the size of red blood cells, there is a serious threat of capillary congestion and thus an adverse result of the treatment. The use of PFCs as artificial oxygen carriers was proposed as early as 1966 by Clark and Gollan.\(^{43}\) From the 1970s through the late 1990s, there were several PFC-based artificial oxygen carriers commercially available. The so-called ‘first-generation’ PFC emulsions (Fluosol\((®)\), Perftoran® and Oxyphorol®), in which artificial emulsifiers were used, had significant though temporary side effects when administered to patients.\(^{42}\) Other limitations of these products were storage (at \(-20^\circ\text{C}\) and their short shelf-life (<6 months, though this is still much longer than blood and blood products). The second- and third-generation PFCs (Oxyfluor\((™)\), FMIQ, Oxygen\((™)\), Therox®, Oxyce®) were emulsified with phospholipids from egg yolk (lecithin) and could be stored for a longer time (18–24 months) at 4 °C.\(^{44}\) All of these products were either initially approved by the FDA but later withdrawn from the market (like Fluosol\((®)\)), or are still waiting to be approved for use in humans (e.g. Oxyce®).\(^{46}\) This means that there are no PFC products currently available for medical use as artificial oxygen carriers.

### 1.4.2 Hemoglobin-based artificial oxygen carriers

The hemoglobin-based artificial oxygen carriers (commonly abbreviated as HBOCs) appear to be good alternatives to PFCs. Evidence suggests that it should be possible to use hemoglobin isolated from the blood of one species as an artificial oxygen carrier in other species.\(^{47}\) Bovine, porcine and equine hemoglobin are cheap and broadly available byproducts of the food industry. The main drawback of hemoglobin is its low stability. Hemoglobin is gradually oxidized to methemoglobin, which cannot bind oxygen. Therefore, the HBOCs have to be stored in oxygen-free conditions, and used immediately after exposure to oxygen. Free hemoglobin in the bloodstream (not entrapped in RBCs or chemically stabilized by other means) has various toxic effects.\(^{48}\) One of the most important of these is the scavenging of nitrogen\(\text{(a)}\) oxide (NO),
which leads to acute vasoconstriction. For this reason, different polymerization and encapsulation techniques have been studied. In 2013, there were two HBOC products commercially available (from the same company), Oxyglobin® and Hemopure®. Both products were based on stabilized and polymerized bovine hemoglobin. Oxyglobin® is a veterinary product intended for use in the treatment of canine anemia. Hemopure® is intended for human use and is currently in human trials in South Africa.

Artificial oxygen carriers could potentially be used not only as a blood substitute for transfusions or treatment for anemia, but also for preservation of organs for transplantation and in tissue engineering. In vitro cell line cultures consist of either cell monolayers or cells in suspension. Typically used cell densities are in the range of \(1–50 \times 10^6\) cells per cm\(^2\). For these cell densities, the oxygen solubility in cell culture media (at normal atmospheric oxygen pressure) is usually high enough to supply a sufficient amount of oxygen. Typical cell density in tissues is approximately 10-fold higher than in cell monolayers. Moreover, tissue is three-dimensional in nature, and oxygen has to diffuse through multiple cell layers to reach cells in the inner regions. This fact, along with high oxygen consumption rates for some tissues (e.g. liver tissue), often renders the oxygen delivery to a tissue by aqueous media without oxygen carriers inadequate, leading to hypoxic damage. The knowledge gained in the area of artificial blood carriers could therefore one day prove to be beneficial for the development of media for (long-term) cell and tissue engineering applications.

2 Oxygen detection

To ultimately control oxygen gradients and even measure oxygen consumption in complex in vitro systems, accurate detection of oxygen in a spatially and temporally resolved manner is crucial. The measurement of dissolved oxygen in blood, medium and tissue can be done in several ways. The iodo metric Winkler titration for the measurement of oxygen is a well-established and precise technique that is considered to be the standard method. However, it is a time-consuming and cumbersome procedure, and reproducibility of the method largely depends on the skill and experience of the analyst. It involves treatment of the sample with Mn\(^{2+}\), OH\(^-\) and KI, after which it is acidified and the I\(^{-}\) titrated. Although it remains of great value in the calibration of other oxygen-measuring instruments, it is not suitable for continuous, on-line measurements in cell cultures, and will not be covered in this article. The two approaches that are widely used and will be discussed here are based on electrochemical and optical principles.

2.1 Electrochemical measurements

The development of the amperometric oxygen probe by Le land Clark in 1956 (ref. 57) and its subsequent integration into the blood-gas machine dramatically improved the care of critical patients. It is a robust and quite reliable measurement technique. The Clark electrode consists of a working platinum (or other noble metal) electrode (cathode) and a reference (usually Ag/AgCl) electrode (anode); a platinum counter electrode is sometimes also incorporated. A schematic representation is given in Fig. 3. The measurement of oxygen is based on an oxygen reduction reaction (ORR) at the working electrode which results in a detectable current. During the ORR, oxygen in the vicinity of the cathode is converted into liquid-phase products according to the following reactions (for basic and acidic media, respectively):

\[
\begin{align*}
O_2 + 2H_2O + 4e^- & \rightarrow 4OH^- \\
O_2 + 4H^+ + 4e^- & \rightarrow 2H_2O
\end{align*}
\]

Amperometric measurements based on this reaction are typically performed at \(-700\) mV to \(-800\) mV vs. Ag/AgCl using a potentiostat to define the working electrode potential. At this potential, oxygen is reduced to hydroxide ions or water, depending on the pH of the environment. For each set-up, a simple cyclic voltammetry experiment can be performed to assess the optimal potential for the reduction. The generation of electrons is proportional to the oxygen being reduced. Therefore, measurement of the generated current can be related to a concentration of oxygen in the sample. However, a major drawback of electrochemical detection of oxygen now becomes apparent. As a consequence of the principle underlying the detection, a diffusion layer extending from the surface of the electrode into the bulk is formed, over which oxygen is depleted – see Fig. 4 and 5. This can, especially for
Macroscopic electrodes but also for microelectrodes, lead to serious problems with respect to the depletion of oxygen in tissue in the vicinity of the electrode, especially if the solution is stationary and not flowing past the electrode\textsuperscript{62} – see Fig. 5. The influence of this effect on the oxygen gradient in small volumes, typically associated with microfluidic devices, is discussed in paragraph 2.1.1. In most Clark-type electrodes, a membrane that allows for selective permeation of oxygen to a small amount of electrolyte around the cathode is used to prevent oxygen depletion of the sample\textsuperscript{60,62}.

The high potential needed to reduce oxygen renders the cathode vulnerable to interfering electro-active compounds, which, especially in biological samples, will be abundant. However, the gas-permeable membrane included in the structure of Clark-type macro- and (some) microelectrodes can prevent diffusion of interfering compounds to the electrode surface, making coating of the electrode itself with a protective membrane unnecessary. However, the same membrane can protect the electrode from the effects of biofouling caused by adherence of biomolecules that impair the diffusion of analyte to the surface of the electrode. Biofouling can increase the response time of and interference at microelectrodes. Layers of adsorbed biomolecules build up during prolonged implantation in vivo, as was shown for amperometric platinum glutamate microsensors in the brain.\textsuperscript{63}

2.1.1 The oxygen diffusion layer. It would be preferable to use microelectrodes without the necessity of a membrane, in order to minimize response time and complexity. However, when measuring oxygen concentrations in a microfluidic culture chamber electrochemically, it is of vital importance that its reduction does not influence the delivery of oxygen to the incubated tissue slice or cell culture. Considering the flowing medium in which the measurements would take place, liquid flow patterns depicted in Fig. 4A are likely. Laminar flow will be present, sliding, as it were, past the electrode. At $\delta \mu m$, the laminar flow rate approaches zero, giving rise to a thin layer of stagnant fluid, known as the diffusion layer. In this layer, the concentration of analyte and product varies as a function of distance from the electrode surface. Analyte concentrations at the electrode surface approach 0 if the reaction is reversible and fast, and increase as the distance from the electrode increases. It is assumed that the concentration of analyte is at a maximum in the bulk regions, independent of whether laminar or turbulent flow patterns prevail. Analyte consumption at the electrode and the formation of a diffusion gradient play no role in these regions. Fig. 5 depicts the linear increase of analyte concentration in the diffusion layer as the distance from the electrode becomes bigger in a system with convective flow past the electrode. Whilst the diffusion layer will extend further and further into the bulk with time in a stationary solution (Fig. 4B), this is not the case with convective transport. Convection, or flow in the case of microfluidic devices, results in the diffusion layer having a

![Fig. 4](https://example.com/fig4.png)

**Fig. 4** Flow patterns at the surface of a microelectrode in a stirred (A) and stagnant (B) solution. $\delta$ is the diffusion layer thickness. In Fig. 4A, the diffusion layer thickness remains constant as analyte is transported from the bulk of the solution to the electrode surface. In Fig. 4B, the solution is stationary, and analyte is thus not actively delivered from the solution bulk to the electrode. As a result, the diffusion layer thickness increases over time, as analyte is depleted on the electrode surface and concentration gradients extend further and further into the bulk of the solution. From Skoog, Principles of Instrumental Analysis, 5E. © 1998 Brooks/Cole, a part of Cengage Learning, Inc. Reproduced by permission. www.cengage.com/permissions.

![Fig. 5](https://example.com/fig5.png)

**Fig. 5** Concentration profile at the interface between electrode and solution during reaction of the analyte A. Also depicted are the effects of increasing negative potentials (X, Y and Z) on the slope of this gradient. Z leads to the limiting current, as it gives the highest turnover of analyte A to the product. $\delta$ is the diffusion layer thickness.
constant thickness. A constant supply of analyte is provided by laminar flow to the outer edge of the diffusion layer. Because analyte is consumed at the electrode, a concentration gradient is established across the diffusion layer as analyte diffuses from the edge of this layer to the electrode. Since the thickness of this layer, \( \delta \), does not change, the concentration gradient is also constant, yielding a steady-state current, as given in eqn (6) for a planar electrode: \(^{64}\)

\[
i = nFAD_i \left( c_a - c_b^0 \right) / \delta \tag{6}
\]

Here, \( i \) is the current in amperes (A), \( n \) the number of moles of electrons involved in the reaction per mole of analyte, \( F \) the Faraday constant, \( A \) the surface area of the electrode, \( D_a \) the diffusion coefficient of the analyte A, \( (c_a - c_b^0) \) the difference between the concentrations of analyte A in the bulk and at the electrode surface, and \( \delta \) the thickness of the diffusion layer. As \( c_b^0 \) becomes increasingly small as a result of the applied potential being increased (see Fig. 5), the current generated at the electrode increases. When the potential applied to the working electrode is large enough, all analyte arriving at the electrode will be immediately consumed and \( c_b^0 \) goes to 0. At this point, the current has reached a constant value and no longer depends on the applied potential. The current is now the diffusion-limited current, \( i_l \). Adapting eqn (6) for this situation of oxygen measurement gives eqn (7): \(^{64}\)

\[
i_l = nFAD_{O_2} c_{O_2} / \delta \tag{7}
\]

Here, \( i_l \) is the diffusion-limited current in mA, \( n \) is the number of moles of electrons involved per mole of analyte (4; see eqn (4) and (5)), \( F \) is Faraday’s constant (96 485 C mol\(^{-1}\)), \( A \) the surface area of the electrode in m\(^2\), \( D_{O_2} \) the diffusion coefficient of oxygen in aqueous solutions at 310.15 K (37 °C) in m\(^2\) s\(^{-1}\), \( \delta \) the thickness of the diffusion layer in m and \( c_{O_2} \) the bulk concentration of oxygen in mol m\(^{-3}\). The diffusion coefficient was calculated according to Wilke and Chang – see eqn (8): \(^{65}\)

\[
D_{O_2} = 7.4 \times 10^{-9} \left( \frac{xM}{\eta} \right)^{1/2} T / \eta^{0.56} \tag{8}
\]

Here, \( D_{O_2} \) is the diffusion coefficient of oxygen through aqueous solutions in m\(^2\) s\(^{-1}\), \( x \) a dimensionless association parameter (2.6), \( M \) the molecular weight of water (18 g mol\(^{-1}\)), \( T \) the temperature of the aqueous solution (310.15 K; 37 °C), \( \eta \) the viscosity of water at 310.15 (0.692 g s\(^{-1}\) m\(^{-1}\)) and \( V \) the molar volume of oxygen (25.6 L mol\(^{-1}\)). \(^{65}\) At 37 °C, \( D_{O_2} \) has a value of 3.24 \times 10\(^{-9}\) m\(^2\) s\(^{-1}\).

As an example, consider a planar, square (275 μm \times 275 μm) electrode with a surface area of \( A = a^2 = (2.75 \times 10^{-4} \text{ m})^2 = 7.56 \times 10^{-8} \text{ m}^2 \). In preliminary experiments in our group, electrodes with this area were immersed in a phosphate buffered solution in equilibrium with 95% oxygen (oxygen concentration is 1.0 mM at 37 °C; see eqn (1) and (2)). The current generated by applying a potential of -700 mV to one of these electrodes was measured to be about 1 \times 10^{-6} \text{ A}. This value for \( i_l \) can be substituted into eqn (7), to estimate a diffusion layer thickness (\( \delta \)) of 9.5 \times 10^{-2} \text{ m}, or 95 μm. This value of \( \delta \) is not large, and so the use of membrane-less, planar microelectrodes of these dimensions for amperometric \( O_2 \) detection will not have negative effects on the oxygen gradient in most microfluidic culture devices. Needle-type microelectrode geometries should also not be a problem, as long as the electrode is placed more than 100 μm away from the cell culture. Nevertheless, attention should be paid to the exact location of electrode integration. The electrodes should not be located in narrow channels that transport oxygen-rich medium or placed too close to the cultured tissue or cells.

2.1.2 Oxygen microelectrodes in microfluidic devices. The fact that the architecture of electrochemical sensors is usually relatively simple, and measurement of current does not require sophisticated instrumentation, makes electrochemical measurement a robust, reproducible method. Micro-machining techniques have dramatically improved over the last twenty years, which has resulted in facile miniaturization of oxygen electrodes. \(^{57,68}\) Besides easier integration into experimental setups, the added value of this is a decrease in costs of the technology.

In Table 2 some examples of oxygen microelectrodes integrated into microfluidic devices are given. Wu et al.\(^{61,69}\) developed a three-electrode oxygen-sensing chip, designed for integration into a microfluidic analysis system (see Fig. 6). The working, counter and reference electrode are all made of sputter-deposited platinum.\(^{61}\) A later version of their chip also comprised three electrodes, but these were sputter-deposited gold electrodes. The gold reference electrode in this case was coated with Ag/AgCl.\(^{69}\) This later design was utilized for in situ measurement of the respiratory activity of adhering cells. The dimensions of the working electrode were 20 μm \times 20 μm, whereas the counter and reference electrode were 200 μm \times 2 mm; all three electrodes were deposited on a glass substrate. The electrodes were covered by an insulating, 24-μm-thick layer of SU-8. A channel was defined in this layer to expose the sensing pads of the electrodes. It was sealed in turn by a 15-μm-thick, oxygen-permeable PDMS membrane, on top of which a thicker additional layer of PDMS was bonded using plasma oxidation treatment. A 3-mm-diameter hole was drilled in this PDMS substrate to serve as a reservoir, with the PDMS membrane forming the bottom of the reservoir. Internal electrolyte (0.1 M KCl/25 mM bicarbonate buffer solution (pH 9.5)\(^{61}\) or 25 mM Tris buffer (pH 10.5) containing 0.1 M KCl and 2% sodium alginate (w/w)\(^{69}\)) was introduced through the PDMS membrane and slabs into the channels via drilled inlets to electrically connect the electrodes. After calibration, the reservoir was filled with 20 to 60 μL samples (e.g. cells), of which the oxygen concentration and consumption was measured. Because of the small electrode surface and the very thin PDMS
membrane, the 90% response times of the electrodes were very good: 8.7 s\textsuperscript{61} and 13.4 s\textsuperscript{69}. The only difference between the two systems was the size of the reservoir (1.5 mm\textsuperscript{61} vs. 3 mm\textsuperscript{69} diameter), the presence of a modification of polar groups in the PDMS membrane,\textsuperscript{61} and the higher viscosity of the alginate-containing electrolyte.\textsuperscript{69} The increased response time in the newer device was attributed to the smaller volume of the membrane. The addition of polar groups (3-aminopropyltrimethoxysilane) to the PDMS membrane facilitated adherence of cells to the membrane. The sodium alginate in the electrolyte, along with the SU-8 layer, prevented cross-talk between the electrodes by limiting the diffusion of oxygen.\textsuperscript{69} Measurements were done in cultures of \textit{E. coli}\textsuperscript{61} and human cervical cancer cells.\textsuperscript{69} However, no integration with a microfluidic total analysis system has been reported yet; only offline measurements are possible.

Krommenhoek \textit{et al.}\textsuperscript{70,71} designed a platform for the monitoring of various parameters of (stirred) yeast cultures. Aiming for high-throughput applications, the sensor chips were designed to fit inside a microowell of a 96-well plate. However, until now only applications of the chips mounted on a rod that can be inserted into larger bioreactors have been reported. The chips (ø 6 mm) comprise a pH-sensing ion-selective field effect transistor, a conductivity sensor to perform impedance spectroscopy (to assess biomass concentration), a miniature temperature sensor, and an ultramicroelectrode array (UMEA) for electrochemical monitoring of oxygen levels. Unlike the previous example above, no gas-permeable membrane was used in the construction of this electrode array. The UMEA was based on a platinum macroelectrode covered with a 1 μm-thick photostructured layer of polyimide. Holes with a radius of 2 μm had been formed in this polyimide layer to create an array of ultramicroelectrodes. The 114 electrodes had a minimal spacing of 50 μm. The advantage of this approach is a very rapid (millisecond range) formation of the oxygen diffusion layer at the electrode surface, with mass transfer of oxygen to the electrode being almost completely independent of convection.\textsuperscript{70}

Another example of electrochemical oxygen detection was presented by Eklund \textit{et al.}\textsuperscript{74,72} A commercially available multiphysimeter, the Cytosensor\textsuperscript{TM}, was modified to measure extracellular pH and \textit{O}_2,\textsuperscript{74} and later also glucose and lactate.\textsuperscript{72} The multiphysimeter consists of two membranes, between which cells can be seeded and medium can be perfused (see Fig. 7). Although they do not present the device as being microfluidic \textit{per se}, the flow chamber only comprised a 2.8 μL volume,\textsuperscript{75} and could hence be regarded as such. The operating cycle consisted of a flow-phase, during which medium was pumped in between the membranes, and a stop-phase, during which pumping was halted. During the stop-phase, the different analytes were measured simultaneously. Acidification was measured potentiometrically with the original electrode from the Cytosensor. Lactate production and glucose consumption were measured \textit{via} platinum

---

**Table 2** Electrochemical oxygen microelectrodes; \textit{W} = working electrode; \textit{C} = counter electrode; \textit{R} = reference electrode

<table>
<thead>
<tr>
<th>Electrodes material</th>
<th>Electrolyte</th>
<th>Membrane</th>
<th>Surface area (mm\textsuperscript{2})</th>
<th>Potential (mV) vs. Ag/AgCl</th>
<th>Response time (s)</th>
<th>Sensitivity (nA ppm\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wu \textit{et al.}</td>
<td>W, C, R: Pt</td>
<td>0.1 M KCl/25 mM bicarbonate buffer</td>
<td>15 μm PDMS</td>
<td>W: 4 × 10\textsuperscript{-4}; R, C: 0.4</td>
<td>-700</td>
<td>8.7</td>
</tr>
<tr>
<td>Wu \textit{et al.}</td>
<td>W, C: Au; R: Ag/AgCl</td>
<td>0.1 M KCl/2% sodium alginate</td>
<td>15 μm amino group-modified PDMS</td>
<td>W: 4 × 10\textsuperscript{-4}; R, C: 0.4</td>
<td>-700</td>
<td>13.4</td>
</tr>
<tr>
<td>Krommenhoek \textit{et al.}</td>
<td>W: Pt array; R: Ag/AgCl</td>
<td>—</td>
<td>—</td>
<td>W: (\textit{n} = 114) 1.26 × 10\textsuperscript{-3}; R: macro/commercial</td>
<td>—</td>
<td>0.96</td>
</tr>
<tr>
<td>Eklund \textit{et al.}</td>
<td>W: Pt</td>
<td>—</td>
<td>—</td>
<td>W: 1.27 × 10\textsuperscript{-2}; R: external</td>
<td>—</td>
<td>71</td>
</tr>
<tr>
<td>Suzuki \textit{et al.}</td>
<td>W: Ag; R: Ag/AgCl</td>
<td>0.1 M KCl</td>
<td>100 μm silicone rubber</td>
<td>W: 6.25 × 10\textsuperscript{-3}; 0.25; R: (\textit{n} = 8) 0.075</td>
<td>-800</td>
<td>50–20</td>
</tr>
<tr>
<td>Koley \textit{et al.}</td>
<td>W: Pt; R: Ag/AgCl</td>
<td>0.1 M KCl soaked</td>
<td>30 μm PDMS</td>
<td>W: 200; R: 100</td>
<td>-1000</td>
<td>40</td>
</tr>
</tbody>
</table>
electrodes coated with their respective oxidase enzymes. The recorded current was a result of the oxidation of hydrogen peroxide formed by the enzymes. Oxygen consumption was measured amperometrically with a 500-μm or 127-μm-diameter platinum electrode, coated with Nafion to prevent fouling from cell constituents. Cells that were seeded on the membrane inserts (Chinese hamster ovary cells and mouse fibroblast cells) were exposed to various compounds, after which the changes in oxygen consumption rate, acidification and energy substrate production and consumption were measured. This is an excellent way of investigating metabolic changes in the cells stemming from these exposures, as both anaerobic and aerobic metabolism are measured. Oxygen consumption rates were initially only reported as changes in measured current, but later a simple, two-point calibration was performed to convert the current to concentrations.

These designs could have a potential application in the amperometric monitoring of oxygen in a microfluidic tissue or cell culture chamber. They possess small electrodes and either do not require a membrane to control O₂ diffusion to the electrodes, or use one that is composed of the same material as many microfluidic devices (i.e. PDMS). This renders them easily miniaturized and integrated. Furthermore, they show relatively good sensitivity and response time.

However, although with the introduction of microelectrodes the temporal and spatial resolution of the electrodes improve, the currents involved are very small, requiring sensitive meters and shielding from electromagnetic interference. To prevent the formation of an oxygen depletion layer in the vicinity of the electrodes, Mitrovski et al., Wu et al. and Koley et al. used a highly oxygen-permeable PDMS membrane. This allows for a very rapid response time of the sensor, while still controlling the diffusion of O₂ to the electrodes. Interestingly, following from the work of Suzuki et al., the layer from the surface of the electrode over which oxygen is depleted stretches out over the gas-permeable membrane (about 190 μm) when large cathode surface areas of 500 × 500 μm are used. Therefore, if electrochemical techniques are to be employed for oxygen monitoring in microfluidic systems, effective miniaturization is of the utmost importance.

### 2.2 Optical measurements

Electrochemical oxygen sensors suffer from the disadvantage that they require a physical, electrically conductive connection between the electrode in solution and the detector. This is not the case with optical methods, which often may be used non-invasively or remotely (i.e. not in contact with solution) to measure solution parameters. Moreover, optical methods do not cause the formation of oxygen-depleted regions, whether in direct contact with solution or not. Most optical oxygen sensors are based on the reversible quenching of the luminescence intensity and decay time of a luminophore by oxygen (see Fig. 8). Quenching is the transfer of energy from an excited luminophore to another molecule, instead of it being emitted as a fluorescent or phosphorescent photon. With oxygen as the quenching agent, the process can be modelled by the Stern–Volmer equation (see eqn (9)).

$$\frac{I}{I_0} = 1 + k_0P_{O_2}\tau$$  \hspace{1cm} (9)

Here, $k_0$ is the quenching rate constant, $P_{O_2}$ the partial pressure of oxygen in solution, $\tau$ and $I_0$ the luminescence lifetime and intensity, respectively, in the absence of oxygen, and $I$ the luminescence lifetime and intensity at the partial pressure of oxygen being measured, respectively. Use of oxygen concentration $[O_2]$ instead of partial pressure is also possible.

Many oxygen-sensitive materials (which are often dyes) exhibit heterogeneity, and a non-linear Stern–Volmer relationship as a result, requiring more complex modeling of their behavior. Most probes, however, will exhibit a linear relationship between $P_{O_2}$ and the luminescence lifetime and intensity for at least an analytically interesting range. For oxygen sensing, phosphorescent probes with a relatively long luminescence lifetime of 1–100 μs are generally used. Fluorescent probes have a higher intensity, but are less suitable for...
The use of a ratiometric method, in which the sensing probe contains both an oxygen-sensitive and a reference (oxygen-insensitive) dye, overcomes some of these problems. The advantage is that with the employment of this reference dye, the emitted intensity measurements can be corrected according to the concentration of the probe, which especially in tissue cultures can be quite unevenly distributed. An example of this is an intracellular probe developed by Hynes and Papkovsky. The probe contains an oxygen-sensitive dye, which produces a photoluminescence signal dependent on oxygen concentration, in addition to a second dye with an oxygen-insensitive emission. This ratiometric method is supported by many standard microscopy and spectroscopy set-ups. Furthermore, complex models incorporating photobleaching and calibration techniques per pixel have been developed to extend the limits of intensity-based oxygen sensing.

A more robust method, however, is luminescence lifetime-based oxygen sensing. Using the same oxygen-sensitive dyes, stable calibration and improved performance are possible, overcoming most problems associated with intensity-based methods. Furthermore, it renders a good contrast between the background and the signal of interest. Lifetime-based sensing of oxygen can be done by measuring the lifetime using pulsed excitation or by measurement of the phase shift between sine-modulated excitation and the emission light, which relates to the lifetime. Fig. 9 illustrates the method of pulse-and-gate time-domain luminescence lifetime detection, in which the excitation light is modulated by a square wave pulse. The detector is gated, resulting in discrete time windows where the intensity of the emission during the decay period of the dye’s luminescence is measured.

The ratio of the data can be used to determine the luminescence lifetime of the indicator:

\[
\tau = \frac{t_2 - t_1}{\ln A_1 / A_2}
\]  

Here, \(\tau\) is the luminescence lifetime, \(t_1\) and \(t_2\) the starting time of the data acquisition windows, and \(A_1\) and \(A_2\) the intensity measured during the gates. By adding a short delay between the end of the excitation and the first data acquisition window, interference by short-lived background luminescence or decay of the excitation pulse light is averted.

As with these two measurement approaches, the oxygen-sensitive dyes themselves can be used in multiple ways, as shown in Fig. 10. Metalloporphyrins are hydrophobic compounds and not soluble in water. In order to be able to sense dissolved oxygen, metalloporphyrins need to be either made soluble (e.g. by addition of a detergent) or embedded in a polymer that provides rigidity, is permeable to oxygen, and is in contact with the sample. Examples of such polymers are organically modified silica (ORMOSIL), poly(dimethylsiloxane) (PDMS), polystyrene and poly(methylmethacrylate). Various ruthenium-based dyes have also been embedded in Nafion. The sensor can in this way be used as a (patterned) thin-film sensor inside, for example, a microfluidic culture chamber (Fig. 10(a) and (b)). It is also possible to use a dye-coated optical fiber, which can
and carry the emitted luminescence back to the detector. Oxygen-sensitive micro- and nanoparticles can be prepared by embedding the dye in a particle matrix (usually silica-based) or doping polymer or silica beads with dye. The particles can be used directly in the sample or can be embedded in another polymer and used as a film (Fig. 10(d) and (e)).

Water-soluble probes, including the ruthenium complexes, are most common, and can be used directly in the sample, making this a very versatile form that is especially useful in imaging of biological oxygen consumption (Fig. 10(f)). The disadvantage of this is that luminescence properties can be adversely influenced by binding with and/or interference by biological components. Furthermore, especially in perfusion systems, a lot of expensive probe would be needed to treat all cell medium. As such, microfluidic biochips have the advantage of small volumes. Of course, as for any measurement in biological samples, it is important to carefully assess possible toxicity of the specific dye used.

2.2.1 Intracellular measurements. Another very interesting application of oxygen-sensitive optical probes is in intracellular measurements. Using probes that have cell-penetrating properties, intracellular gradients, individual cell and tissue consumption rates and in situ oxygenation can be assessed, without damaging the cell or influencing its normal function. The earliest reports of the use of fluorescent probes for intracellular oxygen measurements stem from 1981.

![Fig. 9 Pulse-and-gate time-domain luminescence lifetime detection. The probe is excited by a short light pulse, after which the intensity of emission is measured in two gates (A1 and A2). The calculated decay time is calibrated as a function of the oxygen concentration. Reproduced from ref. 95 with permission from John Wiley and Sons.](image)

![Fig. 10 Possible uses of oxygen-sensitive dyes: a) as a thin film; b) patterned, thin-film; c) dye-coated optical fibers, with and without opaque, optically isolating coating; d) micro/nanoparticles in aqueous medium; e) micro/nanoparticles in thin film; f) water-soluble dye. Reproduced from ref. 76 with permission.](image)
Since then, a wide variety of intracellular oxygen-sensitive probes has been developed. For an excellent overview the reader is referred to two articles by Dmitriev et al.\textsuperscript{77,104} One probe that is of particular interest is the nanoparticle-based probe that comprises a cationic polymer, Eudragit RL-100, and a hydrophobic, oxygen-sensitive dye, PTFPP. Its cell-loading properties, toxicity and analytical performance are well described. Mouse epithelial fibroblasts (MEF), human epithelial carcinoma derived from cervical cancer cells (HeLa), human hepatocellular liver carcinoma (HepG2), non-differentiated and differentiated human neuroblastoma (SH-SY5Y) and rat pheochromocytoma (PC12) cells exhibited good and comparable cell loading with the probe.\textsuperscript{102,104} Quantitative oxygen measurements can be done by detecting phosphorescence in a time-resolved fluorescence plate reader; alternatively, phosphorescence lifetime imaging microscopy (PLIM) can be conducted using confocal microscopy.\textsuperscript{102} The probe has high sensitivity, its luminescence being quenched 1.5-to-15 fold by oxygen, and is capable of penetrating a variety of cell types without observable intrinsic toxicity.\textsuperscript{91,102} Another probe that also uses lifetime imaging techniques to visualize intracellular oxygen gradients was developed by Sud et al., but it uses a fluorescent oxygen-sensitive dye.\textsuperscript{105} A hydrophobic platinum porphyrin probe embedded in micelles for transportation into cells is under development by the Meldrum group.\textsuperscript{106}

The reported results lead to the expectation that the use of these probes in tissue cultures should be relatively easy. However, for systems in which the tissue is perfused and not incubated in stationary medium in wells, it is unclear whether the loading of probes into deeper tissue layers would be sufficient.\textsuperscript{77} If use of these probes is successful, constructing tissue culture systems suitable for monitoring in a confocal microscope becomes very interesting. A microscopy set-up equipped with oxygen and temperature regulation mechanisms would allow observation of luminescence and therefore oxygen gradients during perfusion of the tissue. Experiments with, for example, liver tissue could elucidate the effect of oxygen gradients on tissue functionality, especially during prolonged incubation. This could be done by using different medium oxygenation\textsuperscript{107} and/or the use of oxygen-carrying components in the medium.\textsuperscript{108}

### 3 Defining oxygen gradients through controlled delivery and monitoring

Constant oxygen delivery to a microfluidic channel can be achieved relatively easily. However, cell oxygen consumption will vary, depending on medium conditions (e.g. nutrient concentrations) and cell requirements. As the medium volumes contacting cells in microfluidic perfusion cultures can be quite small, cells can alter oxygen gradients in their immediate vicinity even if oxygen delivery is in principle strictly controlled. The advent of microfluidic cell culture and the possibilities it offers to engineer natural in vitro environments for cells and tissue has sparked an interest in integrating improved oxygen monitoring and control in these systems. As was alluded to above, there are a variety of microfluidic devices incorporating controlled oxygen delivery in the literature. These range from passive oxygen diffusion over PDMS membranes from externally controlled atmospheres to complex in-chip microchannel structures for mixing gases which then diffuse directly into microfluidic channels. Some form of oxygen sensing is often integrated into miniaturized culturing devices; this is especially true when monitoring oxygen consumption is a primary experimental objective. Inherent to the integration of oxygen control is the selection of materials for device fabrication, as gas transport rates into and out of the device will vary greatly from one material to the next. This is especially important in the field of microfluidics, where large surface-to-volume ratios are the norm. The following sections will examine available polymer materials with respect to their gas permeability, diffusion and solubility properties, and present a few relevant examples from the literature of devices incorporating oxygen control.

#### 3.1 Oxygen permeability of polymers used for fabricating microfluidic culture devices

Control of oxygen in a microdevice without the need to oxygenate medium externally can be achieved by selecting a suitable material based on its oxygen permeability. Some common polymers and their relevant properties are listed in Table 3. One of the most common materials used for the fabrication of microdevices for life science applications is PDMS.\textsuperscript{109-112} PDMS is a chemically inert, optically transparent and non-toxic polymer, and is used as a biomaterial in a wide variety of medical components ranging from catheters to ear and nose implants.\textsuperscript{113} The most convenient technique for microfabrication in PDMS is by replication, which involves casting the not yet cross-linked polymer onto a mold or master. The polymer is then allowed to cure, a process during which the polymer crosslinks and hardens. Once cured, the elastomeric polymer layer is removed from the mold, with all the features of the mold faithfully replicated with nanometer resolution in the layer. Using a microfabricated mold with raised micrometer- or nanometer-dimensioned structures, microchannels can be easily formed in this polymer. Oxygen plasma surface modification can then be used to covalently bond layers of PDMS to each other or to glass. These properties make PDMS a popular material for fast prototyping of microdevices, particularly for cell culture purposes. Furthermore, it can be utilized as a component in an active scheme for oxygenation of culture media due to its high permeability for gases and vapors.\textsuperscript{114,115} Oxygen-permeable PDMS membranes inside oxygen-impermeable microdevices can be used for the transfer of oxygen to and from culture medium to establish oxygen gradients within cell culture chambers. The high gas permeability of PDMS could be considered a drawback in some cases, especially when oxygen gradients need to be maintained in very
small volumes of aqueous media. However, the gas permeability of PDMS can be tuned to some extent by modifying the surface with plasma treatment or using certain storage conditions for the cured PDMS. The high permeability to vapors means that the aqueous constituent of medium can evaporate through the PDMS. This effect is particularly problematic in microdevices, where the high surface-to-volume ratios can lead to evaporation-mediated changes in medium composition or even to cell cultures drying out. Another disadvantage of PDMS is the nonspecific absorption of hydrophobic molecules by the bulk PDMS. This is due to the hydrophobicity of the material itself, in conjunction with the high solubility of hydrophobic species in loose PDMS chain networks. Moreover, uncured short PDMS oligomers, as well as the platinum-based curing catalyst, can leak from the bulk of material. The high hydrophobicity can also result in the nonspecific adsorption of proteins and hydrophobic small molecules (e.g., many drug metabolites) to the PDMS surface. Nonetheless, PDMS still remains the most used material for the prototyping of microfluidic biochips.

Another polymeric material with relatively high oxygen permeability is poly(tetrafluoroethylene) (PTFE), generally referred to by its brand name Teflon (DuPont Co). It is most renowned for its exceptional chemical and physical inertness. Moreover, the refractive index of PTFE is very close to or, in the case of amorphous PTFE, even lower than water. The refractive indices of commonly used fluorinated polymers range from 1.32 to 1.38 and can be tuned. These values fall below the refractive indices of most polymers, which have refractive indices in the range of 1.4–1.6, making PTFE and other similar fluoropolymers quite distinctive materials in this regard. PTFE is a common material for porous membranes, particularly in its hydrophilic form. Because of the match between the refractive indices of PTFE and water (the refractive index of pure water is 1.33), it is possible to have a thin, porous membrane that is optically clear (i.e., does not scatter light due to porosity) when wetted. This is a useful property for membranes integrated in microfluidic devices designed for microscopic imaging. The low surface energy of PTFE and similar fluorocarbons makes them suitable as anti-fouling and anti-stick coatings. However, its low transparency and difficulties in processing and bonding make PTFE rather problematic for the fabrication of microfluidic biochips in bulk quantities. PTFE can also be used as a gas-permeable liquid barrier in microdevices.

In contrast to PDMS and PTFE, polyetheretherketone (PEEK) has very low oxygen permeability. Besides exhibiting low adsorption of biomolecules like DNA, it has a high chemical and mechanical resistance. Therefore, other thermoplastic polymers such as polystyrene (PS), cyclic olefin copolymer (COC), poly(methyl methacrylate) (PMMA), and polycarbonate (PC) are more commonly used for microdevice fabrication. These materials have a slightly higher oxygen permeability (see Table 3) than PEEK, are more easily processed and are transparent to visible light. The optical transparency of COC extends down into the UV range. The use of thermoplastic polymers in conjunction with fabrication techniques like injection molding allows the production of microdevices on an industrial scale.

### Table 3  

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Ref.</th>
<th>$P$ (Barrer)</th>
<th>$D$ (m$^2$ s$^{-1}$ cm$^{-1}$)</th>
<th>$S$ (cm$^3$ STP cm$^{-3}$ cmHg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS</td>
<td>155</td>
<td>610</td>
<td>$4.58 \times 10^{-15}$</td>
<td>$2.50 \times 10^{-9}$</td>
</tr>
<tr>
<td>Teflon AF1600</td>
<td>156</td>
<td>154</td>
<td>$1.16 \times 10^{-15}$</td>
<td>$2.40 \times 10^{-3}$</td>
</tr>
<tr>
<td>PE</td>
<td>155</td>
<td>23</td>
<td>$1.73 \times 10^{-16}$</td>
<td>N.D.</td>
</tr>
<tr>
<td>PTFE</td>
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<td>4.2</td>
<td>$3.15 \times 10^{-17}$</td>
<td>$6.30 \times 10^{-4}$</td>
</tr>
<tr>
<td>PS</td>
<td>155</td>
<td>2.9</td>
<td>$2.18 \times 10^{-17}$</td>
<td>$2.76 \times 10^{-4}$</td>
</tr>
<tr>
<td>POM</td>
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<td>$3.15 \times 10^{-17}$</td>
<td>$7.30 \times 10^{-4}$</td>
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<tr>
<td>PEEK</td>
<td>158</td>
<td>0.13</td>
<td>$1.00 \times 10^{-18}$</td>
<td>N.D.</td>
</tr>
<tr>
<td>PVC</td>
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<td>PET</td>
<td>155</td>
<td>0.03</td>
<td>$2.25 \times 10^{-19}$</td>
<td>$9.20 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

### 3.2 Oxygen control in microfluidic devices for culturing tissue and cells

As elaborated earlier, it is possible to use electrochemical and optical measurements in microfluidic systems to monitor oxygenation of medium during incubation of tissue or cells. Several different ways of achieving oxygenation have been described, including approaches exploiting oxygen monitoring.

#### 3.2.1 Oxygen control in non-perfused microfluidic cell culture

The most basic form of oxygen delivery is by passive diffusion from an externally controlled atmosphere through a gas-permeable material directly into the culture microchamber. PDMS is often the material of choice due to its high gas permeability. In stagnant, microwell-type culture systems (no perfusion of medium), this can be a very convenient approach. The sensors in the microwell system proposed in Krommenhoek et al. for the incubation of yeast were discussed earlier. Here, oxygen will simply diffuse into the medium present in the microwell system. Another way of controlling oxygen concentrations in microwells was reported by Oppegard et al. Rather than placing uncovered, cell-seeded microwell plates in a hypoxic chamber to
equilibrate cell medium with controlled low-oxygen atmospheres, a microfluidic insert to exert oxygen control in individual wells in a 6-well plate was developed. Transwell inserts with a porous membrane bottom were suspended in wells. These were filled with medium to form so-called Boyden chambers, which can be regarded as chambers within wells. MDA-MD-231 cells, an invasive breast cancer cell line, were seeded onto the immersed membrane surface of each chamber. A microfluidic insert, termed a “hypoxic device” and formed in the shape of a pillar, was then placed in each well. The hypoxic device served to hermetically seal the well to prevent diffusion of oxygen into it from the environment. However, gases could be infused into microchannels formed in the pillar of the device, to then diffuse over a PDMS membrane into the Boyden chamber in close proximity to the cell layer. This allowed for equilibration with the desired oxygen (or other gas) concentration being infused. Reaching an oxygen concentration of 1% from an initial concentration of 21% took about 20 minutes, which was significantly faster than when Boyden chambers were used in a hypoxic chamber. This made induction of intermittent hypoxia easily achievable. Validation of the device was done with planar fluorescent oxygen sensors underneath the membrane. A good example of on-chip diffusion of oxygen as a way to control oxygen in a culture was reported by Zanzotto et al. A simple microbioreactor system for the incubation of bacteria was developed. It comprised a glass base, a PDMS body containing a 5-to-50 \( \mu \)L culturing well (depending on the diameter used) and a 100-\( \mu \)m-thick PDMS aeration membrane to allow oxygen diffusion into the culture well. The PDMS well contained two holes with embedded pH and oxygen-sensitive foils for optical measurement of these parameters. Furthermore, optical density was measured to assess the growth of the bacteria. The device was shown to be comparable to regular 500-\( \mu \)L bioreactors in terms of growth profile, dissolved oxygen and pH profiles over time, and energy uptake and metabolite formation over time. Because of the aeration membrane and the low cultivation volume, oxygenation of the medium in the well could be easily controlled. In a later design by Zhang et al., a magnetic stirrer was added to improve the oxygenation of the bacteria culture.

3.2.2 Oxygen control in perfused microfluidic cultures.

One can also make use of diffusion to control oxygenation of a culture in perfused systems. Leclerc et al. published a PDMS-based system in which 4 chambers for hepatocyte culture were supplied with oxygen by means of a central oxygen chamber that was perfused with air. The channels that were used to perfuse the culture chambers with medium were separated from this oxygen chamber by only 300-\( \mu \)m-thick PDMS walls, allowing rapid equilibration of medium with oxygen. Cultures of cells seeded in the device reached cell densities comparable to macro-scale bioreactors. Van Midwoud et al. developed a comparable system that relied on the oxygenation of medium through PDMS. In this case, not cells, but precision-cut liver slices, were incubated in a PDMS biochip, featuring 6 individually addressable culture chambers of 25 \( \mu \)L each. The whole device was placed in an incubator environment of 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \). The culture chambers in the device were enclosed on all sides by PDMS, with two 250-\( \mu \)m-thick PDMS membranes, the “breathing windows”, forming the top and bottom surfaces of the chambers. This allowed for rapid equilibration of the medium that was used to perfuse the chambers, as was confirmed with a commercial electrochemical microsensor placed at the outlet of the device for 24 hours. On-line oxygen monitoring in a platform for 3D liver culture was reported by Domansky et al. They developed an array of 12 perfusable, open-well bioreactors containing scaffolds onto which rat hepatocytes and hepatic sinusoidal endothelial cells were seeded (see Fig. 11). Each bioreactor contains 3 mL of medium and consists of two 15 mm-diameter wells, one containing the scaffold with cells (reactor well) and one functioning as a medium reservoir. The medium is circulated by an integrated diaphragm pump between the two wells, through the porous scaffold in the reactor well \textit{via} a microfluidic channel underneath the wells. Optical fibers (2 mm diameter) covered with a ruthenium-based oxygen sensitive dye can be inserted vertically into each well through the lid of the device. These sensors were used to measure oxygen levels in the medium in both the reactor and reservoir wells. The gathered data was subsequently used to validate an already proposed model of oxygen distribution in the device. With this information, further improvement of the incubation method could be achieved.

3.2.3 Establishing oxygen gradients in microfluidic culture devices.

In cases where gradient oxygenation in the culture is desired, simple diffusional-supply approaches similar to those discussed above in paragraph 3.2.2 can be used. Malda et al. used a glass-based 5-\( \mu \)m-diameter microelectrode to assess oxygen concentration in tissue-engineered, 3D cartilage constructs, cultivated in a stirred diffusion cell. By inserting the electrode in the constructs from the top and gradually removing it in 100-\( \mu \)m steps, an assessment of 3D oxygen gradients in the tissues could be made. The results were compared to those obtained in native tissue, and were found to closely match the gradients observed there, apart from at the beginning of cultivation. The measurements were used to improve models for oxygenation of tissue-engineered and native cartilage. In smaller systems, the establishment of oxygen gradients usually requires more complicated structures and oxygen delivery systems. Jaeger et al. developed a bioreactor specifically designed to mimic oxygen gradients observed in tumors (as described in 1.1). In this device, tumor cells in Matrigel® (a gelatinous protein mixture produced by mouse sarcoma cells and resembling extra-cellular matrix) were cultivated on a silicone hydrogel layer. This layer was patterned with micropillars 25 to 100 \( \mu \)m in diameter and 200 to 250 \( \mu \)m high (Fig. 12). Oxygen flowing through a channel underneath the silicone layer diffused through the pillars into the Matrigel cell culture and was consumed by the cells there. The cell culture was contacted by medium, which in turn was contacted by a stream of nitrogen and \( \text{CO}_2 \). Using optical oxygen probes, finite element modeling...
and close observation of tumor-cell growth patterns, the effect of local oxygen gradients on tumor cells was studied. A significant drop of the oxygen concentration was observed over a region of about 100 μm around each micropillar, which is also reported in in vivo studies of oxygen gradients in tumor tissue. This makes this device very interesting for preclinical drug studies in cancer research.

Lo et al. studied the effects of oxygen gradients on cell cultures. They seeded Madin–Darby Canine Kidney cells in a 1-mm-diameter reservoir with a 100-μm-thick PDMS bottom, through which oxygen from gradient generators underneath could diffuse. These generators made use of either diffusion between parallel microchannels or mixing in a network (see Fig. 13). The parallel microchannels (Fig. 13-A) generated a gradient as a result of diffusion between two gas inlet channels, one transporting 100% O₂, and the other 0% O₂ (the two horizontal channels extending from left to right from “In” to “Out” in the figure). In the network generator (Fig. 13-B), mixing of the 100% and 0% O₂ gases occurred in an intricate pattern of channels by splitting and recombining the two input gas streams. By placing a commercially available, oxygen-sensitive fluorescent film on the PDMS membrane covering the generators, the oxygen gradients could be visualized (Fig. 13-C and D). In this way, levels of reactive oxygen species that were consistent with hypoxia or oxidative stress could be realized, and the effects on the cell culture studied. Combining this simple, yet effective design for oxygen gradient generation and the use of an oxygen-sensitive thin film (as was also reported by Eddington in an array of open microwells) is thus very promising for monitoring oxygen gradients on cell or tissue cultures.
Lam et al.\textsuperscript{150} presented a PDMS-based microfluidic system incorporating a similar gradient system. Here, 20-μm-high, 100-μm-wide microchannels were used for mixing oxygen and nitrogen. By altering the relative flow rates of the gases and using the high gas permeability of PDMS, precise regulation of the dissolved oxygen concentration in adjacent microchannels perfused with culture medium could be achieved. The device was able to facilitate a very broad range of oxygen concentrations (0 to 100% O\textsubscript{2}, or about 0–1000 μM) in these channels. Tuning of the oxygen concentration was possible by changing the dimensions of the channels in the mixer network. An array of Pt-porphyrin-based oxygen sensors was used to monitor the oxygenation of the device. Validation of the system was done by growing 3 species of bacteria with varying oxygen demands and one mammalian cell type (murine embryonic fibroblast cells). A comparable system was published by Thomas et al.\textsuperscript{151} Here, gas-equilibrated water was pumped through 250-μm-wide control channels in a PDMS device. From these channels, the gases diffused through a 200- or 80-μm-thick layer of PDMS into a 1000-μm-wide central chamber containing a dextran solution. The gas could either be oxygen or nitrogen, and thus precise oxygen concentrations or gradients could be formed. Gas-equilibrated water instead of just the gas itself was used to avoid evaporation of the solution. The device was sealed at the bottom by a PDMS membrane that contained the porphyrin-based, oxygen-sensitive dye PtTFPP, which is also used for intracellular measurements, as described in Dmitriev et al.\textsuperscript{77} Using this dye, oxygen gradients in the whole device could easily be visualized. The device exhibited a wide range of obtainable oxygen concentrations, and the possibility to create oxygen gradients over the central chamber. Incubating cells or tissue in the central chamber would render this a simple device to study the effects of these concentrations during incubation.

Another way to deliver precise concentrations of oxygen to a cell culture was described by Maharbiz et al.\textsuperscript{152} Here, the device comprised a Ti/Pt electrode pattern in microchannels filled with electrolyte. Electrolysis of water into oxygen and hydrogen was facilitated in a tunable fashion, after which the bubbles of gas were transported to a cell culture. When a bubble left the electrode area over a silicone barrier towards the culture region, new electrolyte was aspirated into the electrode area from a reservoir. The device would be capable...

Fig. 13 The intricate channel patterns used for generating oxygen gradients used by Lo et al. The oxygen diffuses through a PDMS membrane on top of these structures into a 1-mm-diameter reservoir where cells can be seeded. A) The gradient is established through diffusion between the two parallel microchannels B) the gradient is established by mixing in a network. C) and D) Display the respective oxygen concentrations at different positions in the gradient structure as imaged by an oxygen-sensitive film. Reproduced from ref. 149 with permission from The Royal Society of Chemistry.
of supporting a wide range of cell types, with oxygen demands from 0 to 10 μmol h$^{-1}$.

Finally, Weise et al. reported a device that allowed the measurement of oxygen consumption of HepG2 cells, grown in monolayer and three-dimensional cultures in a perfused microbioreactor. Inside the polycarbonate (a polymer with low gas permeability) bioreactor, the cells were seeded on a MatriGrid® membrane. Two optical oxygen sensors were placed below (upstream) and above (downstream) this membrane. By perfusing medium through the membrane with cells, and subtracting the read-outs of the sensors, oxygen consumption could be measured. It was found that the vitality and growth kinetics of the monolayer and perfused three-dimensional culture was comparable, but the oxygen consumption of the 3D cultures were lower than that of the monolayer. A similar example was recently reported by Harink et al. The medium is oxygenated before it enters a glass microfluidic device by allowing oxygen to diffuse over the walls of the perfluoralkoxy alkane (PFA) tubing used to perfuse the device with medium. This allows for a large degree of control and the ability to study the behavior of cultures under hypoxic conditions. As the device could be used for incubation and simultaneous imaging in a microscope, viability and hypoxia assays could easily be performed. The device was specifically designed for use in regenerative medicine and stem cell research, where strict (hypoxic) oxygen conditions often need to be applied.\(^9\)^\(^{10}\)

4 Conclusions and outlook

Microfluidic biochip platforms have proven to be versatile incubation devices for cell cultures and tissue. However, to further improve and profit from the advantages of microfluidic perfusion over the traditional static culturing techniques, the incorporation of sensors to monitor relevant parameters is required. Oxygen is one such parameter, as an essential part of the metabolism of each cell, but also as a regulatory parameter in both cells and tissue. Both an increased understanding of its role, and a precise control over its concentration in accordance to this, would potentially benefit the viability and functionality of incubated tissue or cells. Considering the ease with which different techniques discussed in this review can be miniaturized, implementing oxygen control into these culturing devices seems like a logical step, and some examples of this have been discussed. Of course, one should consider how these measurements could affect the incubated tissue or cells and their direct environment in order to select the most suitable approach for implementation.

Oxygenation measurements, especially in combination with assessment of expression of hypoxia-related factors (e.g. HIFs), will deliver valuable information for the improvement of tissue perfusion systems and cell culturing devices. Hopefully, an increased understanding and control over oxygenation in in vitro models will lead to an increased viability and functional in vivo resemblance. This would lead to the widespread acceptance of these systems as valid platforms for the study of organ functionality, pathology, metabolism and toxicology studies of novel drugs, and many more applications in the organ-on-a-chip field.

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References

Artificial Oxygen Carrier: Its Frontline


