3D CELL CULTURE WITH INTEGRATED OXYGEN CONTROL AND MEASUREMENT

Samantha M. Grist, Selim Gawad, Carmen Bayly, Linfen Yu, Lukas Chrostowski, and Karen C. Cheung

Department of Electrical and Computer Engineering, The University of British Columbia, Vancouver, British Columbia, Canada

INTRODUCTION

Tissue hypoxia (or inadequate oxygen levels) in cancerous tumours has been linked with resistance to radiation therapy and many anticancer drugs [1]. Although there is a naturally-occurring oxygen gradient in many tissues [2], current plate-based assays for cell-based testing of cancer treatments do not permit the creation of gradients in oxygen concentration, requiring large numbers of cells and samples to determine the effects of oxygen levels on the efficacy of cancer treatments.

Double [3] and single-layer [4] microfluidic oxygen gradient generators using gases, gas-equilibrated liquids, and oxygen-controlling chemical reactions [5, 6] (in control channels separated from the cell culture channel by oxygen-permeable membranes) have been previously demonstrated. Designs using gaseous control can encounter problems with bubbles and pervaporation [4], while those using gas-equilibrated liquids require high flow rates [4] (700 µL/min), complicating the use of syringe pumps for long-term culture.

Optical oxygen sensor films can be integrated into microfluidic devices to measure oxygen levels *in situ*. These sensors rely on the reversible quenching of the luminescence of an indicator compound (encapsulated in a polymer or sol-gel matrix material) by molecular oxygen. The quenching of the luminescence intensity may be measured by acquiring images of the sensors; quenching can be modeled by the Stern-Volmer equation [7]:

$$\frac{I_0}{I} = 1 + K_{SV} pO_2, \quad (1)$$

where I_0 is the sensor intensity in the absence of oxygen, I is the sensor intensity at oxygen partial pressure pO_2, and K_{SV} is the Stern-Volmer quenching constant. An alternative sensor type with improved robustness to excitation and measurement system inhomogeneities uses both an oxygen-sensitive (sensing) and an oxygen-insensitive (reference) luminescent compound both encapsulated in a polystyrene film [8]. Instead of measuring the overall intensity, the intensity ratio between the emission intensities of the two dyes is measured to find the oxygen level.

Another disadvantage of many cell-based drug testing platforms is their reliance on two-dimensional (2D), monolayer-based cell culture. Our group has demonstrated 3D cell culture in alginate core-shell beads as a means of inhibiting cell breakthrough and growth outside of the microculture [9] with good cell viability and proliferation after 5 days and beyond.

In this work, we demonstrate a single layer microfluidic oxygen gradient generator that aims to combine the advantages of gas and liquid control lines, similar to a multi-layer oxygenator developed by Wood *et al* to study sickle cell disease [10]. The generator uses gas control channels with liquid-perfused 100-µm side channels between the gas and cell culture channel to inhibit bubble formation in the cell culture channel. We integrate this system with 3D cell culture as well as a phosphorescent optical oxygen sensor film in order to measure the oxygen levels inside the device. Finally, we discuss improvements to our platform to extend it to the study of temporal changes in oxygenation as well as the use of ratiometric sensors for improved robustness.

METHODS

Microfabrication

Our intensity-based optical oxygen sensors used platinum octaethylporphyrin ketone (PtOEPK) indicator in polystyrene films, and were patterned on a glass substrate with a

laser cutting process developed by our group [11]. Ratiometric sensors employing Platinum (II)-5,10,15,20-tetrakis-(2,3,4,5,6-pentafluor-phenyl)-porphyrin (PtTFPP) as the sensing dye and Macrolex Fluorescent Yellow (MFY) as the reference dye in polystyrene films (like those demonstrated by Ungerbock *et al*. [8]) were fabricated using the same process.

The single-layer PDMS microfluidic devices were fabricated through standard soft lithography and bonded to the glass substrate with integrated sensors. Figure 1 illustrates the cell culture device and experimental setup.

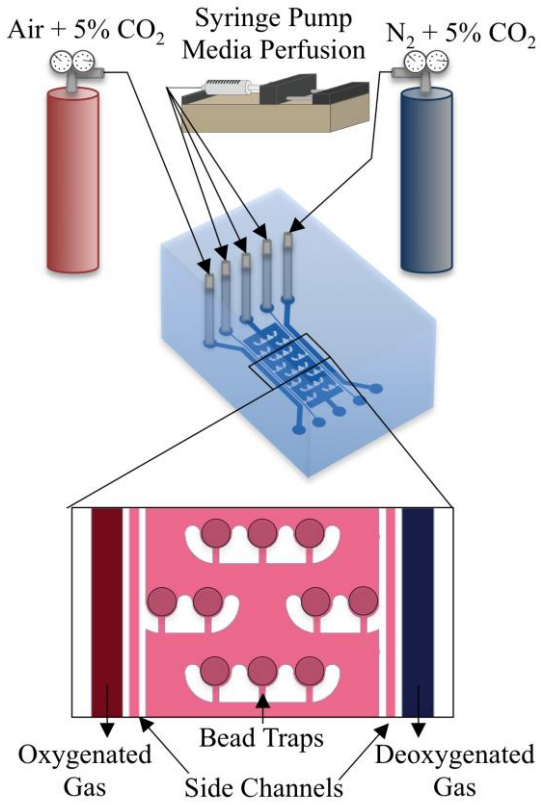

Figure 1: Illustration of the experimental setup. Air with 5% CO_2 is supplied to one side of the cell culture channel while nitrogen with 5% CO_2 is supplied to the other.

Sensor calibration

The sensors were calibrated in fluid by supplying water bubbled with oxygen, air, and nitrogen to the microfluidic channel enclosing the sensors while supplying the same gas to the gas control channels and the ambient environment around the chip. Images of the sensors were acquired at each oxygen level and the resulting intensities were fitted to the Stern-Volmer equation (Eq. 1) to obtain the sensor calibration constants. Gaseous calibration was also performed by supplying the gases directly to the channel enclosing the sensors and repeating the same procedure.

Bead preparation, loading, and culture

Alginate core-shell beads containing MCF-7 breast cancer cells in 1.5% alginate were microfluidically generated using methods previously demonstrated by our group [9]. The 400 µm beads were cultured in media inside standard cell culture flasks for several hours before loading into the microfluidic culture chips with 400 µm bead traps. The chips were first immersed in sterile water and degassed in a desiccator to remove any bubbles, before switching the water to DMEM cell culture media in advance of loading the beads into the chips. The beads were loaded via manual pipetting, and extra un-trapped beads were removed by connecting the chip outlet to a syringe pump and applying negative pressure. This negative pressure caused the media in which the chip was immersed to flow through the channel, carrying away un-trapped beads. Once the extra beads were removed, the flow rate was reduced to 0.5 µL/min for continuous perfusion culture. The bubble removal channels were also perfused with media via negative pressure at a flow rate of 0.2 µL/min.

The setup was cultured inside a microscope stage-top incubator (Live Cell Instrument Chamlide). The gas control channels of Fig. 1 were connected to nitrogen containing 5% CO_2 and air containing 5% CO_2 (both at flow rates of approximately 20 mL/min), and the ambient environment in the chamber around the chip was perfused with humidified air containing 5% CO_2. Hypoxic conditions could be created by adjusting the input gas compositions.

Automated oxygen control system

We have developed a gas control system using an Arduino controlling solenoid valves and reference O_2 and CO_2 sensors to mix varying quantities of air, N_2, and CO_2, store them in small tanks, and supply them to the device in a programmable fashion. This system

facilitates the control of time varying oxygen profiles by switching between the tanks.

To demonstrate the capabilities of the system, we supplied gases directly to the microfluidic channel enclosing ratiometric optical oxygen sensors and sensed these gaseous oxygen levels (using the gaseous calibration data for the same sensors). We ran a 12-hour experiment where the following demonstrative time-varying gas profile was repeated every 140 minutes: 2% oxygen was supplied for 35 minutes, followed by 0% for 30 minutes, 0.5% for 35 minutes, 0% for 20 minutes, and 0.5% for 20 minutes. The chip was temperature controlled during the experiment using the same microscope stage top incubator used for the cell culture tests.

RESULTS

Bead trapping and oxygen measurement: spatial O_2 gradient

Bubble formation in the cell culture channel was not observed over the 4 day culture period at 37°C. Figure 2 depicts the trapped beads and oxygen levels, along with an image (a) of the chip filled with food dye to visualize the microfluidic channels. Figure 2 (b) shows the trapped alginate core-shell bead, with the cell-laden 1.5% alginate core outlined by the dotted line and the 1.5% alginate shell outlined by the dash-dotted line. Figure 2(c) shows the measured oxygen gradient across the width of the cell culture channel, as controlled by supplying 0% and 20% oxygen to the right and left gas control channels, respectively. The oxygen levels were measured by PtOEPK/polystyrene sensor patches near the hydrodynamic traps for the alginate beads. These measured oxygen levels were overlaid on the brightfield image of the beads in traps.

Automated gas switching: cycling O_2 levels

Figure 3 presents the measured gaseous oxygen levels inside the microfluidic channel during the 12-hour automated gas switching experiment, measured using the ratiometric (PtTFPP/MFY/polystyrene) sensors as both a ratiometric measurement and an intensity-only measurement (measuring the signal from only the PtTFPP intensity without consideration of the reference dye).

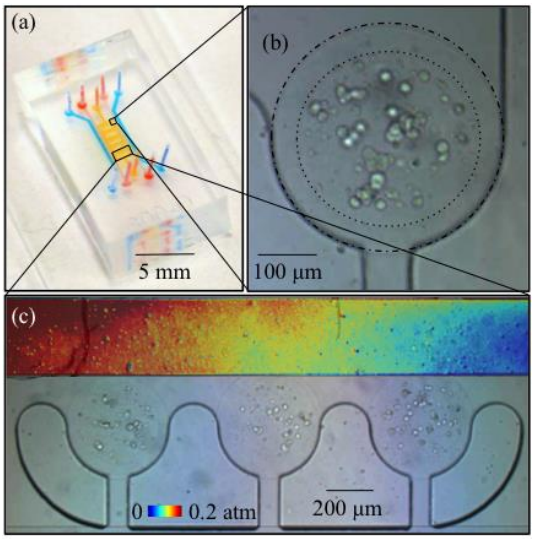

Figure 2: (a) Oxygen gradient generator chip with food dye inside highlighting the oxygen control gas channels (blue), cell culture channel (yellow), and side channels (red). (b) Alginate core-shell bead immobilized in a flow-through bead trap; the 1.5% alginate core with loaded MCF-7 cells is highlighted by the dotted line while the 1.5% alginate outer shell is highlighted by the dashed line. (c) Measured dissolved oxygen (pO2) gradient across the cell culture channel with trapped alginate core-shell beads loaded with MCF-7 cells.

The oxygen measurements show that the system is able to supply a stable gas supply to the chip, with reliable switching between tanks. Using either this oxygen gradient generator chip or a multilayer chip, we can create complex O_2 environments including temporal as well as spatial variations in O_2 level within the 3D cultures. These environments will act as more realistic models of the tumour microenvironment than a static O_2 level or simple spatial gradient.

It is also clear from Figure 3 that the ratiometric sensors provide a much more stable measurement than the intensity measurements alone, as the ratiometric measurement controls for system inhomogeneities and light system fluctuations (as well as partially accounting for photobleaching, which is likely the source of the slight upward drift in the measurement as the

sample was constantly exposed during the 12-hour experiment).

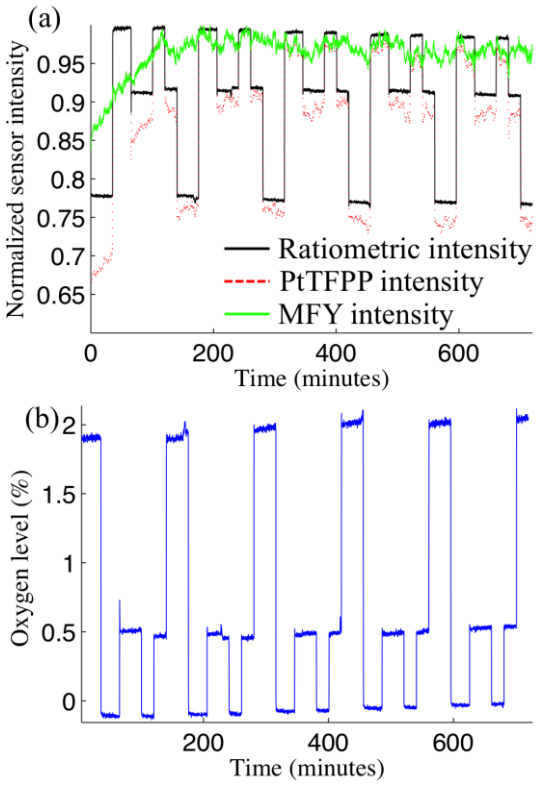

Figure 3: (a) Comparison of normalized intensity (I/I_{max}) and ratiometric intensity of the sensing dye, reference dye, and ratio of the intensities of the sensing and reference dyes. The ratiometric intensity shows a much more stable response than the intensity measurement alone. (b) Measured oxygen levels (using ratiometric sensor) during 12 hour gas switching experiment.

CONCLUSION

We have demonstrated a microfluidic cell culture environment integrating 3D, bead-based micro-cultures with oxygen control and measurement. We show integration of oxygen control and measurement with cell-laden alginate bead culture and also demonstrate the capabilities of a gas mixing, switching, and control system to supply the microfluidic oxygen control devices with time-varying oxygen levels. Future work will involve using this platform to model the natural O_2 gradient within tumours (spatial O_2 gradients) as well as the intermittent hypoxia that can also exist in tumours (cycling the O_2 levels), and study the effects of these phenomena on tumour cells and the effectiveness of anticancer drugs.

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