Oxygen sensitive microwells†

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Oxygen tension is critical in a number of cell pathways but is often overlooked in cell culture. One reason for this is the difficulty in modulating and assessing oxygen tensions without disturbing the culture conditions. Toward this end, a simple method to generate oxygen-sensitive microwells was developed through embossing polystyrene (PS) and *platinum*(II) *octaethylporphyrin ketone* (PtOEPK) thin films. In addition to monitoring the oxygen tension, microwells were employed in order to isolate uniform clusters of cells in microwells. The depth and width of the microwells can be adapted to different experimental parameters easily by altering the thin film processing or embossing stamp geometries. The thin oxygen sensitive microwell substrate is also compatible with high magnification modalities such as confocal imaging. The incorporation of the oxygen sensor into the microwells produces measurements of the oxygen tension near the cell surface. The oxygen sensitive microwells were calibrated and used to monitor oxygen tensions of Madin–Darby Canine Kidney Cells (MDCKs) cultured at high and low densities as a proof of concept. Wells 500 μm in diameter seeded with an average of 330 cells exhibited an oxygen level of 12.6% whereas wells seeded with an average of 20 cells per well exhibited an oxygen level of 19.5%, a 35.7% difference. This platform represents a new tool for culturing cells in microwells in a format amenable to high magnification imaging while monitoring the oxygen state of the culture media.

1 Introduction

Microwell cell culture allows precise control of cellular micro-environments by physically isolating a defined number of cells in discrete wells. This construct simplifies the analysis of individual cell response compared to standard (or bulk) cell culture. Standard cell culture produces measurements that are averages of millions of individual responses and can reveal misleading results on individual cell behavior due to heterogeneity even among the same cell type.[1] Controlling these interactions is vital for applications ranging from drug resistance to tissue engineering.[2,3] Unlike Petri dishes, microwells standardize the number of cell–cell contacts by limiting the number of cells in close proximity. The high volume of wells allows many experiments to be performed in parallel. These features have been exploited in many systems. In stem cell culture, microwells are an important tool to monitor multipotency of a stem cell,[4] perform high throughput screening assays,[5] and generate various sized embryoid bodies.[6,7]

Microwells have been constructed in a vast multitude of formats and configurations including, but not limited to poly-dimethylsiloxane (PDMS),[8,9] hydrogels,[4,5,10] polystyrene (PS),[9,11,12] and photoresists.[5,8,9] PDMS is optically transparent, non-toxic, and moldable making it useful in microwell fabrication.[13] The microwells can be formed directly from a master, or a PDMS microstamp is used to form wells in uncured PDMS.[8,9] However, PDMS is not an ideal substrate for cellular investigations due to the polymer's hydrophobicity, which absorbs many bioactive compounds.[14] Polyethylene glycol (PEG) hydrogels are

another popular microwell material composed of PEG–DA prepolymer that is molded into microwell arrays by PDMS stamps and subsequently crosslinked by UV light.[4,5] PEG microwells can also be fabricated by selectively crosslinking PEG using a photomask.[10] PS is another substrate that is increasingly used in microwell work due to its extensive use in cell culture and is the standard material in cell culture dishes. Thick PS films can be formed by hot embossing with a PDMS master under a load to create microwells.[9] Another method heats patterned pre-stressed polystyrene sheets to create molds for PDMS or PEG microwells.[11] Our technique builds on these previously successful methods by incorporating an oxygen sensor, *platinum*(II) *octae-thylporphyrin ketone* (PtOEPK), into PS to form oxygen responsive microwells on a thin substrate compatible with high magnification imaging.

The ability to monitor oxygen is an important yet often overlooked aspect of cell culture. Oxygen consumption is one indicator of metabolism and can reveal changes in the cell and its environment. Cell metabolic rate also influences other processes such as the production of reactive oxygen species and proliferation. Incorporating oxygen sensors into cell culture substrates can provide a real time analysis of oxygen levels at the micro-environmental level which can relay the cell's condition. Measuring oxygen levels is also important as oxygen tensions vary dramatically between tissue types *in vivo*. It is common to see oxygen tensions of 90–110 mmHg (12–14%) O_2 for brain,[15] 27–49 mmHg (3.6–6.4%) O_2 for bone marrow,[16] and 30–90 mmHg (3.9–12%) O_2 for liver.[17] It is essential to characterize various tissues' oxygen tensions when conducting oxygen-dependent experiments. Furthermore, monitoring oxygen tension in cell culture is useful to determine if the cells are exposed to the appropriate oxygen levels which can have an effect on the experimental outcome.

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There are several variations of *in situ* oxygen sensors. Clark electrodes were previously used to measure oxygen tension of cell monolayers.[18] Unfortunately, these probes consume oxygen which changes the oxygen tension exposed to the cells. Also, the probes are not easily integrated into the cell culture setup for continuous measurement and are prone to fouling due to repeated cell exposure. Currently, commonly used sensors involve oxygen sensitive fluorophores embedded in a substrate. One method incorporates platinum porphyrin dye (PtTFPP) into PDMS thin films that are sandwiched between Teflon and another layer of PDMS. The sensor detects changes in the oxygen tension through the PDMS layer where cells are attached.[19] Others have used nanosensors composed of oxygen-sensitive ruthenium(II) embedded in polystyrene nanobeads which are attached to cells to monitor oxygen consumption directly.[20]

In this paper we produced oxygen-sensitive microwells that house cells for culture and detect oxygen in a format compatible with high resolution imaging. PtOEPK, a fluorophore that is quenched in the presence of oxygen reducing the intensity, is embedded in PS for immobilization[21] and is embossed with a PDMS stamp to form the microwells. PS becomes the cell substrate. Well depth is varied by changing the concentration of solvent in the PS solution. PS in contact with the PDMS master reduces the sensor thickness of approximately 75 μm making it compatible for high resolution microscopic imaging of cells. The microwell design produces areas between the wells that are not in contact with the cells which prevents fouling of the sensor. The sensors have the ability to monitor changes with a limited number of cells as a function of oxygen over a large area simultaneously.

2   Materials and methods

2.0   Polystyrene/PtOEPK patterning

A 35% w/w toluene/PS solution was composed of PS pellets (200 000 M_w, Sigma-Aldrich, MO) dissolved in toluene (Sigma-Aldrich, MO). The solution was tightly sealed in a glass bottle to prevent toluene evaporation and was placed on a shaker for 24 hours for complete dissolution. PtOEPK was added at 1 mg mL^{-1} of PS/toluene solution and was dissolved. A cover glass was cleaned with acetone, methanol, and isopropanol and dried under nitrogen. A 400 μL volume of solution was pipetted onto a circle cover glass with a diameter of 25 mm and was set aside for 24 hours for complete toluene evaporation (Fig. S1d, ESI†). The PS/toluene solution was exposed to light during the evaporation process to reduce the extent of photobleaching during experiments. The PDMS stamp was placed in contact with the PS-coated cover glass and placed on a 100 °C hot plate for 15 minutes. A weight of 3.0 kg was placed on the stamp to press the PDMS into the heated substrate (Fig. S1e, ESI†). The PDMS was cooled to room temperature and removed, revealing the microwells (Fig. S1f, ESI†). The microwells were then released from the cover glass (Fig. S1g, ESI†). The microwells were prone to curling after removal. To create a flat substrate, the microwell discs were placed on an 85 °C hotplate and gently flattened. Next, the wells were plasma treated with a corona discharge device (BD-20AC, Electro-Technic, IL) to promote cell

attachment; however, this step can be skipped if non-adherent cells will be used. For ease, the microwells were placed in a 6-well plate. A millilitre of PDMS was added to the bottom of the 6-well plate to "glue" the microwells to the plate and prevent movement. The microwells were sterilized in 70% ethanol for 20 minutes and were exposed to UV light at 254 nm in a biosafety cabinet for 4 hours prior to use in cell culture experiments.

2.1   Cell culture and staining

Madin–Darby Canine Kidney (MDCK) cells (ATCC, MD) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% (vol/vol) Fetal Bovine Serum (Thermo Scientific HyClone, UT) and 1% of penicillin (10 000 U) and streptomycin (10 mg mL^{-1}) (Sigma-Aldrich, MO). The cells were incubated at 37 °C with 5% CO_2. Cells were seeded by pipetting a concentration between 0.5 and 1×10^7 cells mL^{-1} on a square cover glass the width of the substrate. A cell loading technique was utilized to seed cells into the wells while removing excess cells from the surface as described elsewhere.[22] Wells containing media and cells were then placed into the incubator and subsequent medium was not added until the cells attached to prevent dislodgement. Hoechst dye (Sigma-Aldrich, MO) was added at a concentration of 3 μg μL^{-1} to stain and count cells trapped in microwells. PtOEPK cytotoxicity was evaluated with a live/dead viability/cytotoxicity assay (Invitrogen, CA). MDCK cells were stained with a rhodamine-phalloidin (Invitrogen, CA) to image the actin cytoskeleton.

2.2   Oxygen sensor calibration and microscopy

Oxygen sensitive microwells were calibrated by determining the corresponding fluorescent intensity at a particular oxygen level. Gases containing 21%, 10%, and 0% O_2 (100% N_2) were exposed to the microwell surface during fluorescent image acquisition, and the intensities were recorded. Images were obtained at the junction between four wells (Fig. 3) to measure intensity changes. Ten measurements were made at various locations on a cover slip of microwells. Photobleaching was controlled by limiting the number of data points collected during the experiment to reduce the time microwells were exposed to the excitation light. An inverted fluorescent microscope (Olympus IX71, NY), a Hamamatsu camera (C8484-03001, Japan) fitted with a filter with an excitation/emission of 595/760 nm with a 620 nm dichroic mirror (Chroma, VT), and Metamorph (v7.63.10) were used for image acquisition and analysis. To determine sensor sensitivity, a Stern–Volmer plot was produced by correlating percent oxygen with the intensity ratio (I/I_o) where I is the intensity at a certain oxygen level and I_o is the intensity in the absence of oxygen. Sensor sensitivity was determined with equation the Stern–Volmer curve and was used to calculate the percent oxygen perceived by the microwell.

3   Results

3.0   Microwell patterning with PDMS molds

PtOEPK-embedded PS was utilized when creating the microwell sensor. Fig. 1(a) shows a bright field image of 500 μm diameter microwells on a glass cover slip. PtOEPK/PS embossing is
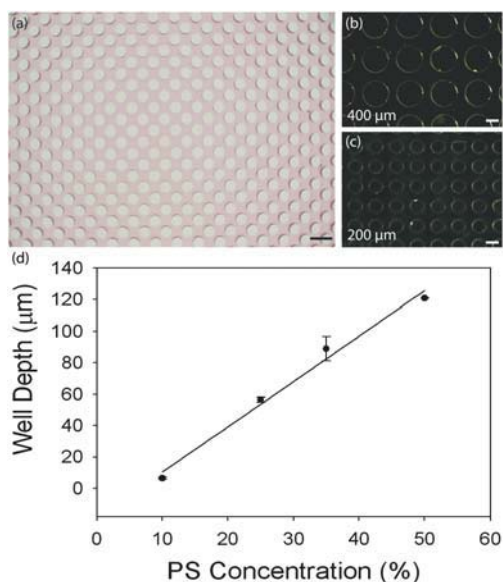
Fig. 1 PS/PtOEPK thin films were embossed to form uniform 500 μm diameter microwells. (a) A bright field image of the oxygen-sensitive microwells. Scale bar is 1 mm. Well diameters vary by changing the PDMS stamp diameter depending on the application. Wells of (b) 400 μm and (c) 200 μm are shown. (d) Well depth was altered by changing the PS concentration. Scale bars are 200 μm. The well depths for 10, 25, 35, and 50 percent were 6.5 μm, 56.4 μm, 91.8 μm, and 120.9 μm respectively. The data represents the mean of three experiments, and the error bars are the standard deviation.

a flexible technique that is adaptable to specific experimental conditions. To demonstrate this, well sizes were altered by using PDMS stamps of different diameters (Fig. 1(b) and (c)). Sensor thickness was altered by varying the concentration of PS dissolved in toluene with less PS producing shorter wells. Fig. 1(b) shows the corresponding well depth for a particular PS concentration. PtOEPK/PS is 75 μm thick at the bottom of wells, which is compatible with high magnification microscopy, such as the confocal microscope.

3.1 Cellular experiments with microwells characterization

Microwells were seeded with a range of cell concentrations and the average number of cells trapped is displayed in Fig. 2(a). Concentrations of $1–5 \times 10^4$ cells mL^{-1}, $1–2.5 \times 10^5$ cells mL^{-1}, and $1–2 \times 10^6$ cells mL^{-1} trapped 101 ± 24, 36 ± 15, and 9 ± 5 cells respectively. Trapped cells exhibited normal growth and proliferations as confirmed in Fig. 2(c) and (f) which displays cells 1 hour and 2 days after seeding (stained with calcein AM). To determine the cytotoxicity of PtOEPK, a live/dead assay was performed on cells growing in the microwells. After a week in culture, cells trapped in oxygen sensitive microwells shown in Fig. 2(d) and (g) maintained viability confirmed by the uptake of only the calcein AM dye and limited uptake of the EthD-1 dye. A control was created by trapping cells in microwells without PtOEPK as shown in Fig. 2(e) and (h). Microwells with and without the sensor had 93% and 95% viability respectively confirming the suitability of wells for short term cell culture. Fig. 2(b) shows a confocal image of Madin–Darby Canine

Kidney (MDCK) cells stained with rhodamine-conjugated phalloidin to visualize the actin. Thus, the microwells are also adaptable to high magnification microscopy.

3.2 PtOEPK characterization

The concentration of PS in toluene affects the intensity readings of the sensor. For our application, a 35% (w/w) solution of PS and toluene was used to achieve deeper wells to contain the cells. However, the concentration and the resulting thickness used are dependent upon the size of the cells and application. Fig. 3(a) and (c) displays changes in fluorescent intensity of 35% (w/w) microwells exposed 0% oxygen (100% nitrogen) and 21% oxygen. Oxygen measurements were performed in the center of the four wells. A Stern–Volmer plot describes the relationship between a chemical species (PtOEPK) and its quencher (oxygen),[23] and this was used to determine the change in intensity relative to oxygen and PS concentration in Fig. 3(b). At 35%, the Stern–Volmer slope was 0.0753, and a concentration of 21% oxygen yielded a 2.5 increase in the intensity as compared to 0% O_2.

3.3 Microwell sensor oxygen measurements

To determine the microwells ability to detect changes, the oxygen levels of trapped cells were monitored in two different conditions. One cover slip with 500 μm diameter microwells was seeded with MDCK cells at a high density (330 cells per well) and another cover slip at a low density (20 cells per well). MDCK cells were selected due to their high aerobic activity.[24] Oxygen sensitive microwells were calibrated as previously described to determine the Stern–Volmer curve. The measurement locations used during calibration were re-measured with the addition of cells. Cover slips were placed in a six well plate and stored in an incubator. Fluorescent images were captured after 12 hours. High density wells had an oxygen level of $12.6 \pm 0.77\%$ whereas low density wells exhibited an oxygen level of $19.5 \pm 0.47\%$ which was 35.7% higher than the high density seeding as shown in Fig. 4. Thus, microwells with a lower cell density resulted in a lower rate of oxygen consumption which maintained a higher oxygen level.

4 Discussion

An advantage of the technique presented here is the simple fabrication due to the combination of the sensor and substrate. Previous methods require the deposition of multiple layers of sensor and a substrate for cell attachment requiring intricate fabrication or multiple photolithographic steps to fabricate the designs.[9] PS dissolved in toluene makes embossing manageable since the solution is easily deposited as a thin film onto a cover glass and very minimal heat and pressure are required to generate the patterns. The microwells are also reusable provided the samples properly sterilized. Common sterilization techniques such as ethanol and UV light are used before cell culture and appear to not have an effect on the PtOEPK's ability to detect oxygen in culture. However, the microwells were only reused a maximum of 3 times. The microwells' integrity is not known for subsequent reuses. Plasma treated PS is compatible with adherent cell culture eliminating the need to adsorb proteins and other components for cell attachment. However, suspension cells do not require plasma treatment reducing the adhesion to the
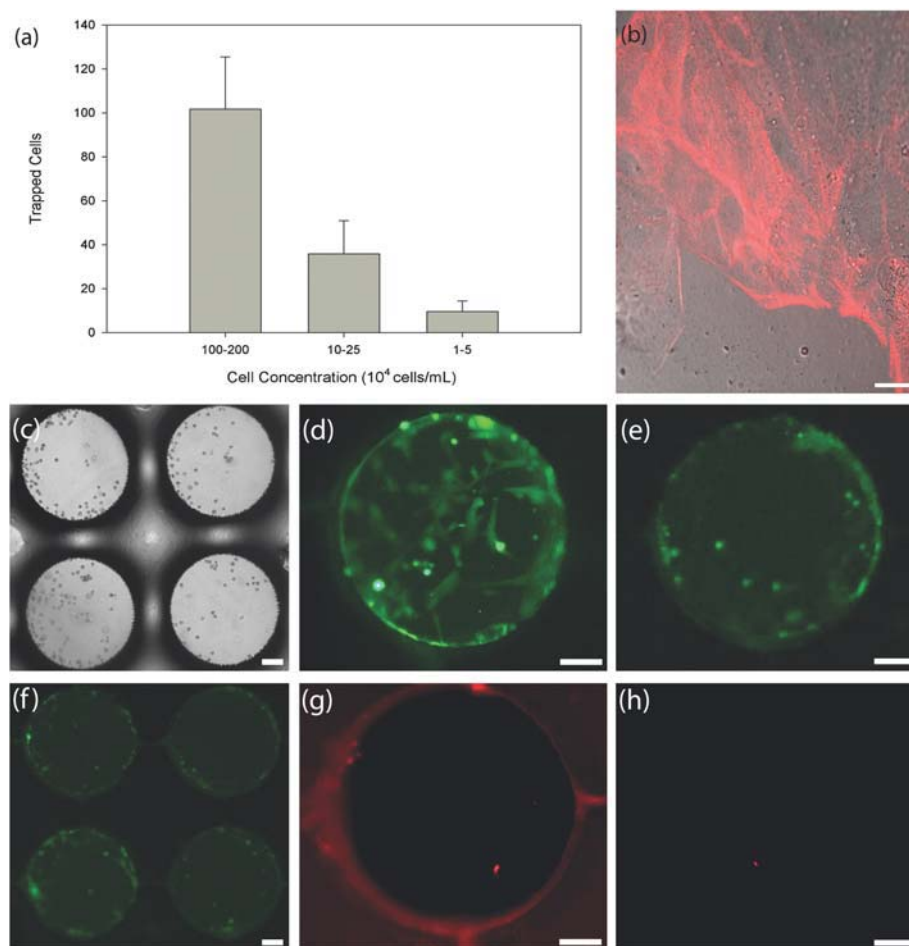
Fig. 2 (a) Microwells seeded with cell concentrations of $1–5 \times 10^4$ cells mL^{-1}, $1–2.5 \times 10^5$ cells mL^{-1}, and $1–2 \times 10^6$ cells mL^{-1} trapped 101 ± 24, 36 ± 15, and 9 ± 5 cells respectively. (b) The actin cytoskeleton of a MDCK cell was stained to display the microwell compatibility with high magnification microscopy. Scale bar is 20 μm. (c) Live/dead assay was performed on cells growing in the microwells. Trapped cells were imaged (c) 1 hour and (f) 2 days after seeding. Cells were confined to the wells and exhibited normal growth and proliferation. Scale bar is 100 μm. MDCK stained with (d) calcein AM (live) and (g) EtD-1 (dead) revealed limited uptake of EtD-1 confirming PtOEPK's low toxicity for short term cell culture. (PtOEPK excitation wavelength overlaps with EtD-1 causing the microwell to fluoresce.) A control was created by trapping cells in microwells without PtOEPK as shown in (e) and (h). Microwells with and without the sensor had 93% and 95% cell viability respectively. Scale bars are 100 μm.

substrate. MDCK cells seeded in the wells exhibited normal attachment and spreading as shown in Fig. 2(b) and (c) when pretreated with oxygen plasma. Since the cytotoxicity of PtOEPK was unknown, a live/dead assay determined that PtOEPK was safe for a short term cell culture period of 1 week. However, for experiments longer than 1 week, the PtOEPK may leach out and cytotoxicity would have to be reassessed.

This technique is easily adaptable to various experimental conditions. Well depth was determined by the PS concentration used, and well diameter was varied by the diameter of the PDMS stamp. It is a robust technique and the PDMS stamp can easily be reused up to 15 times without deterioration of the pattern. The microwell design created distinct locations for oxygen measurements on the chip between the wells where the sensor is in the proximity (within 500 μm) of the cells without being directly exposed to the cells which would generate optical interference of the fluorescence intensity. Well depth can be easily adjusted by altering the dissolution conditions for a particular cell type as larger cells require deeper wells for trapping. This adaptability creates a variety of applications that would benefit from oxygen

monitoring ranging from single cell analysis to developing standardized embryoid bodies both of which have been previously demonstrated. Previous studies have also measured the stability of PtOEPK and its low susceptibility to photobleaching which make it ideal for extended experiments.[25] Most importantly, the oxygen sensitivity can be locally measured by measuring the intensity between four wells. A 2 cm^2 area on a substrate contains over a hundred sensor regions, making the ability to gather multiple data points across a large substrate a simple and straightforward task.

A cell's oxygen tension plays a critical role in cell behavior. Thus, measuring oxygen tension can provide additional information about how a cell responds to various oxygen concentrations or how a change in oxygen affects a cell process. This is demonstrated in Fig. 4 where MDCK cells at high density had a 35.7% decrease in oxygen tension than low density wells. The ability to monitor the oxygen tension is especially important in cell processes involving growth and development. Oxygen is an important element in embryonic stem and progenitor cell development where areas of low oxygen drive differentiation.[26]
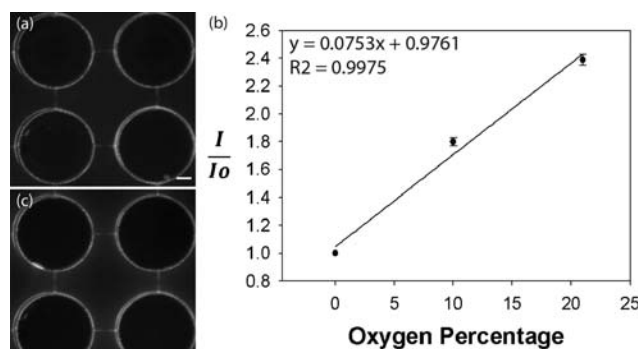
Fig. 3 Microwells were exposed to (a) 0% oxygen (100% nitrogen) and (c) 21% oxygen and changes in fluorescent intensity were monitored. Oxygen measurements were performed in the center of the four wells. A Stern–Volmer plot describes the relationship between a chemical species (PtOEPK) and its quencher (oxygen), and this was created to determine the change in intensity relative to oxygen and PS concentration in (b). At 35%, the Stern–Volmer slope was 0.0753, and a concentration of 21% oxygen yielded a 2.5 increase in the intensity as compared to 0% O_2. Scale bars are 100 μm.
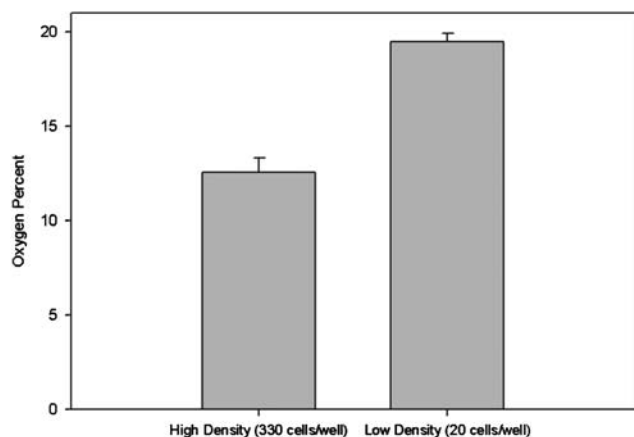

Fig. 4 The oxygen concentration was measured at the microwells' surface. High density microwells exhibited an oxygen level of 12.6 ± 0.77% whereas low density wells exhibited an oxygen level of 19.5 ± 0.47% which was 35.7% higher than the high density seeding. The heights of the bars are the mean of three independent experiments with 6 oxygen measurements at each experiment with error bars representing the standard deviation.

Oxygen sensitive microwells could monitor the degree of differentiation at different levels of oxygenation to determine optimal parameters.

5 Conclusion

The method reported in this paper provides a simple and efficient way to construct oxygen sensitive microwells. PS is a suitable cell culture material, and PS film thickness is easily adjustable by simply changing the concentrations. The ability to tailor the geometric parameters of the wells while incorporating an oxygen sensor makes it flexible for various experiments and other applications. The microwells allow simultaneous monitoring of oxygen levels during experiments. Oxygen-sensitive microwells could be a simple and useful biological tool that can be incorporated into the standard biomedical research lab.

Acknowledgements

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