Title: Dispersible oxygen microsensors map oxygen gradients in three-dimensional cell cultures. Supplemental Information

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Methods and Materials

Generation of PDMS beads

PDMS beads were generated using microfluidic flow-focusing devices typically used for droplet generation. Specifically, we used a 3-to-1 converging flow-focusing micro-channel (100 μm high channels) device to fabricate PDMS beads. However, the device design can be modified to generate beads of different sizes[1]. Microfluidic flow-focusing devices used the same master fabrication, and PDMS layer preparation as previously presented[2]. The PDMS layer had inlets and outlets punched into them by biopsy punches. This was done prior to plasma bonding the layer onto a glass slide. Metal, blunt-ended needle tips (Jensen Global, Santa Barbara, CA, USA) were used to connect tubing to the device: Tygon tubing (Saint-Gobain™ Tygon™ R-3603 Clear Laboratory Tubing, Saint-Gobain Performance Plastics, Akron, OH, USA) was inserted into the continuous flow inlets, and PTFE Tubing (Cole-Parmer, Vernon Hills, IL, USA) was inserted into the dispersive flow inlets. The connections were sealed by coating with uncured 1:10 – PDMS, which was subsequently allowed to cure at 60 °C.

After fabrication, the devices were prepared for the process of producing beads by applying a corona treatment, to enhance the hydrophilicity of the microchannels. Then, immediately afterwards, a surfactant solution of either 0.1% (w/v) Pluronic F108 (BASF Co., Ludwigshafen, Germany) or 0.5% (w/v) Sodium Dodecyl Sulfate (Sigma Aldrich, Saint Louis, MO, USA) in distilled water was flowed through the tubing and channels. For flow focusing, a surfactant solution (0.1% Pluronic F108 or 0.5% SDS) was flowed through the side channels, to form the continuous phase, and a 1:1 mixture of toluene and 1:10 PDMS pre-polymer solution (Sylgard 184, Dow Corning, Midland, MI, USA) was flowed through the middle channel, to form the dispersive phase. The two flows were differentially moderated using two different syringe pumps (KD Scientific, Holliston, MA, USA). The flow rates were slowly ramped up for the continuous flow, and down for the dispersive flow, until the final flow rates were achieved. The final volumetric flow rates ranged from 0.15 to 0.35 μL/min for the dispersive flow, and 50 to 75 μL/min for the continuous flow. The flow-rate ramping steps for both flows were carefully controlled to minimize the time needed to initiate convergence of the two liquid phases, and to maintain stable focusing-flow in the microchannel device.

The low surface energy of the surfactant solution helped to maintain the PDMS micro-droplets in the outlet reservoir after they had been formed. After being collected at the outlet reservoir into glass scintillation vials, the PDMS microdroplets were cured at 60 ºC overnight, forming solid PDMS microspheres. Experimental observations indicated that the size of the PDMS microspheres did not change substantially (≤ 1 μm) upon curing the microdroplets, (data not shown). The microsphere size was determined using image analysis conducted via MATLAB's

image processing toolbox. Circular borders for PDMS microspheres were found using MATLAB's *imfindcircles* function, and used to calculate the microsphere diameter.

Infusion of ruthenium into PDMS for O_2 sensing

After fabrication, the beads were washed with distilled water. The distilled water solution was then removed, and 5 mg/mL of tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) dichloride (Alfa Asar, Ward Hill, MA, USA) in dichloromethane (Sigma, Saint Louis, MO, USA) was introduced into the scintillation vial. The beads were allowed to incubate in the ruthenium-dicholoromethane solution for 24 hours at room temperature, in the dark. The majority of the ruthenium-dicholoromethane solution was removed from the scintillation vial, and 5 mg/mL of ruthenium in isopropanol was applied onto the beads for 30 minutes to dilute the dichloromethane (at least 1:4 dilution). The isopropanol was then removed, and the beads were washed with distilled to remove organic solvents. Final washes were done in 0.1% Pluronic F108 to minimize bead sticking.

Calibration of PDMS microsensors

Phase-shift measurements (phase fluorimetry) were taken of the oxygen-microsensing beads using a microscope that had a fluorescence attachment with a 5 W LED, 455 nm monochromatic light source (Mightex Systems, CA) to excite the beads in a pulsatile manner. The LED was driven by a function generator (33220A, Agilent Technology, Inc., CA, USA), with a square wave at a 100-kHz frequency and 50% duty cycle. A 590 nm long-pass filter was used, to minimize extraneous noise. A silicon PIN photodiode with a preamplifier (PDA36A, Thorlabs, Inc., NJ, USA) was used to capture fluorescent emission. The PIN photodiode was set to a gain of 30 dB, and was connected to a lock-in amplifier (SR830, Stanford Research System, Inc., Sunnyvale, CA, USA) to assess the output signal. The system was controlled by a personal computer with a LabVIEW graphic user-interface program, and data were acquired through a GPIB interface (LabView and GPIB-USB-HS, respectively, National Instruments, Co., Austin, TX, USA). This setup is similar to that already described by others[3]. The beads were analyzed at 21% (room air), 15%, 10%, 5%, 2.5% and 0% O_2. The measurements were taken by flowing a pre-mixed combination of N_2 and O_2 gas (Cryogenic Gases Inc., Detroit, MI, USA) into a custom-made purging chamber, except when equilibrating with air, which was allowed to equilibrate to ambient conditions. The beads responded to the environment and stabilized less than 10 seconds after the gas started to flow into the purging chamber, with the specific time depending on the size of the beads. For calibration purposes, a minimum of a minute of gas flow was used. All the measurements also captured intensity data for the beads, these were used for the intensity-based measurements.

Assessing live/dead (cytotoxicity)

HS-5 cells were cultured with beads for 36 hours and 120 hours on tissue-culture plates (Corning, NY). The impact of ruthenium-loaded microbeads on cell viability was assessed using the LIVE/DEAD cytotoxicity kit (Invitrogen, Waltham, MA, USA). Samples were washed with dPBS (Gibco, Carlsbad, CA, USA), and then incubated with a 2 µM calcein AM and 4 µM ethidium homodimer in dPBS for 30 minutes before aspirating and washing with dPBS. Images

were collected around and away from the beads using a digital camera (Canon EOS Rebel T3i, Canon, Japan), and then analyzed by manual counting.

Secondary measurements for comparison of cell-patterned hydrogels measurements

The oxygen-consumption rates (OCR) and oxygen-level measurements made with our custom oxygen microsensors were compared with measurements made using a commercial device. The OCR measurements were determined using a Seahorse XFe Extracellular Flux Analyzer for 10 wells of MDA-MB-231 eGFP cells within a collagen gel. Trypsinized MDA-MB-231 eGFP cells were mixed with neutralized type-I bovine collagen (BD Biosciences, San Jose, CA, USA) to create a suspension of 7.5 x 10^6 cells/mL in 2 mg/mL of collagen. 50 µL of the cell-collagen solution were dispensed into a Seahorse XF96 Cell Culture Microplate well, at a similar cell density (3.50 x10^4 cells/mm^2) to that of the cell patterning, and allowed to polymerize for 30 minutes at 37 °C. Subsequently,150 µL of cell culture medium were dispensed over the polymerized gel. Measurements were taken after 25 hours of culturing the cells within the collagen gel. OCR measurements were obtained over six hours in cycles of ten minutes, each cycle consisting of a seven-minute measuring phase and a three-minute mixing phase.

An Ocean Optics NeoFox system, using a PI600 Oxygen Sensor Probe was used to measure oxygen levels in the cell-culture media, blank collagen gels, and collagen gels containing cells. The PI600 Oxygen Sensor Probe was fixed to a 45° angle, consequently the samples were plated in a 6-well to facilitate both the penetration of the probe into the sample, and the measurements. To minimize excessive use of materials, the wells first had a PDMS ring with a 6-mm hole placed into them. The 6-mm region was coated with a thin layer of collagen, and allowed to incubate for 2 hours at 37 °C. It was then washed with PBS. Cells were seeded at a cell density of 3.50 x10^4 cells/mm^2. After the cells attached to the plate, the cell-culture medium was removed, and a 100 µL of cell culture media with 2 mg/mL neutralized type-I bovine collagen were dispensed above the cell layer, and allowed to polymerize for one hour at 37 °C. The PDMS ring was removed from around the collagen sample, and the well was filled with cell culture medium. Measurements were taken after 24 hours of culturing the cells in collagen discs. A two-point calibration was done with the Ocean Optics NeoFox system in DMEM culture medium, with DMEM at ambient conditions and N_2-purged DMEM. Lifetime measurements of 0.384 µs as 0.284 mol/m^3 (max solubility at 20 °C) was used for oxygen level conversion.

Results

Generation of oxygen microsensors

A microfluidic flow-focusing system (Figure SI 1A) was used with appropriate flow rates to fabricate monodispersed beads (Figure SI 1B, C). The bead size could be adjusted by modifying the device architecture[1] (*i.e.*, the aperture of the dispersive flow) or the ratio between the continuous and dispersive flow rates[4]. The PDMS microbeads were infused with ruthenium (Figure SI 1D) to produce the dispersible oxygen microsensors.

Calibration of the microsensor, and assessment of lifespan

The ruthenium-infused microbeads were calibrated (using two modalities: intensity-based quenching, using the Stern-Volmer relationship[5], (Figure SI 2A) and phase fluorimetry (Figure SI 2C). Consistent with the litereature[6,7], the intensity-based measures gave large variations,

particularly at higher oxygen levels. Conversely, phase-shift measurements (Figure SI 2C) presented minimal variations at the different oxygen levels measured. The experiments were repeated across 5 batches of beads, demonstrating reliable and consistent measurement capabilities.

To determine the shelf-life of our sensors, we monitored the stability of the phase fluorimetry signal. The reduction of signal intensity and the corresponding change in the phase shift response is plotted as a function of time in Figure SI 3 and Table SI 1. We found that, provided the signal intensity remained above 20 μV, the phase-shift measurements remained reproducible for at least a year (Figure SI 2D) when cycling between the two extremes of interest: normoxic (21% O_2) and anoxic (0% O_2).

Compatibility between microsensor and cell culture

The microsensors' biocompatibility was assessed by performing live/dead assays on cells cultured with the microsensors. We exposed multiple cell types to our microsensors, and we did not see any alterations in the cell viability. Specifically, we quantified the viability of cultured HS-5 cells at 36 and 120 hours, and saw no appreciable cell death within cell cultures exposed to the oxygen microsensors as compared to the control (Figure SI 4), similarly HEK 293T and MDA-MB 231 cells demonstrated no appreciable death (data not shown) reaching 100% confluency on similar length time scales and became difficult to quantify.

Secondary measurements for comparison of cell-patterned hydrogels measurements

To evaluate the oxygen-consumption rate (OCR) of MDA-MB-231cells seeded within collagen gels at a similar seeding density to our cell-micropatterning work with a commercially available system, we used a Seahorse XFe Extracellular Flux Analyzer. The measurements obtained for the OCR (Figure SI 5A) were on average 271.63±2.72 pmol/min. Oxygen-cycling data from four samples is plotted (SI 5B) to highlight the dramatic drop of oxygen within just 7 minutes, during the measurement phase of the OCR evaluation.

To determine the oxygen levels in the three different regions relevant to our cell micropatterning work, we used an Ocean Optics NeoFox system with a PI600 Oxygen Sensor Probe. Values ranged from 0.066 to 0.022 mol/m^3 in the collagen gel with cells seeded within it. It proved difficult to determined where, in relation to the cells, the measurements were being taken within the collagen gel. Most of the measurements were taken some distance away from the cells. In addition to the cell-laden collagen gel, one gel-only sample and two samples with cell-culture medias were assessed (Figure SI 6). However, the destructive nature of the probe limited the ability to sample multiple times within the same gel samples. Nonetheless, the values obtained were similar to our microsensor measurements. This validates our measurements, while also highlighting challenges of using this type of oxygen sensor, and emphasizes the need for a system such as our dispersible microbead sensors.

References

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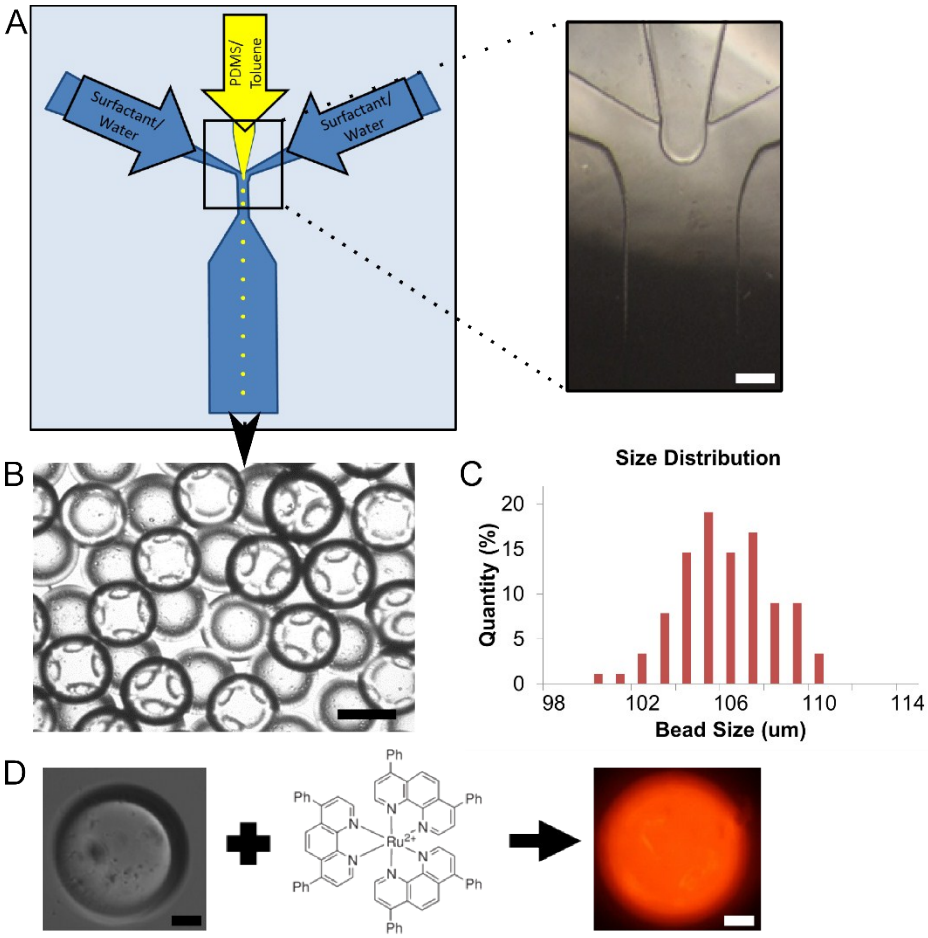

Figure SI 1. *Sensor microbead generation.* A) A schematic illustration of the flow-focusing device illustrating how a dispersive flow of PDMS-toluene interacts with a continuously-flowing surfactant-water solution to form spheres. The inset shows an image of the dripping behavior within the device, scale bar = 100 µm. B) An array of the resultant monodispersed beads, scale bar = 100 µm. C) An example of the size distribution taken from 100 beads, using MATLAB image analysis to determine bead size and bin into 1 µm bins. D) The beads were infused with tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) dichloride to generate an oxygen microsensors that fluoresced red with decreasing intensity in the presence of increasing oxygen, scale bar = 25 µm.

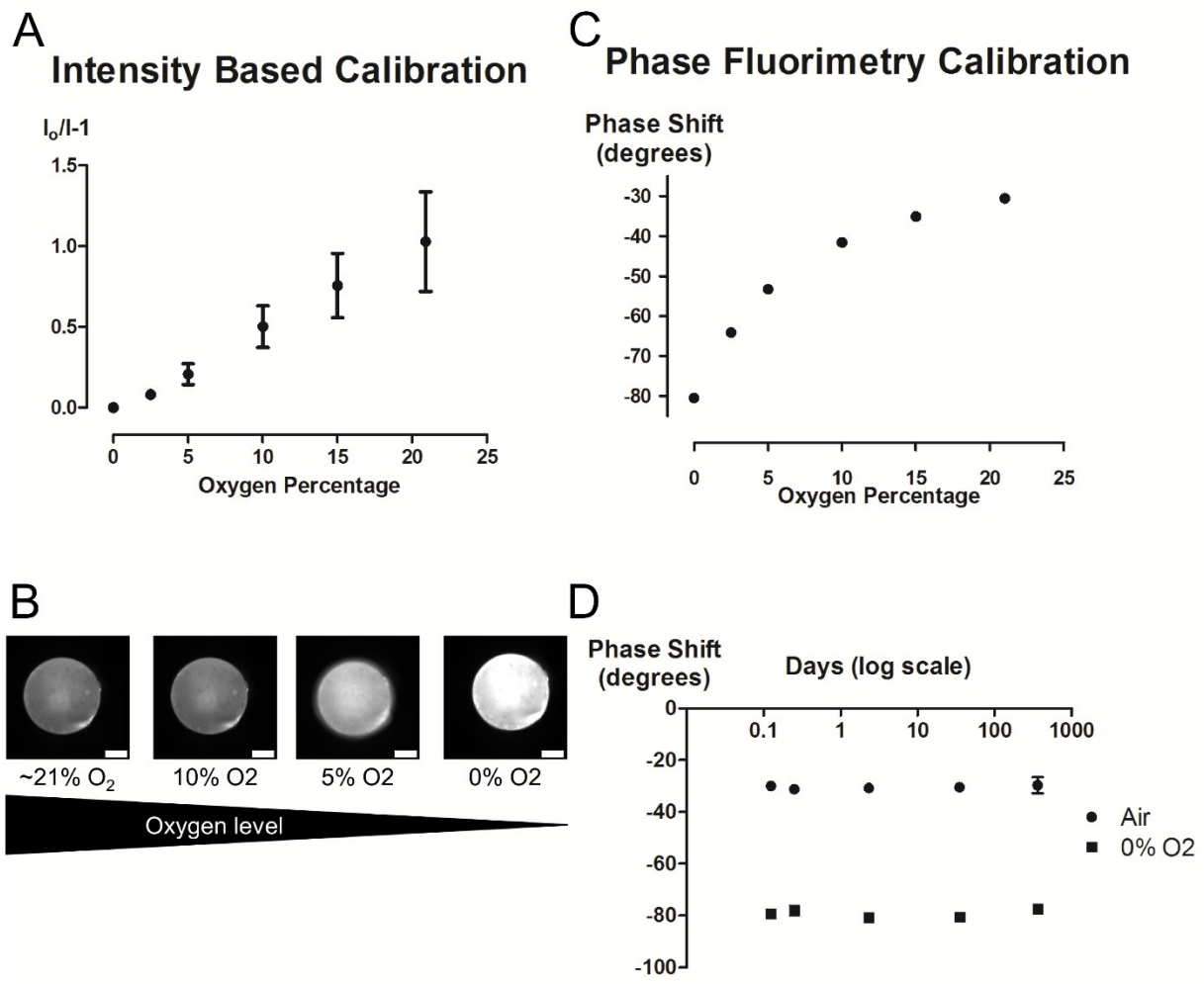

Figure SI 2. *Response of microsensors to oxygen concentration.* A) Intensity-based quenching calibration curve in response to different oxygen levels plotted according to Stern-Volmer relationship (I_o/I-1); the error bars represent one standard deviation (n=20). B) Images of one microsensor's response to changes in oxygen. C) Phase-fluorimetry calibration curve, the error bars represent one standard deviation, however, they cannot be seen at this scale (n=20). D) Phase fluorimetry demonstrates stability in phase shift between normoxic and anoxic conditions, data from beads monitored over the course of one year, error bars presented are standard deviation, however, all except one cannot be seen at this scale (n=5).

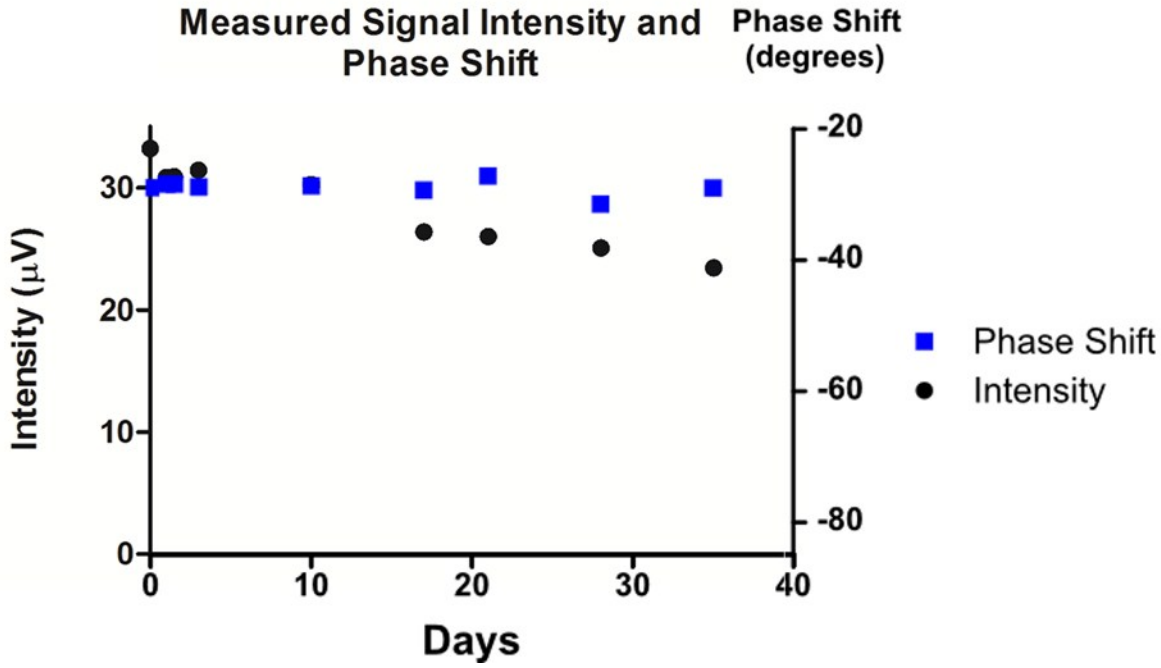

Figure SI 3. The reduction in the microsensor intensity from a single bead over 35 days compared to the change in the phase-shift signal. All data were collected at 21% oxygen, in ambient conditions. The microsensor was exposed to intermittent light as it was kept in a microscopy room.

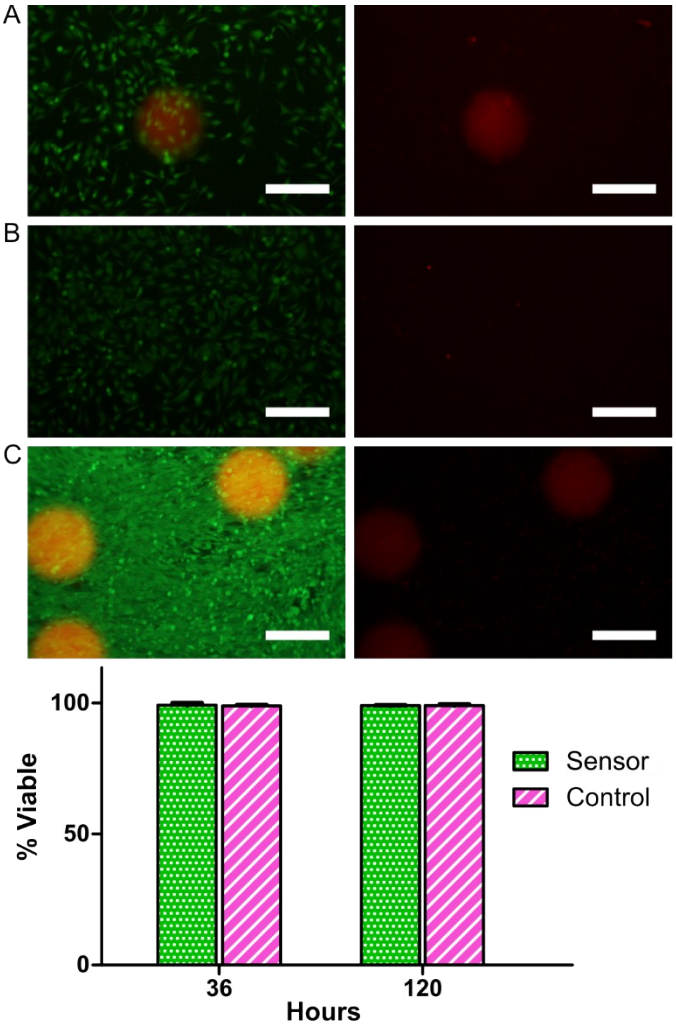

Figure SI 4. Live/dead stain for HS-5 cells, cultured for A) 36 hours with microsensors, B) 36 hours without microsensors (control) and C) 120 hours with microsensors. Live cells were stained green with calcein AM (left images) while dead cells were stained red with ethidium homodimer (right images). Quantified percent viability present for microsensor treated samples or control samples, with no significant difference between with or without microsensors.

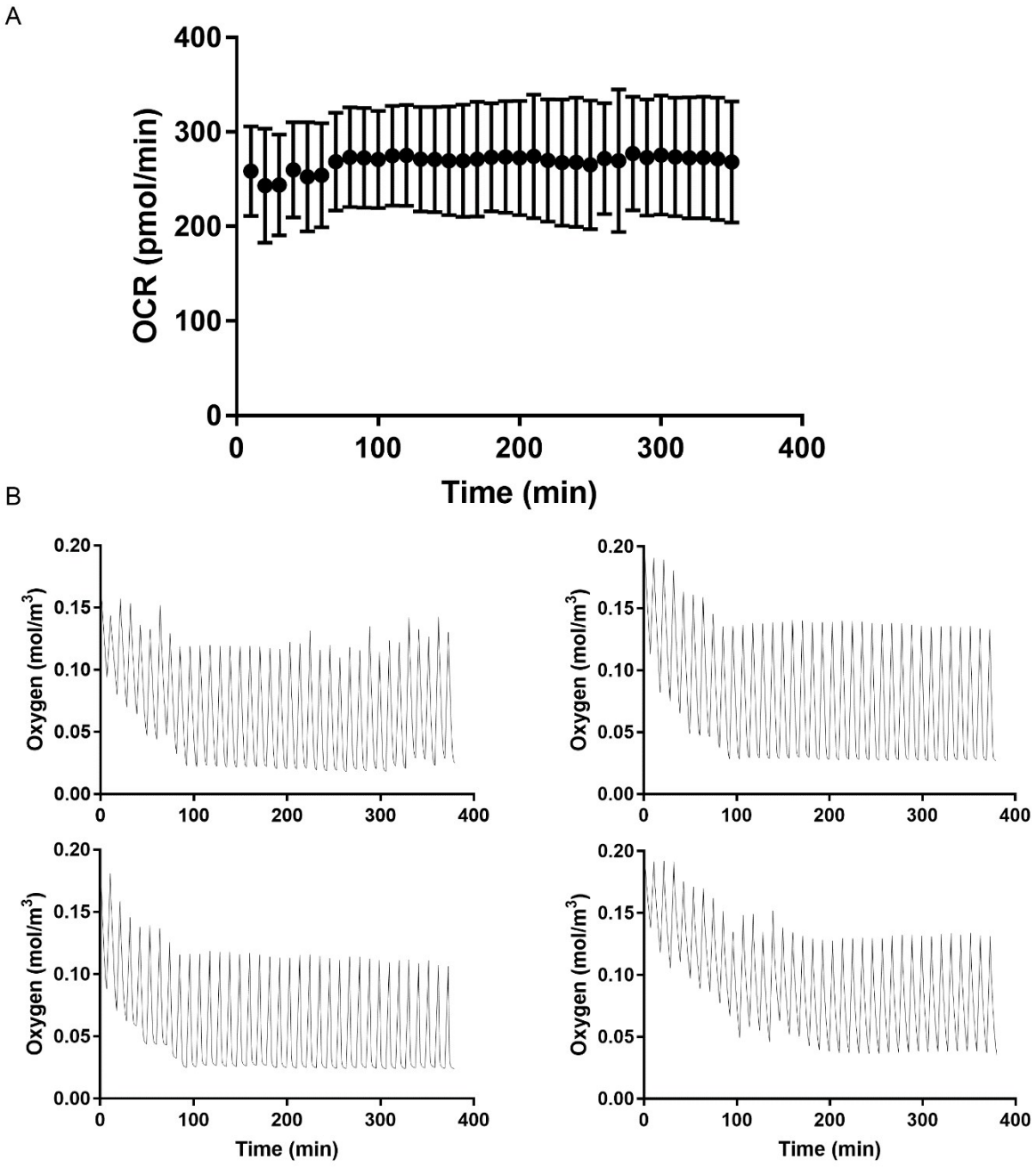

Figure SI 5. A) Oxygen consumption rate for MDA-MB-231 cells cultured within a collagen gel using Seahorse XF^e Extracellular Flux Analyzers. 35 measurements were taken over 6 hours at 10 minute intervals. Error bars presented are one standard deviation, n = 10 well samples were assessed. B) Oxygen concentration is presented for 4 of the 10 samples, to show representative temporal dynamics of oxygen while measuring oxygen consumption. Measurement time length is 7 minutes with a negligible oxygen transfer rate to determine oxygen consumption by the cells cultured.

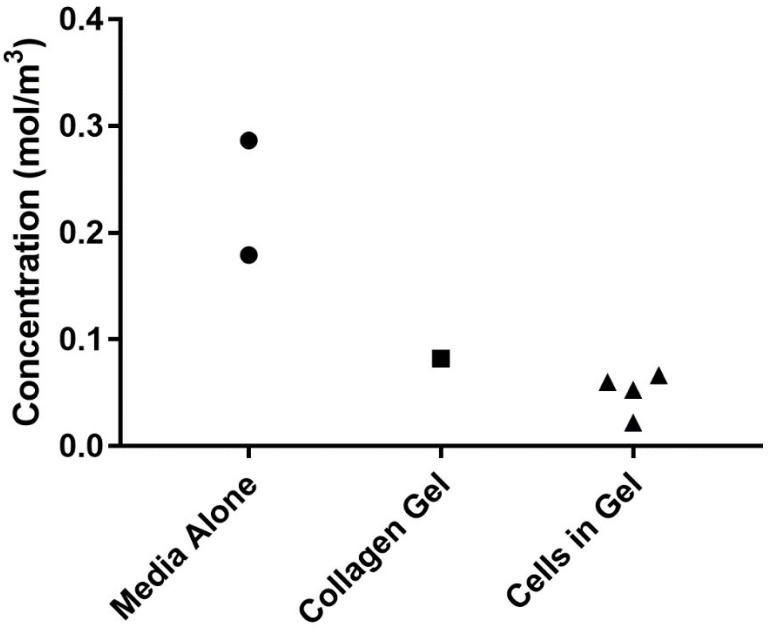

Figure SI 6. Oxygen levels obtained using Ocean Optics Neofox, to assess cell culture media, collagen gel, and cell-laden collagen gel.

	Intensity Reduction	Phase Shift Change
Average % Difference	22.5	2.4
Standard Deviation	15.0	1.3

Table SI 1. Percent intensity reduction and percent phase shift fluctuations of microsensors after one week of light exposure. Percent difference between day 1 and day 7, were determine for n = 15 microsensors. The intensity values were measured in μV and the phase-shift values were measured in degrees (°).