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A microfluidic gradient generator to simulate the oxygen microenvironment in cancer cell culture

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ABSTRACT

Standard cancer lab models lack many attributes of the in-vivo cancer microenvironment. Oxygen levels for example are not commonly controlled in 2D cell-culture well plate experiments. However, low O_2 (hypoxia) in particular is common in cancerous tissue due to high proliferation rates of cancer cells and inadequate vasculature. Hypoxia is also associated with cancer recurrence and drug resistance. We report a microfluidic system capable of exposing a 2D cell-culture to a dissolved oxygen gradient ranging from hypoxia (< 5 mg/L) to hyperoxia (40 mg/L) for over 30 min, measurable in real-time using an integrated sensor film. The film incorporates a photostable, non-cytotoxic oxygen-sensitive fluorescent dye, which exhibits a linear response and high contrast ($I_0/I_{100} = 12$) within the range of interest, was integrated onto glass substrates as a cell culture substrate. To demonstrate the applicability of the platform, *Ishikawa* cancer cells were cultured on the platform and exposed to linear cross-stream oxygen gradients. The platform provides a valuable tool for the culture of cancer cells in an in-vivo like microenvironment and will enable more accurate screening of new anti-cancer drugs.

1. Introduction

The Importance of Oxygen Conventional culture models, such as two-dimensional (2D) cell mono-layers in culture plates or wells, lack many environmental features that are present in-vivo. This is concerning given the inhomogeneous oxygen microenvironment present in cancer tumours.

Hypoxia is often present in-vivo due to high proliferation rates and inadequate or absent vasculature [1]. Hypoxic conditions have been correlated with the development of aggressive phenotypes, cancer recurrence [2], and shorter life span before recurrence [3].

Hypoxia is difficult to define as the oxygen tension in various tissues throughout the body varies. Table 1 shows the concentration of oxygen of several types of healthy tissue and their cancerous counterparts [4], indicating that disease can have as little as 5% the oxygen of healthy tissue. Hypoxia in endometrial cells is considered 1–1.5 mg/L [5].

The absence of oxygen control in anti-cancer drug discovery and screening systems is an issue that needs to be urgently addressed. The lab model needs to be upgraded to include at least some of the nuances of the 3D environment. Naturally, this has led to the use of 3D cell culture, and 2D culture with environment control on the macro- or microscale.

3D cell-culture The use of microscale 3D cell clusters [6,7] and 3D hydrogels encapsulated cells [8] has been on the rise due to the ability of these technologies to better reproduce the 3D microenvironment and thus provide more indicative results in relation to drug efficacy [9]. While these methods are 3D, the oxygen inhomogeneity is uncontrolled, size reproducibility can be poor [10], and it is difficult to visualise the inner hypoxic areas until after the microtumour/3D scaffold has been fixed and sectioned, or the cells lysed.

There are many advantages to environmental control on-chip, such as fast and relatively cheap prototyping for lab-on-a-chip devices, rapid and controlled cycling of temperature, media and concentration gradients. Unique to oncology is the opportunity to investigate the use of rare primary cells towards future personalised drug screening processes. Specifically for the 2D culture on-chip, it is possible to observe cell-cell interactions, migration and other biological processes in a microchannel towards a real tumour-on-a-chip model.

2D cell-culture on-chip To date, a limited number of studies have used oxygen control and sensing alongside 2D cancer cell-culture and drug testing on the microscale [11,12]. Two-dimensional systems allow for real-time imaging of cells, while microfluidics can be used to

Table 1
Oxygen concentration present in healthy tissue and their diseased counterparts. Adapted from [4,5].

Tumour type	Median O_2 (mg/L) healthy	Median O_2 (mg/L) diseased
Head and neck	6.9	2.0
Lung	5.1	1.0
Pancreatic	6.9	0.4
Cervical	6.9	0.4
Prostate	7.0	2.7
Endometrial	19–20	1–1.5

simultaneously mimic aspects of the 3D microenvironment and to guide cell-cell interactions. The added advantage over 3D cultures is the level of control and the ability to incorporate or exclude attributes of the 3D environment as desired.

Oxygen scavenging/generating reactions for oxygen control [11] or gas channels separated by a thin membrane, either in 2D [12,13] or 3D [14] have been commonly used to control O_2. For oxygen measurement, oxygen sensitive dyes [12], or fluorescent films [15,16] can be used. The dyes are dissolved in the cell-culture media, with effects of dye uptake on drug response, cell viability, proliferation and metabolism mostly unknown. The films are fabricated by typically encapsulating a sensing compound in a solid-state matrix, and do not come into contact with the cells or media.

Drug screening and discovery Lab model systems that mimic the 3D environment, when used in drug screening, could reduce the occurrence of false positives and negatives. In addition to that, the visibility of cells allows quick assessment of drugs without the complex lysing, or sectioning and staining process necessary for 3D cultures. Additionally, with the utilisation of high throughput parallelisation possible with microfluidic devices, the process could be sped up considerably.

2D culture on chip with oxygen control In this work, we present a reusable, reversibly-sealed microfluidic system capable of producing an oxygen gradient, as illustrated in Fig. 1. Non-invasive oxygen sensing functionality was embedded into the cell-culture substrate and off-chip flow and gas control was employed. The oxygen sensor, previously developed in our group [15,16] consist of a polystyrene (PS)-based matrix incorporating platinum(II) octaethylporphyrin ketone (PtOEPK) as an oxygen-sensitive fluorescent dye. This allows intensity-based fluorescent imaging as a function of oxygen concentration gradients through adherent cells in real time [17]. Regarding the microfluidic chip, a reusable, resealable approach was used to allow further biological analysis. A plug and play approach was used to provide gas control for ease of prototyping and system modification. We demonstrate applicability of the platform by culturing *Ishikawa* endometrial cancer cells and exposing the culture to cross-stream oxygen gradients on-chip.

The reported system enables the generation of a gradient between the extremes of hyperoxia and hypoxia. The gas control system, at present, ensures near-maximum saturation of the cell-culture media with oxygen/nitrogen for hyperoxia and hypoxia, respectively.

2. Materials and methods

The geometry of the microfluidic chip used to generate an oxygen gradient is shown in Fig. 2a. Two inlet channels converged on a 1 mm wide, 100 μm high, 3 cm long cell-culture channel, which then diverges to three 333 μm wide outlet channels. Standard photolithography and replica moulding techniques were used to form a mould and subsequent microfluidic chips. Briefly, a dehydrated silicon wafer was laminated (Sky 335R6, DSB) with a 100 μm negative-tone dry film of photoresist (SUEX, Microlaminates) at 60 °C. The channel geometry was designed using L-Edit (Mentor Graphics) and a chrome-covered glass plate was exposed (μPG, Heidelberg), developed and etched to reveal this pattern. The photoresist was then exposed to 675 mJ/cm^2 of ultraviolet (UV) light (MA-6, Suss Microtec) through the mask patterns with 20 rounds of 10 s exposure and 60 s of no exposure between rounds. The wafer was subsequently heated at 60 °C (100 °C/h ramp) for 5 min, 90 °C (100 °C/h ramp) for 10 min, then cooled to room temperature (15 °C/h ramp). Mould development was then performed by immersion in Propylene glycol monomethyl ether acetate (PGMEA) for 10 min and rinsed with isopropyl alcohol.

Polydimethylsiloxane (PDMS) prepolymer was mixed with curing agent (10:1 w/w, Sylgard 184, Dow Corning) and desiccated before pouring into a 5 mL syringe and further desiccated. The degassed PDMS was then injected onto the precut Si/SU-8 mould set into a casting station (Microfluidic ChipShop), and cured at 80 °C for 4 h. The casting station top plate with PDMS was then peeled off the mould, and transferred to the chip holder, shown in Fig. 2b.

The sensor film substrate was prepared by dissolving PS pellets and PtOEPK powder into toluene. 7% w/w of PS/toluene was first prepared and left for the PS to dissolve for > 24 h. Powdered PtOEPK (Frontier Scientific, O40969) was then added (1 mg/mL) and placed in an ultrasonic bath for 15 min at room temperature.

The sensor films were spin-coated onto glass microscope slides that had been dehydrated for > 24 h at 185 °C and cleaned in 100% oxygen plasma (Emitech, K1050X) for 10 min at 100 W. Approximately 200 μL of sensor solution was pipetted onto each microscope slide in the first stage of a two-stage spin program: (i) 4000 rpm for 5 s (1000 rpm ramp) and (ii) 8000 rpm for 55 s (500 rpm ramp). After spin coating, the films were stored in the dark while the toluene was allowed to evaporate (> 24 h) then annealed for 90 s at 100 °C.

Each inlet stream, oxygenated (hyperoxic) and nitrogenated (hypoxia), had its own line of components, as shown in Fig. 3. Each line consisted of rigid, low gas-permeability polytetrafluoroethylene (PTFE) tubing (ID 1/16″), flanges and connectors, flow restrictor, bubble trap, flow sensor (FS1, Elveflow) and homemade gas exchanger. A pressure-driven flow controller (OB1, Elveflow) provided fluid flow. An oxygen
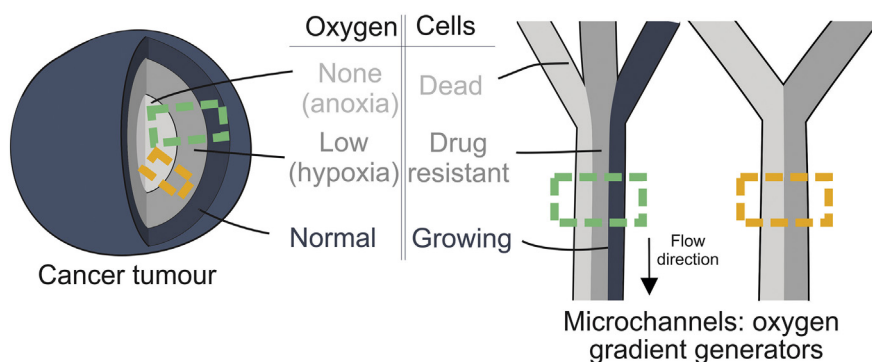

Fig. 1. A schematic representation of the inhomogeneous oxygen concentration gradients present in the cancer microenvironment alongside the equivalent microfluidic oxygen gradient generators.