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A microwell array device capable of measuring single-cell oxygen consumption rates

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Abstract

Due to interest in cell population heterogeneity, the development of new technology and methodologies for studying single cells has dramatically increased in recent years. The ideal single cell measurement system would be high throughput for statistical relevance, would measure the most important cellular parameters, and minimize disruption of normal cell function. We have developed a microwell array device capable of measuring single cell oxygen consumption rates (OCR). This OCR device is able to diffusionally isolate single cells and enables the quantitative measurement of oxygen consumed by a single cell with fmol/min resolution in a non-invasive and relatively high throughput manner. A glass microwell array format containing fixed luminescent sensors allows for future incorporation of additional cellular parameter sensing capabilities. To demonstrate the utility of the OCR device, we determined the oxygen consumption rates of a small group of single cells (12 to 18) for three different cell lines: murine macrophage cell line RAW264.7, human epithelial lung cancer cell line A549, and human Barrett's esophagus cell line CP-D.

Keywords

Optical Sensor; Oxygen Sensor; Oxygen Consumption Rate; Single Cell; Phosphorescence; Lab-on-a-chip; Microfabrication; Array

Introduction

Historically, the majority of cellular response studies have been performed on bulk populations of cells. Recent findings have revealed cell-to-cell heterogeneity among previously assumed homogeneous populations[1–6] and that measuring the average response to a stimulus does not accurately reflect individual cell responses. Identifying single-cell heterogeneity can be accomplished by measuring cellular parameters such as gene and protein expression, ion gradients, membrane integrity, and oxygen consumption rates on a cell-by-cell basis across an entire population. The capabilities of a single cell array device would allow real-time phenotyping and stimulation response experiments, revealing insights into cellular heterogeneity. The Microscale Life Sciences Center (MLSC) at the University of Washington, Arizona State University, and the Fred Hutchinson Cancer Research Center is developing lab-on-a-chip technology to analyze cellular populations at the single cell level[7] in order to reveal heterogeneous behaviors and pathways that individual cells undergo. The MLSC has a focus

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on heterogeneity of cellular life and death processes, which are important in understanding diseases such as heart disease, stroke, and cancer.

The rate at which individual cells consume oxygen has been chosen as a critical parameter to measure because of its direct correlation to the central physiological process of ATP production in eukaryotic cells. Cellular ATP levels reflect mitochondrial activity and the amount of energy that is available for metabolic processes such as the synthesis of proteins, cell division and homeostasis. Mitochondrial function can change acutely, as in the early response of cells that undergo apoptosis[8], and chronically, in diverse neuropathies, diabetes, aging and cancer. [9] Since ATP and energy metabolism are intimately linked with life and death decisions in these diseases, measuring heterogeneity in single cell oxygen consumption should allow for a better understanding of how cells respond to therapeutic strategies.

Our approach for measuring oxygen consumption rates non-invasively is to diffusionally isolate single cells in an array of ~80 pL microwells containing cell media and monitor the oxygen concentration around each cell using luminescent oxygen sensors. A close-packed microwell array containing individual cells and sensors sealed in each microwell allows for all microwells and contents in the microscope field of view to be monitored in parallel, enabling high-throughput measurements.

A handful of methodologies have been developed to measure the oxygen consumption rates of single cells, however each has associated drawbacks. Microrespirometer methods[10], which measure the decreasing size of an air bubble next to a single-cell organism in a capillary, while novel for its time, are not suitable for high throughput experimentation, are prone to measurement errors, and lack the desired resolution. Detection methods utilizing electrochemical microsensor probes[11] to measure the flux of molecular oxygen in near proximity to single cells[12–14] has proven to be useful in showing dynamic oxygen concentration fluctuations corresponding to other physiological events. The main drawbacks to this method are sensor drift, detection limit, electrode fouling, and the difficulty in positioning the sensor in very close proximity to the cell ($< 1 \mu\text{m}$). [15] Although methods that alleviate the associated problems of varying cell-probe distances with an impedance feedback technique have been developed[16], the experimental platform is not well-suited for a high throughput lab-on-a-chip device and does not quantitatively yield the number of oxygen molecules consumed, but rather the flux near the cell. Another technique for measuring the oxygen consumption in the near proximity of individual yeast cells coated with fluorescent oxygen nanobeads has also been presented.[17] While the method is relatively high throughput (~100 cells in parallel), it still only measures oxygen flux. In addition, the beads may interfere with normal cell behavior, and certain cell types such as macrophages can consume the beads, as we have found in our own studies. In the case of intracellular phosphorescence-based oxygen detection strategies[18,19], the nature of the measurement produces reactive singlet oxygen internal to the cell that may adversely affect its viability. Finally, measuring sensor fluorescence intensity rather than lifetime or phase yields sub-optimal results because of dependency on amount of sensor present.[20]

Microwell arrays of various formats have recently become a very popular tool for monitoring the dynamics and cellular responses of individual cells[21–25]. While well-established flow cytometry devices have been in use for several decades, they only allow for single timepoint measurements. Arrays enable multi-timepoint and continuous monitoring experiments, which may be important for cellular processes with extended duration. Also, the array format may cause less stress than existing weir, docking, electrophoretic, tethering or optical trapping strategies by reducing physical, electrical and/or optical forces needed to capture and retain cells. The known structure of an array also alleviates the need for complex cell-locating analysis software encountered with random cell placement. While arrays have been made of several

types of materials, glass has extremely low autofluorescence levels and has the desirable properties of being optically transparent and oxygen impermeable, which are necessary for oxygen consumption rate measurements.

Materials and methods

Principle of operation

A glass chip with nine 4×4 arrays of etched microwells submerged in growth medium is randomly seeded with a dilute solution of cells yielding 0 to 2 cells per microwell. Each microwell on the chip contains an ringed-shaped oxygen sensor made of polystyrene beads embedded with a platinum phosphor and attached along the bottom circumference (Fig. 1A). After cell seeding, a glass lid attached to the end of a piston is pressed onto a single 4×4 array making contact with a raised lip encircling each microwell (Fig. 1B). The lips create a vacant area between the microwells allowing for fluid dispersion when the lid is lowered and reducing the chance of any debris or cells outside of the wells interfering with the glass-on-glass seal surrounding each microwell. The lid blocks any diffusion of oxygen into or out of the microwell containing the cell(s).

The miniature cell incubator (MCI) maintains suitable environmental conditions for the cells while they are trapped in the microwells (Fig. 1C). It holds approximately 3 mL of cell medium and 3 mL of gas. A sterilized gas mixture enters the MCI via a port through the reservoir wall creating a positive pressure inside to minimize contaminants. A quartz window below the chip prevents chip flexure when the lid is pressed down and allows for optical access to the microwells from underneath using an inverted microscope. A temperature of 37 °C is maintained by a set of feedback-controlled heaters mounted inside a custom built environmental chamber enclosing the entire microscope.

When the glass lid is pushed down onto the top of the chip and a seal is made, the cells residing in the microwells are diffusionally isolated from each other, and oxygen neither leaves nor enters. By using the oxygen sensor inside each microwell to track the oxygen concentration of the cell medium over time, the oxygen consumption rates can be determined. Custom-made automation and image processing software enabling a time-domain sensing technique takes advantage of the properties of phosphorescence to monitor all of the sensors in the field of view in parallel.

Microwell arrays and oxygen sensors

Microwell arrays and oxygen sensors are fabricated as previously described.[26–29] The procedure is repeated here due to several changes in technique and geometric configuration. 3-inch borosilicate glass wafers (Erie Scientific, Portsmouth, NH) are cleaned with a piranha etch and a 70°C RCA-1 solution, after which 150 Å of Cr and 3000 Å of gold are thermally evaporated onto one side of the glass surface. AZ1512 photoresist (Clariant Corporation, Sommerville, NJ) is spun onto the wafers. They are then soft baked at 90 °C for 1 min and exposed through a transparency mask on an Oriel 3-inch aligner. The photoresist is developed in a AZ300 MIF developer (Clariant Corporation, Sommerville, NJ) for 30 s and hard baked at 110 °C for 5 min. Gold is etched with Gold Etch TFA (Creekside Technologies, Snohomish, WA) for 120 s, and the chrome is etched with Chrome Etch TFD (Transene Co., Danvers, MA) for 10 s. Remaining photoresist is washed away with acetone. Blue-Tack (Semiconductor Equipment Corporation, Moorpark, CA) is used on the backside of the wafers to protect it during the hydrofluoric acid (HF) etching of the microwells. Exposed glass is etched in 49% HF (VWR, San Francisco, CA) for the required time to achieve a microwell depth of approximately 25 µm. The lithography process is repeated for a second mask that creates 3-µm lips around the outer edge of each well. Remaining gold and chrome are removed and the

wafers are cleaned in acetone, isopropanol, and DI water. This fabrication process yields 32 1×1 cm chips per wafer each containing nine arrays of 16 microwells (144 wells per chip). Each microwell in an array is approximately 65 μm in diameter, 25 μm deep, 80 pL in volume, and spaced 300 μm apart center-to-center.

Phosphorescent oxygen sensors are added to each microwell as follows. Microwell array chips are plasma-etched for 1 min to ensure glass surface hydrophilicity. 2 μL of 1-μm FluoSpheres® Platinum carboxylate-modified microspheres (Invitrogen, Eugene, OR) rinsed in deionized water are pipetted onto the chip and carefully dragged by hand across the surface using a wiper made of Polydimethylsiloxane (PDMS). The droplet is dragged back and forth over the microwells until no liquid remains. The chips are left to dry for a few minutes after which excess beads on the surface of the lips are gently removed with transparent adhesive tape. Upon drying, the beads form a ring around the bottom edge of each well. Lastly, the chips are put on a 120 °C hotplate for 10 min to slightly melt the beads and assure their adherence to the glass in aqueous experiments.

Data collection and image processing

Oxygen sensor data are collected using a technique exploiting the phosphorescent properties of the platinum phosphor sensors called the optimized rapid lifetime determination (ORLD) technique.[30,31] While some methods directly measure the quenching-dependent intensity of the emitted light, the ORLD method measures a quenching-dependent ratio of two overlapping emission integration bins following an excitation pulse (Fig. 2). In our case, two images of the entire sensor array are captured with a digital camera capable of precision gating (Andor iStar, South Windsor, CT). The sensors are excited via a 150 μs pulse from a 395 nm super-bright LED (Innovations in Optics, Inc., Woburn, MA), and the emission is filtered with a 650 ±20 nm bandpass filter (Thor Labs, Newton, NJ) before entering the camera. Integration bins A and B are 30 μs and 300 μs in width and delayed 1 μs and 8.5 μs from the turn-off of the excitation light source respectively.

A custom image-processing program determines the ratio of integrated light captured with the camera from the two images for all sensors in the array and converts the ratio to an aqueous oxygen concentration value using a calibration curve. Automation hardware/software and image-processing software are used to collect and process several image sets throughout the duration of an experiment, yielding the change in oxygen concentration in the sealed microwells.

The automation hardware/software and image-processing software are similar to our previously described methods[26,27], except that a 395 nm super-bright light emitting diode (LED) is used for the excitation light source and the oxygen detection technique is the ORLD rather than the on-off frequency domain (OOFD) method. The ORLD method has proven to be three times faster in both data collection and processing and has a larger dynamic range without sacrificing any sensor performance (data not shown).

Miniature cell incubator

The miniature cell incubator (MCI) (Fig. 1C) is a miniaturized lab environment that maintains suitable conditions for cultured living cells. The stage mount contains a 3.0 mm thick quartz window that provides a rigid support for the microarray in addition to optical access from underneath. A white Polyoxymethylene structure 1.2 cm in thickness surrounding the window and a PDMS gasket make up the medium well, which creates a volume of 6 mL for medium and a gas mixture (5% CO₂, 20% O₂, N₂ balance, humidified). A stainless steel chip holder keeps the microarray chip securely in place during an experiment. A thin clear polycarbonate cover with an access hole for the piston rests on top of the Delrin medium well. The gas mixture

flows (~50 mL/min) into the MCI through the side and exits through the space around the piston maintaining positive pressure and reducing the chances of airborne contamination.

Piston

A taper is added to one end of a ¼-inch polycarbonate rod resulting in a flat tip 2 mm in diameter. The tip is polished with 600-grit polishing paper and cleaned with ethanol. A 2.2 mm diameter circle of 100 µm-thick cured PDMS (Dow Corning, Midland, MI) and a 3×3 mm square 500 µm-thick piece of borosilicate glass is attached to the tip with liquid PDMS and cured in an oven at 70 °C for one hour. The PDMS layer acts as a cushion to compensate for any small off-axis misalignment between the piston and chip surface.

Lid actuator apparatus

The lid actuator is designed to press the lid down onto the microwells in a controlled and precise manner. The device is rigidly mounted to the microscope body on the front and back sides of the stage. The device is adjustable in the X-Y direction so that the piston can be aligned with the objective. Because of this, the microscope stage can be moved and the piston will always be centered above the microwells in view. When the crank is turned clockwise, the threaded rod exerts a downward force on an aluminum block resting on top of a spring, which supplies some cushion when pushing down the lid. A load cell mounted underneath the spring allows for real-time measurements of the force being applied. This enables accurate force control and repeatability from experiment to experiment. The actuator force needed to seal the microwells is approximately 10 lb (4.5 kg). The speed at which the lid is moved up and down is kept at a minimum (approximately 100 µm/s) to reduce turbulence above the array surface, reducing cell mobility and assuring that the cells maintain their location in the microwell array throughout the entire experiment.

Cell culture and reagents

The murine macrophage cell line RAW264.7 and human epithelial lung cancer cell line A549 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells are cultured at 37 °C in 5% CO₂ in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal calf serum (Cambrex, Walkersville, MD), 5 mM HEPES (Invitrogen, Carlsbad, CA), 0.2 mg/mL L-glutamine (Invitrogen, Carlsbad, CA), 0.05 mM β-mercaptoethanol (Sigma-Aldrich, St Louis, MO), 50 mg/mL gentamycin sulfate (Invitrogen, Carlsbad, CA) and 10,000 U/mL penicillin and streptomycin (Invitrogen, Carlsbad, CA). Cells are harvested by mechanical streak. Live cells are counted by hemocytometer with 0.1% trypan blue (Invitrogen, Carlsbad, CA). Cells are passaged at 80% confluence by trypsinization.

CP-D Barrett's esophagus cell lines[32] that were GFP-labeled and adapted to serum-free medium (Maley lab, Wistar Institute, Philadelphia, PA) are cultured at 37 °C, 5% CO₂, in Keratinocyte Serum-Free Medium (KSFM), supplemented with 50 mg/L BPE, 5 µg/L rhEGF, 50 U/mL penicillin and 50 mg/L streptomycin (Invitrogen, Carlsbad, CA). Cells are maintained in log-phase (50% confluency) and detached by 0.05% trypsin (Invitrogen, Carlsbad, CA) incubation, quenched with 0.01% FBS in PBS and counted via Z1 series Coulter counter.

Microwell array chips are first cleaned with Micro-90 glass cleaner followed by a milli-Q water rinse, plasma treated for 1 min, sprayed with 75% ethanol, and dried with nitrogen gas in a sterilized container. The chip is placed in one well of a 24-well tissue culture plate. For A549 and RAW264.7 cells, 1 mL of cell suspension with approximately 10,000 living cells in DMEM without antibiotics is loaded in the well. The cells are incubated at 37 °C, 5% CO₂ for 1 hr allowing them to settle and attach to the microwell array surface or the bottom of the microwell. For CP-D cells, similar methods are used, except that instead 30,000 cells in 2 mL of KSFM

are seeded in 6-well plates containing cleaned microwell array chips and grown for 18 hr prior to experimentation.

Before an oxygen consumption experiment, the cells are washed twice with 1 mL Hank's balanced salt solution (Invitrogen, Carlsbad, CA). They are then stained with 10 $\mu\text{L/mL}$ of 2.5 μM cell permeable Calcein AM live-cell dye and 2 $\mu\text{L/mL}$ of 500 μM SYTOX Orange cell impermeable DNA-binding dye (Molecular Probes, Eugene, OR) dissolved in HBSS for 30 min at 37 °C in a 5% CO_2 incubator. Calcein AM is a cell-permeable dye that has been used to determine cell viability in eukaryotic cells.[33,34] In viable cells, nonfluorescent Calcein AM passively crosses the cell membrane into the cell and is converted to green-fluorescent Calcein by intracellular esterases. The Calcein is highly charged and retained by cells with intact membranes. SYTOX Orange enters cells with damaged membranes and binds to the DNA. It has been used extensively as a dead cell indicator[35,36]. After staining, the cells are washed twice with 1 mL DMEM and resuspended in 1 mL DMEM without antibiotics for imaging and data collection. Calcein staining is omitted in CP-D cells, due to existing stably-integrated fluorescent EGFP protein. A fluorescent microscope (Zeiss Axiovert 200M LSM Meta 510, Thornwood, NY) is used to image fluorescence of Calcein (also EGFP in CP-D) and SYTOX at 488 nm excitation/530 nm emission and 543 nm excitation/570 nm emission respectively, before and after each oxygen consumption experiment.

Results and Discussion

Characterization of sensors

Sensor analytical characteristics were investigated on a 4×4 microwell array submerged under a thin layer of Dulbecco's Modified Eagle Medium (DMEM) cell culture medium at 37 °C and an atmospheric pressure of 0.998 atm. Sensor output and variability is quantified by determining the average output and standard deviation of 100 consecutive data points for a single sensor in the array at partial pressures of 20%, 10%, and 0% oxygen bathed over the cell medium. Sensor drift is quantified by determining the slope of a least-squares fit line to 250 consecutive data points (6.25 min) for a single sensor in the array at a constant partial pressure of 20% oxygen bathed over the cell medium. The results are summarized in Table I and Fig. 3.

The double quenching site Stern-Volmer calibration curve[37] most accurately models the quenching behavior of our sensors.[26] Sensor variability decreases with less aqueous oxygen concentration for two reasons. First, with less oxygen to quench the phosphorescent emission, the signal to noise ratio of the light detected by the camera increases. Secondly, because of the non-linearity and shape of the calibration curve (Fig. 3A) a given variability in ratio translates into a larger variability in concentration at higher oxygen concentration levels. R_0 and R are the unquenched (zero oxygen concentration) and quenched ratio respectively.

Cell culture medium such as the DMEM used in our experiments contain a variety of chemical compounds such as amino acids, glucose, inorganic salts, and vitamins that tend to foul and cause undesirable results with microelectrode devices. Despite the fact that the cell medium contains salts, the salinity is considered negligible for the aqueous oxygen concentration calculations[38] based on almost identical sensor responses for both deionized water and cell medium equilibrated with the different gas concentrations. The sensor step response to changing oxygen concentration levels is shown in Fig. 3B.

While the response time of phosphorescent quenching is instantaneous for all practical purposes, our ability to detect rapid changes in oxygen concentration is limited by the 0.44 s needed to collect image data as required by the ORLD method. Because our oxygen drawdown experiments are on the order of 5 to 10 minutes with oxygen concentration changing from 7.5

to 3.25 ppm for microwells containing single cells during this time, the 0.44 s resolution is more than sufficient. The average image-processing time for a single oxygen measurement for all sixteen sensors in the field of view is 1.46 s. Real-time monitoring of the experiments is limited to one measurement every 2 s or 30 measurements/min, while the maximum data collection rate with postprocessing is 120 measurements/min.

The sensor drift is essentially zero, which is a noteworthy advantage over microelectrode-based oxygen sensing. However, it has been observed that when the lid is pressed down on the microwell array there is the slight downward drift of oxygen concentration in the microwell over time. Based on several observations, it is hypothesized that this effect is caused by highly-reactive singlet state oxygen chemically combining with the polystyrene bead sensor matrix and thereby being “consumed” by the polystyrene as previously described as an “induction effect”. [39] The singlet state oxygen is an intermediate state of the oxygen molecules during the interaction with the phosphor under excited conditions, and since the sealed microwell is a closed system, this slight effect becomes more and more noticeable with smaller microwell volumes. This effect is quantified and subtracted out of the observed cell oxygen consumption data.

Characterization of microwells

SEM images of the microwells etched in the borosilicate glass chip with sensors deposited are shown in Fig. 4A-C at various magnifications. To quantify microwell characteristics such as depth, diameter, lip height, and volume, a measurement technique involving molding, optical profiling, and image processing has been developed. Knowing the volume of each microwell is of utmost importance since the value is used to calculate the actual moles of oxygen molecules consumed by the cell(s) in each microwell, by multiplying the oxygen concentration change over time by the volume and dividing by the molecular weight of oxygen.

A PDMS mold of a glass chip containing the microwell arrays is made followed by deposition of a thin (~50 nm) layer of silver. The resulting inverted micro-bumps are scanned with an optical profilometer (Wyco NT3300, Tuscon, AZ). The inversion of the wells and the silver coating are necessary for obtaining a quality 3-D digital image from the profiler. Without these steps, full reconstruction of the well profile is impossible because the sidewalls of the high aspect ratio structures reflect too little light into the profilometer, resulting in data loss. After profiling, the images are inverted and any missing data points are interpolated and restored.

The pre-processed images are then imported into a custom image-processing program written in MATLAB and plotted (Fig. 4D). The microwell characteristics are determined in the following manner: First, using three points to identify a plane, the top surface of the lip and the chip surface are defined. The lip height is the distance between these two planes and the microwell depth is the distance between the bottom of the microwell and the plane defining the top of the lip surface. Next, the well domain is defined using an edge filter that locates the circumference of the well, and the diameter is calculated. Finally, the volume is determined by integrating the volume between the bottom surface of the well and the plane defining the top of the lip surface over the domain of the well.

This newly-developed measurement technique has given insight into the stochastic nature of the wet etch technique used to produce the microwell array chips. Because the microwell volumes in a given array vary significantly (~6.5%) from a mean volume of approximately 80 pL, it is necessary to measure each microwell that contains a single cell for the final oxygen consumption rate calculation. Other techniques that might generate microwells with variances below one percent, such as dry etching or the use of alternative glass substrates, would greatly reduce the time required for data analysis by omitting this volume measurement step.

OCR of single cells

To demonstrate the utility of the microwell array device, the OCR of single cells from the following cell lines were experimentally determined: murine macrophage cell line RAW264.7, human epithelial lung cancer cell line A549, and human Barrett's esophagus cell line CP-D. Cells were cultured, prepared and seeded onto the microwell arrays for experimentation. A typical image of a microwell array containing live/dead-stained cells and oxygen sensors obtained with a fluorescent microscope is shown in Fig. 5A.

At the start of an oxygen consumption measurement, the glass lid was pressed down on top of the microwell array containing randomly-seeded cells and submerged in cell media with 10 lb (4.5 kg) of force, and the automation program collected a batch of oxygen sensor images every 17.5 s for 5.5 min following the ORLD method protocol. After the last image was acquired, the lid was raised, and the image-processing program processed the image batch to produce a plot of aqueous oxygen concentration versus time. The expected separation of oxygen concentration over time inside the sealed microwells containing zero, one, or two viable cells is shown in Fig. 5B. The slope of the lines fit to the data points for each microwell yields the oxygen concentration depletion rate (ppm/min) inside that microwell. Subsequent microwell volume obtained as described in the methods section for each unique microwell containing a single cell is used to convert the concentration depletion rates to single cell oxygen consumption rates (fmol/min). The slight non-zero slope of the zero-cell microwells illustrates the "induction effect" described above.

Our goal of determining the OCR for at least ten single cells for each cell line was accomplished after repeating an experiment three times on ten unique arrays for a total of 30 6-minute experiments yielding three repeat measurements on each single cell. Approximately 85% of the microwells containing single cells expressed a repeatable OCR defined as having a standard deviation of less than 25% of the mean rate (N=3). Only repeatable oxygen consumption rate measurements on each cell meeting this criterion were included in the final data set. The 15% of single cell OCR measurements that varied significantly over the three repeats was most likely due to a cell drastically changing its state (death or division) or a bad data point caused by an inadequate seal. The OCR histograms (Fig. 5C) for RAW264.7, A549, and CP-D cell lines contain 14, 18, and 12 average single cell measurements respectively. The mean rate and standard deviation for the three cells lines was calculated as 0.61 ± 0.23 , 0.91 ± 0.39 , and 1.32 ± 0.50 fmol/min/cell. These single cell respiration rates fall within the spectrum of average rate measurements taken on large populations of human cells using bulk techniques.[40–42]

The OCR variation that we observe between cell types reflects the different metabolic rates of the cell lines. These differences are due to inherited and regulatory changes as a result of developmental adaptations tissues undertake in response to evolved cellular roles in tissue physiology. Certain adaptations related to oxygen consumption rates have been shown to have important implications for resistance to particular forms of cytotoxic stress such as hypoxia or to drugs targeting specific metabolic functions.[43–45]

Heterogeneity of OCRs within a cell line is a function of mitochondrial mass and activity. Mitochondrial mass can display heterogeneity as a result of unequal mitochondrial distribution upon division. Mitochondrial activity has also been observed to change as an early response in cells during different parts of the cell cycle[46], in response to changing nutrient conditions [47], and in cells about to undergo apoptosis.[8] Neoplastic and cancer cell lines are known to develop mitochondrial genome and genetic instability, which may contribute to increased OCR diversity. It is interesting to note in our limited dataset that the two cell lines known to be more genetically unstable, lung tumor cell line A549 and Barrett's neoplasia cell line CP-D, also display higher heterogeneity. Understanding the extent of phenotypic heterogeneity in cancer cells will be vital to understanding and predicting resistance to various therapeutic challenges.

Conclusions

The ability to measure oxygen consumption rates at the single cell level will allow for correlations between preexisting physiological state and cellular outcomes of importance to disease, such as progression toward cancerous and inflammatory states. Heterogeneity observed in various oxygen and other metabolic markers will enable better assessment of other parameters of importance, for instance, resistance to therapy. We have developed a microwell array device capable of measuring the oxygen consumption rates of single cells with fmol/min resolution under controlled conditions. To demonstrate the operation of the device, we measured the OCR of a small group of single cells from three different cell lines. The next step is to use this tool for studying single cell heterogeneity and stimulus/response-based OCR. Future modifications and incorporation of additional extra-cellular analyte sensing capabilities such as pH, Ca^{2+} , Mg^{2+} , and CO_2 will further enhance single cell analysis throughput and discovery potential.

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Biographies

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Fig. 1.

The oxygen consumption rate (OCR) device. (A) The cells residing in the microwells with the oxygen sensor are diffusionally sealed from the external surroundings when the glass lid is pushed down onto the top of the microwell array. A phosphorescent oxygen sensor enables the monitoring of the oxygen concentration inside the microwell as the cell consumes oxygen. (B) Each glass chip contains nine 4x4 microwell arrays providing nine different areas for potential experiments. Each array is sized so that all 16 microwells are within the field of view using a 10X microscope objective and all 16 locations can be investigated in parallel. A thin PDMS layer is placed between the piston and the glass lid to provide a means for the lid to self align to the chip surface if there is any off-axis tilt in the piston. (C) The miniature cell incubator

(MCI) provides a suitable environment for the cells residing in the microwells. Positive pressure inside the covered reservoir from a constant flow of conditioned gas through an inlet port (not shown) reduces the possibility of airborne contamination. The MCI is kept at 37 °C by a set of feedback-controlled heaters mounted inside a custom built box enclosing the entire microscope (not shown).

Fig. 2.

Optimized rapid lifetime determination (ORLD) method. Two camera exposure bins, A and B, integrate the sensor emission at two overlapping intervals after an excitation pulse. The ratio of B/A is inversely proportional to the oxygen concentration of the medium in contact with the oxygen sensors.

Fig. 3. Oxygen sensor calibration and characterization. (A) Three standard gas mixtures of oxygen (20%, 10%, and 0%) bathing cell medium over the microwell array were used to produce a non-linear Stern-Volmer calibration curve. R_0 and R are the unquenched and quenched ratio (ORLD method output) respectively. (B) Sensor response to injection of same three gas mixtures into the miniature cell incubator at 2.5 min intervals.

Fig. 4. 100-μm diameter and 30-μm deep microwells. (A) SEM image of 4×4 microwell array. (B) SEM image of a single microwell containing self-assembled oxygen sensor in the shape of a ring along the sidewall of the microwell. Some beads tend to clump and attach to the microwell in areas other than the side wall. (C) SEM image of 1-μm polystyrene beads making up the oxygen sensor matrix. (D) 3-D image of a microwell reconstructed from an optical profiler image used in calculating microwell characteristics.

Fig. 5.

Experimental Results. A) Each microwell in this glass 4×4 array contains a red ring-shaped oxygen sensor and either zero, one (circled), or two mouse macrophage RAW264.7 cells. Eight of the sixteen microwells trapped the randomly-seeded live cells, which appear as green dots. B) An OCR experiment performed on the array yields a plot of oxygen concentration versus time for each sealed microwell in the array after a lid has been lowered. This typical result shows separation of oxygen concentration depletion rates inside microwells containing zero, one, or two cells. Sensors are numbered from left to right and top to bottom C) The average OCR of three different cell lines using the microwell array device was determined to be 0.61,

0.91, and 1.32 fmol/min for mouse murine macrophage RAW264.7, human epithelial lung cancer A549, and human Barrett's esophagus CP-D cells respectively.

Table IAnalytical characteristics of the oxygen sensor^a

Property	Value
output at 20% partial pressure	6.93 ± 0.25 ppm (n=100, same sensor)
output at 10% partial pressure	3.51 ± 0.15 ppm (n=100, same sensor)
output at 0% partial pressure	0.0 ± 0.016 ppm (n=100, same sensor)
drift	-0.00314 ± 0.0083 ppm/min (n=4, same sensor)
acquisition time per measurement	0.44 ± 0.012 s (n=50, same sensor)
processing time per measurement	1.46 ± 0.043 s (n=50, same sensor)
maximum post-processing rate	~120 measurements/min
maximum real-time rate	~30 measurements/min
detection range	0.0 to >7.5 ppm

^aMost data are presented as mean $\pm$ 1 standard deviation.