A New Approach for Measuring Single-Cell Oxygen Consumption Rates

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Abstract—A novel system that has enabled the measurement of single-cell oxygen consumption rates is presented. The experimental apparatus includes a temperature controlled environmental chamber, an array of microwells etched in glass, and a lid actuator used to seal cells in the microwells. Each microwell contains an oxygen sensitive platinum phosphor sensor used to monitor the cellular metabolic rates. Custom automation software controls the digital image data collection for oxygen sensor measurements, which are analyzed using an image-processing program to yield the oxygen concentration within each microwell versus time. Two proof-of-concept experiments produced oxygen consumption rate measurements for A549 human epithelial lung cancer cells of 5.39 and 5.27 fmol/min/cell, closely matching published oxygen consumption rates for bulk A549 populations.

Note to Practitioners—In the field of biology, the majority of cellular response studies have been performed on large populations of cells, providing only the average cellular response to a stimulus. Recent advances have revealed cell-to-cell heterogeneity among previously presumed homogeneous populations and have suggested that this heterogeneity is an important factor for understanding many diseases such as cancer, heart disease, and stroke. It may be necessary to monitor the response of individual cells within a population to a given stimulus. Identifying and tracking the progression of detrimental variants that have slipped by the safety mechanisms of the body is key to developing the next generation of patient treatments. These outliers can be identified by several measurable parameters including oxygen consumption rates, which is the focus here.

Index Terms—Biochemistry, frequency-domain analysis, image processing, oxygen, phosphorus compounds.

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I. INTRODUCTION

THE DEVELOPMENT of a single-cell metabolic rate monitoring system with fmol/minute resolution is essential to the mission of the Microscale Life Sciences Center (MLSC) at the University of Washington, a National Institutes of Health (NIH) National Human Genome Research Institute (NHGRI) Center of Excellence in Genomic Science (CEGS), www.life-on-a-chip.org [1]. This mission includes the development of a high-throughput multiparameter integrated system to monitor in parallel cellular parameters such as respiration rates, gene expression, protein expression, cytokine expression, membrane integrity, and ion gradients.

The evolving technology, when complete, will permit real-time investigations of cellular heterogeneity using experiments that examine cell damage and death pathways including neoplastic progression (cell proliferation) as seen in cancer or pyroptosis (pro-inflammatory cell death), as seen in heart disease and stroke. The premise driving fmol/min measurement resolution is that cell behavior may not be distributed simply about the mean of a large population; outliers may, in fact, tip the scale to either cell proliferation or cell death.

Advancements in single-cell analysis systems are emerging [2]–[7]; however, real-time high-throughput multiparameter single-cell measurements have remained elusive. Such a system would logically incorporate microfluidics, cell trapping, environmental control, automation software, fluorescence microscopy, and data analysis. Oxygen was selected as the initial parameter of interest due to the inherent difficulty associated with this measurement at the required detection resolutions. The technological development needed to enable these advances lays a strong foundation upon which to incorporate subsequent parameters.

Oxygen sensor systems based on the quenching-dependent emission from phosphor embedded polymers are becoming increasingly popular [8]–[13]. Absorption of visible or UV radiation from a laser, for example, excites the phosphor into a short-lived singlet state whereupon it nonradiatively relaxes to a lower level triplet state. Light is often emitted as the electron relaxes from the triplet state back to the ground state. The ensemble emission of the phosphor can be empirically represented by the following exponential decay function:

$$I(t) = I_0 \exp(-t/\tau) \tag{1}$$

where I(t) is the intensity of the phosphorescence as a function of time, I_0 is the maximum intensity at time t = 0, and the lifetime τ is the average time the phosphor stays in the excited state following excitation. The lifetime of the decay of phosphorescence is typically on the order of microseconds to seconds compared with typical fluorescence lifetimes of nanoseconds [14].

Oxygen sensing is achieved because the triplet state of the phosphor is at a similar energy level as the triplet ground state of molecular oxygen. The presence of oxygen provides a molecular orbital overlap that allows for a nonradiative relaxation pathway for excited electrons within the phosphor molecule. This additional decay path manifests itself as a shortening of the observed lifetime. While other oxygen detection experiments have measured the exponential decay curve directly [15], it is also possible for the sensor to be excited with sinusoidal intensity modulated light and to measure the phase shift of the emission. Emission from a sinusoidally driven sensor follows with the same frequency, but is delayed in phase and reduced in amplitude relative to the excitation. Measuring the phase shift rather than the exponential decay has the important benefits of improved signal-to-noise ratio, greater sensor output dynamic range, shorter excitation exposure times, and allowing for experimental optimization techniques.

In order to assign a value to the observed decrease in lifetime or phase, it is necessary to model the decay. A first approach might be to model the phosphorescence with only a single-component lifetime or phase as described by a linear Stern–Volmer equation referred to here as Model 1

$$\frac{\tau_0}{\tau} = \frac{\Phi_0}{\Phi} = 1 + K_{\rm SV}[q] = 1 + K_{\rm SV}[O_2]$$
(2)

where τ_0 and Φ_0 are the unquenched emission lifetime and phase, respectively, while τ and Φ are the same quantities in the presence of different quencher concentrations, $K_{\rm SV}$ is the Stern–Volmer constant, and [q] is the quencher concentration [16]. Since the main quenching agent present in the microwells is oxygen, [q] can be replaced by the aqueous concentration of oxygen $[O_2]$.

In many cases, the phosphorescent quantum yield is not modeled well by the linear Model 1 Stern–Volmer relationship. A downward deviation from the linear model is observed, and as a result of the nonlinearity, it is necessary to calibrate following a different model. A commonly used power law quenching model, referred to here as Model 2 is

$$\frac{\tau_0}{\tau} = \frac{\Phi_0}{\Phi} = 1 + K[O_2]^m$$
(3)

where K and m are the fitting parameters [17]. Model 2, while purely empirical, improves calibration, but has no physical or chemical significance beyond provision for improved calibration fits.

A third Stern–Volmer equation models two distinct quenching sites in the sensor. Both sites are quenchable, but they have different quantum yields and will be referred to here as Model 3

$$\frac{\tau_0}{\tau} = \frac{\Phi_0}{\Phi} = \frac{1}{f_{01}/(1 + K_{\rm SV1}[O_2]) + f_{02}/(1 + K_{\rm SV2}[O_2])}$$
(4)

where f_{01} and f_{02} are the fraction of the total emission from each component under unquenched conditions and K_{SV1} and



Fig. 1. An array of microwells $150 \ \mu$ m in diameter and $50 \ \mu$ m deep are etched into a borosilicate glass chip. Each microwell contains randomly seeded living cells and a platinum phosphor optical sensor in the shape of a ring fused to the bottom perimeter of the well.

 K_{SV2} are the associated Stern–Volmer quenching constants for each component [17].

In order to practically utilize a Stern–Volmer calibration model, it is necessary to experimentally calibrate at known oxygen concentrations. The abundance of oxygen in an aqueous solution is commonly expressed as a molar concentration $[O_2(aq)]$ by

$$[O_2(aq)] = K_H \cdot PO_2 \tag{5}$$

where $K_{\rm H}$ is Henry's constant and PO_2 is the partial pressure of oxygen. Equation (5) states that the concentration of oxygen dissolved in water is directly proportional to Henry's constant multiplied by the partial pressure of oxygen in the gas in contact with the water.

According to Benson and Krause, Jr., who empirically fit experimental data for temperature ranging from 0 °C to 45 °C, Henry's constant for oxygen in water as a function of temperature is approximately

$$K_{\rm H} = \exp\left[0.2945329 - \frac{5596.17}{T} + \frac{1049668}{T^2}\right] \tag{6}$$

where $K_{\rm H}$ is Henry's constant in M/atm and T is temperature in Kelvin [18]. An equation is also presented in [18] to include the effects of salinity, but for this study the salinity has been considered to be negligible. After a number of sensor lifetime or phase readouts at known temperature and partial pressure are collected and fit to an accurate Stern–Volmer model, any sensor readout can be converted to aqueous concentration of oxygen.

II. EXPERIMENTAL

A. Microwell Array

Several 1 × 1 cm chips containing an array of microwells are etched on 3-inch borosilicate glass wafers using standard photolithography and etching techniques (Fig. 1). The microwells are 150 μ m in diameter, 50 μ m deep, and spaced 200 μ m apart center-to-center. A 3 μ m high lip encircles the top of each



Fig. 2. Illustrative side view of microwell, sensor, and lid (not to scale). The cells residing in the microwell with the sensor are sealed off from the external surroundings when the gold oxygen barrier is pushed down onto the seal ridge. Sensor excitation and imaging occurs through the bottom of the chip.

microwell. The sensors at the bottom circumference of each microwell consist of a fused matrix of polystyrene beads embedded with a platinum phosphor [19]. The wafer level deposition allows for efficient creation of numerous wells with sensors in parallel.

B. Lid Actuator

Sealing each microwell is essential for metabolic rate experiments. Each well, which contains one or more cells, a sensor, and cell media needs to be diffusionally isolated from the external surroundings to measure oxygen metabolism. The sensor monitors the decreasing aqueous oxygen concentration of the cell media as cells consume oxygen. If the microwell is not sealed, exterior oxygen could refresh the microwell oxygen supply, and the respiration rate of the cell could not be accurately determined. A gold oxygen barrier is pushed onto the seal ridge of the microwell by a piston to make a seal, while the compliance layer compensates for any off-axis tilt between the contacting surfaces (Fig. 2).

The lid actuator is designed to press an oxygen barrier down onto the microwells (Fig. 3). A 24-well tissue culture plate is modified to accommodate two securely attached anchors on either side of a well containing the microwell array and cell media. The spanning bar transfers the force generated by the wing nuts through the piston down to the oxygen barrier. The piston has a flat polished tip to which the compliance layer and oxygen barrier are attached.

C. Microscope and Environmental Chamber

An environmental chamber is needed to maintain a constant and controllable temperature for the cells under investigation, which is of critical importance when following biological protocols. Temperatures adequate for cell assays range approximately from 35 °C to 37 °C. A commercially available device (PeCon GmbH, Erbach, Germany; TempContol 37-2 digital, heating unit, humidifier system, and microscope stage chamber) that mounts onto the stage of an inverted microscope (Zeiss, Thornwood, NY; Axiovert 200M) accommodates the placement of the lid actuator apparatus inside. The environmental chamber and temperature control system help maintain the required thermal environment for biological experiments.



Fig. 3. A cut-away view of the lid actuator apparatus shows the main components comprising of a 24-well plate, two anchors, two wing nuts, a spanning bar, and a piston. A force is applied to the oxygen barrier on top of the microwells when the wing nuts are turned down, which seals each microwell underneath the piston.



Fig. 4. Data collection overview. The OOFD method generates a kinetic series of F frames. Each frame in the kinetic series ($Q \times Q$ pixels) contains a phasesensitive image of the N sensors (each frame in this illustration contains four circular sensors). A sequential collection of K kinetic series contains all the data necessary to estimate the oxygen concentration in each microwell over the span of an experiment.

D. Data Collection

A frequency-domain technique for experimentally determining the phosphorescence phase has been developed called the on-off frequency-domain (OOFD) method [20], which is a modification of a method described by Shonat *et al.* [9]. In the OOFD method, a series of images of the sensors is acquired. The sensor is pulsed on and off with laser light at a fixed pulse width W, and the timing of each camera exposure is delayed by Φ_D with respect to the laser (Fig. 4). At an initial phase delay of zero, a $Q \times Q$ pixel digital image of the sensors is acquired representing the first of F frames in a single kinetic series. Subsequent phase-delayed images are similarly obtained and concatenated to the end of the growing kinetic series until the Fth frame is obtained. Each frame contains images of the same N sensors that differ in phase-dependant intensity. Each



Fig. 5. The automation hardware enables excitation and recording of the phasesensitive emission of the sensors in the microwell array.

of the K kinetic series yields one oxygen concentration point in time for each sensor. Image processing follows (described in the next section), which extracts the individual sensor areas for each kinetic series and plots the integrated intensities of each sensor as a function of phase. A fourth-order polynomial is fit to the data, whereupon the phase Φ is determined by taking the derivative of the fit polynomial and finding the root that corresponds to the phase. This phase, of course, changes with different oxygen quenching levels.

The data collection hardware enabling the oxygen sensors is shown in Fig. 5. The function generator (Tektronix, Richardson, TX; AFG 310 Arbitrary Function Generator) is set to produce a square wave (5 Hz, 50% duty cycle). The delay generator (Berkeley Nucleonics Corporation, San Rafael, CA; Model 555 Pulse/Delay Generator) produces two in-phase square pulses (50 μ s pulse width) when triggered by the function generator. One pulse is sent to the laser (Power Technology Inc., Little Rock, AR; Diode Laser PPMT LD1539 405 nm), while the other pulse is sent to the camera (Andor, South Windsor, CT; iStar DH734-18F-73 Imaging ICCD). The pulses are continually sent and serve to synchronize the laser and the camera.

The personal computer (Dell, Round Rock, TX; Precision 370-P4 2.80 GHz, 1.00 GB RAM) contains the hard drive and the Andor camera Peripheral Component Interconnect (PCI). The camera controller PCI connects to the camera via a shielded bundle cord. There is also a 15 pin D-Sub auxiliary port on the PCI used to trigger the external shutter controller (Uniblitz, Rochester, NY; Shutter Driver/Timer Model VMM-T1) via the automation program. The normally-closed external shutter opens with Transistor-Transistor Logic (TTL) high. The microwell array sitting in the lid actuator device is placed on the inverted microscope stage (Zeiss, Thornwood, NY; Axiovert 200M).

The laser light used to excite the platinum phosphor sensors passes through a $5 \times$ beam expander and an optical diffuser used to reduce the optical density and spread out the Gaussian profile. It reflects off a dichroic and illuminates the sensors



Fig. 6. The automation software controls the timing of the excitation and the collection of digital data by the camera. An external shutter is only opened while a kinetic series is taken. Otherwise, it remains closed for background image acquisition and to keep laser light from reaching the microwell array when unnecessary.

after traveling through a $10 \times$ objective (Zeiss, Thornwood, NY; A-Plan 10X/0.25 Ph1Var1 1020-863). No excitation filter is necessary since the laser is fixed at the desired 405 nm excitation wavelength. The phosphorescence emission of the sensors travels back through the objective, passes through the dichroic, which blocks any laser light, through a 585 nm long pass filter (Omega, Brattleboro, VT; XF3090 585 ALP) and enters the camera. While it is true that it is possible to damage cells with near-UV laser light, 405 nm or lower excitation wavelengths for live-cell probes and sensors are commonplace [21]–[24], and there are many commercially available cellular stains that are excited in the UV to near-UV range. By reducing exposure times and using diffuse excitation light, possible side effects to cell heath are minimized.

The automation of the sensor data acquisition is orchestrated by software written in a procedural programming language supplied with the camera called AndorBasic. Referring to Fig. 6, the automation steps are explained. First, the relevant acquisition parameters outlined in Fig. 4 are defined (Step 1) including: intensifier pulse width W, phase delay between subsequent frames Φ_D , number of frames in kinetic series F, and number of kinetic series in the kinetic series batch K.

Next, several camera settings are defined (Step 2). The trigger mode is set to external. The data type is set to "background corrected" to subtract a background image including any dark noise or fixed pattern noise that is present from each image in the kinetic series. The number of accumulations is set and specifies how many multiple scans of each frame in the kinetic series are added together in computer memory. The gain is set to define how much the emission signal is amplified within the camera. The binning is set to specify how many neighboring pixels on the CCD should be combined to make the resulting $Q \times Q$ image. The program then enters a loop that collects K kinetic series. Since the frames of the kinetic series are background corrected, a background image must be collected (Step 3) before the frames of the kinetic series are acquired. The external shutter is then opened (Step 4) to let the pulsing laser light through to the sensors, and the kinetic series is acquired (Step 5). The external shutter is immediately closed (Step 6) to prevent the laser light from reaching the sensors and cells. The kinetic series is then converted to ASCII format and saved on the hard drive with a number appended to the end of the file name that matches the loop index (Step 7). All kinetic series needed for the experiment are collected in this manner by going through the loop K times.

E. Cell Culturing and Seeding

A human epithelial lung cancer A549 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured as attached cells in Dulbecco's Modified Eagle's Media (DMEM) with 4.5 g/L, 2 mM glutamine, and Penicillin-Streptomycin (Cambrex Bioproducts, Walkersville, MD; catalog # 12-614, 17-602, 17-602). The media was supplemented with 10% Fetal Clone III (Hyclone, Logan, UT; catalog # SH30109.03) in 5% CO₂ at 37 °C. Cells were harvested using 0.25% trypsin in versene (Cambrex Bioproducts, Walkersville, MD; catalog # 17-160, 17-711).

Microwell array chips were placed in individual wells of a 24-well tissue culture plate and incubated at room temperature in 70% ethanol for 10 min. Ethanol was decanted and each array was rinsed with sterile Dulbecco's phosphate buffered saline (Invitrogen, Grand Island, NY; catalog # 14200-075), diluted to $1\times$, adjusted to pH 7, and rinsed a second time in the modified growth media that was used for cell culture. 0.5 mL of the same modified growth media was added to each well of the 24-well tissue culture plate containing a microwell array chip. The microwells were seeded by adding 100 μ L of cell suspension (approximately 500–10 000 cells) to each well containing a chip. The cells were incubated 16–20 h at 37 °C allowing time for them to settle and attach to the microwell array surface and the bottom of the microwells depending on where they settled.

Before the data collection, the cells were stained with a cell permeable DNA stain, Hoechst 33342, (Molecular Probes, Inc, Carlsbad, CA; catalog # H1399) by adding 6.0 μ L to the well from a 1 mM stock solution in distilled water. Adding the Hoechst dye before the experiment allowed enough time for the dye to permeate the cells for imaging at the end of the experiment. After imaging the cells with the Hoechst dye, 3 μ L from a 1 mg/mL stock solution of propidium iodide (PI) (Sigma-Aldrich, St Louis, MO; catalog # P4170) was added to the well. Images of the PI stained cells were acquired after 15 min.

III. IMAGE PROCESSING

The image processing program [written in MATLAB and implemented in MATLAB Version 7.0.0.19920 (R14)] processes the multiple kinetic series collected during an experiment and produces a plot of oxygen concentration versus time for each sensor. It consists of six major components: A) *Locate Sensors*, which determines the sensor locations in a kinetic series; B) *Integrate Intensity*, which integrates the pixel intensity values of each sensor in each frame of the kinetic series; C) *Estimate Phase*, which determines the phase for each sensor by fitting a curve to the integrated intensity data and locating the peak; D) *Process All Kinetic Series*, which processes every kinetic series collected during an experiment and stores the phase data in a matrix; E) *Convert Phase*, which converts all phase values to aqueous oxygen concentration with a calibration curve; and F) *Plot*, which produces the final plot of oxygen concentration versus time for each sensor. Each of these components are now discussed in detail.

A. Locate Sensors

First, the ring-shaped sensors imaged in a kinetic series are located. Let $\zeta_{Q\times Q}^{f}$ represent a kinetic series, where $f = 1, \ldots, F$ is the frame index and Q is the number of pixel rows and columns in each frame. Each frame is a phase dependent intensity map of the sensors, which remain in the same spatial position. Let $\xi_{Q\times Q}$ be a binary map containing a ring located in the image center

$$\xi_{Q \times Q}(\mathbf{X}) = \begin{cases} 1, & \text{if } \alpha^2 \le \left(x - \frac{Q}{2}\right)^2 + \left(y - \frac{Q}{2}\right)^2 \le \beta^2\\ 0, & \text{otherwise} \end{cases}$$
(7)

where $\mathbf{X} = (x, y)$ are the pixel locations within the image domain and α and β are inner and outer radius of the ring in pixels, respectively. The N sensors are located by convolving the first frame of a kinetic series $\zeta_{Q \times Q}^1$ with sensor filter $\xi_{Q \times Q}$ and searching for the N peaks in the resulting image. Let $\Gamma_{Q \times Q}$ be the resulting convolution image

$$\Gamma_{Q \times Q} = \zeta_{Q \times Q}^1 * * \xi_{Q \times Q}.$$
(8)

Let \overline{l} be a vector of length N with components l_1, l_2, \ldots, l_N containing the sensor location coordinates

$$l_n = \mathbf{X}_n \tag{9}$$

where n = 1, ..., N is the sensor index and \mathbf{X}_n is the pixel location in $\Gamma_{Q \times Q}$ corresponding to the *n*th local maxima. Once the sensor coordinates are determined, a filter set is made, which contains *n* binary images of a ring located at each sensor position. Let $\Psi_{Q \times Q}^n$ be a filter set, where n = 1, ..., N is the frame index. Each frame is a binary map of a ring centered at the *n*th location in \vec{l}

$$\Psi_{Q\times Q}^{n}(\mathbf{X}) = \begin{cases} 1, & \text{if } \alpha^{2} \leq (x - l_{n,x})^{2} + (y - l_{n,y})^{2} \leq \beta^{2} \\ 0, & \text{otherwise} \end{cases}$$
(10)

where $\mathbf{X} = (x, y)$ are the pixel locations within the image domain and α and β are inner and outer radius of the ring in pixels, respectively.

B. Integrate Intensity

Next, the integrated intensity for each sensor in each frame of the kinetic series is determined. Let $I_{N \times F}$ be an N row by F column matrix containing the integrated intensity for each sensor in each frame of the kinetic series. Each location in $I_{N \times F}$ equals the integration of the Hadamard product of the *n*th frame of the filter set $\Psi_{Q\times Q}^n$ with the *f*th frame of the kinetic series $\zeta_{Q\times Q}^f$

$$I_{n,f} = \int_{\Omega} \Psi_{Q \times Q}^{n} \cdot \zeta_{Q \times Q}^{f} \tag{11}$$

where n = 1, ..., N is the sensor index, f = 1, ..., F is the kinetic series frame index, and Ω is the image domain.

C. Estimate Phase

Then, the phase for each sensor in the kinetic series is estimated. Let \overline{d} be a phase delay vector of length F with components d_1, d_2, \ldots, d_F

$$d_f = \Phi_D(f-1) \tag{12}$$

where $f = 1, \ldots, F$ is the kinetic series frame index and Φ_D is the phase delay between consecutive frames in a kinetic series. The program performs a fourth-order polynomial least-squares error fit of the integrated intensity in the *n*th row in $I_{N\times F}$ to the phase delay vector \vec{d} . That is, it finds parameters $\alpha_1, \alpha_2, \alpha_3, \alpha_4$, and α_5 of the equation

$$I_{n,f} = \alpha_1 d_f^4 + \alpha_2 d_f^3 + \alpha_3 d_f^2 + \alpha_4 d_f + \alpha_5$$
(13)

that minimizes the total square error between the data in each row of $I_{N\times F}$ and \vec{d} , where $n = 1, \ldots, N$ is the sensor index and $f = 1, \ldots, F$ is the kinetic series frame index. A continuous fourth-order polynomial function denoted $g_n(\Phi)$ with parameters $\alpha_1, \alpha_2, \alpha_3, \alpha_4$, and α_5 is created for each sensor, where $n = 1, \ldots, N$ is the sensor index. Let \vec{v} be a vector of length Nwith components v_1, v_2, \ldots, v_N containing the estimated phase Φ for each sensor. Each phase value is estimated by taking the derivative of g and finding the appropriate root in g', which is between 0° and 90°

$$v_n = \Phi : g'_n(\Phi) = 0, \quad 0 < \Phi < \pi/2$$
 (14)

where $n = 1, \ldots, N$ is the sensor index.

D. Process All Kinetic Series

The program now repeatedly processes and stores each K kinetic series collected during an experiment. Let $J_{K\times N}$ be a K row by N column matrix containing the estimated phase for each sensor in all kinetic series

$$J_{k,n} = v_n \tag{15}$$

where k = 1, ..., K is the kinetic series index and n = 1, ..., N is the sensor index.

E. Convert Phase

Each phase value in $J_{K \times N}$ must be converted to an aqueous oxygen concentration. In this example the conversion is accomplished after fitting calibration data to a three-point Model 3 Stern–Volmer calibration curve (4).

Let \overline{p} be a vector of length 3 with components p_1 , p_2 , and p_3 containing three partial pressure calibration points 0%, 10%, and 20%, respectively. Let \overline{c} be a vector of length 3 with components c_1 , c_2 , and c_3 containing three aqueous oxygen concentration values (ppm) corresponding to the three partial pressure calibration points in \overline{p} calculated from (5) and (6) combined

$$c_a = p_a \kappa \psi \exp\left(0.2946329 - \frac{5596.17}{\eta} + \frac{1049668}{\eta^2}\right) \quad (16)$$

where a = 1, ..., 3, κ is the molecular weight of O₂ (mg/mol), ψ is the atmospheric pressure (atm), and η is the media temperature (K). Let \vec{q} be a vector of length 3 with components q_1 , q_2 , and q_3 containing three average phase calibration points experimentally determined at each partial pressure in \vec{p} . Let \vec{n} be a vector of length 3 with components n_1 , n_2 , and n_3 containing the y axis of the Stern–Volmer plot

$$n_a = q_1/q_a \tag{17}$$

where a = 1, ..., 3. Next, the program performs a model 3 Stern–Volmer (4) least-squares error fit of the calibration phase data vector \vec{n} to calibration concentration vector \vec{c} . That is, it finds parameters $f_{01}, K_{SV1}, f_{02}, K_{SV2}$ of the equation

$$n_a = \frac{1}{f_{01}/(1 + K_{\rm SV1} \cdot c_a) + f_{02}/(1 + K_{\rm SV2} \cdot c_a)}$$
(18)

that minimizes the total square error between data vectors n and \vec{c} fit to (4). All phase values in matrix $J_{K \times N}$ are then converted to concentration values and stored in matrix $O_{K \times N}$ by solving the following equation for O:

$$\frac{n_1}{J_{k,n}} = \frac{1}{f_{01}/(1 + K_{\rm SV1} \cdot O_{k,n}) + f_{02}/(1 + K_{\rm SV2} \cdot O_{k,n})}$$
(19)

where k = 1, ..., K is the kinetic series index and n = 1, ..., N is the sensor index.

F. Plot

Finally, a plot of oxygen concentration versus time for each sensor is made. Let \vec{t} be a time axis vector of length K with components t_1, t_2, \ldots, t_K

$$t_k = T(1-k) \tag{20}$$

where k = 1, ..., K is the kinetic series index and T is the time between consecutive kinetic series acquisitions during an experiment. Plotting each column in $O_{K \times N}$ versus \vec{t} yields a graph showing the aqueous concentration of oxygen in each microwell as a function of time.



Fig. 7. Calibration curves. Investigation of our oxygen sensor using the PMT, MLE, and OOFD acquisition methods suggests that quenching may be consistent with the assumptions of a Model 3 Stern–Volmer calibration. In addition, a three-point calibration suffices for an accurate calibration. All five calibration data points are shown, but only the two endpoints and the middle oxygen concentration points were used for the curve fit.

IV. RESULTS AND DISCUSSION

A. Sensor Calibration

The lifetime and phase for partial pressure O_2 levels of 0%, 5%, 10%, 15%, and 20% bubbled into 3 mL of water in contact with the microwell array were experimentally determined using the OOFD method, as well as a PMT and MLE method. The PMT and MLE methods have been included in this study to aid in validating our new methodologies and are generally described in [25] and [26], respectively. All data were taken from a single sensor from the microwell array at 31.7 ± 0.3 °C and 1.0 atm. Each calibration point is the mean value of 30 consecutive lifetime or phase estimates. The three Stern–Volmer models (2–4) were then fit to the five calibration points for each of the three acquisition methods.

After a visual comparison between the three different Stern–Volmer models fit to the calibration data, Model 3 appeared to be the best fitting for all three acquisition methods. This qualitative finding is in agreement with a more rigorous calibration study on transition-metal complex oxygen sensors [17]. In addition, a three-point calibration (0%, 10%, and 20% PO_2) gives an almost identical fit as the full five-point calibration for all three acquisition methods and suffices for an accurate calibration (Fig. 7). Further reference of Model 3 calibration implies a three-point calibration.

Several suspected reasons for the observed downward curvature of the data, as shown in Fig. 7, have been reported [12], [17], [27]–[29]: multiple lifetimes of the phosphor, heterogeneity of the sensor matrix (site heterogeneity), systematic errors in quantitative emission intensity using gated emission and excitation, and/or oxygen permeability of the sensor polymer. The two quenching sites modeled by the Model 3 Stern–Volmer equation are most likely the phosphor on the surface of the beads and the phosphor in the bulk of the beads [8]. The sites on the



Fig. 8. The nuclei of A549 human epithelial lung cancer cells (L: live, A: apoptotic) are stained with a cell membrane permeable DNA-binding fluorescent dye Hoechst 33342. The cells were also stained with PI (not shown—no PI uptake observed) to help facilitate in classifying the cell state. (a) Three of the nine microwells contain a small number of cells as indicated by the dye. The outlines of the sensors in the bottom of the microwells are also faintly visible. (b) Microwell #5 contains one apoptotic cell. (c) Microwell #6 contains two apoptotic cells and three live cells. (d) Microwell #7 contains one apoptotic cell and one live cell.

surface are more readily accessible to the free oxygen compared with the sites buried in and bonded to the polystyrene.

B. Proof-of-Concept Experiments

The results presented in this section provide fundamental validation for the single-cell oxygen consumption system by highlighting two proof-of-concept biological experiments. Several microwell arrays were randomly seeded with a dilute solution of human epithelial lung cancer cells as described above. Two were chosen that appeared to have a minimum number of cells (0–6) trapped in each microwell.

Each experiment began with adding Hoechst 33342 dye to the well containing the microwell array chip. The lid with the gold oxygen barrier was then pressed onto the top of the microwells with a force estimated to be in the range of five to ten pounds. Shortly thereafter, the data collection process was initiated. The data were processed by the image processing program to produce a plot of the oxygen concentration, as a function of time for each microwell. The cells stained with the Hoechst dye were then imaged 30 min after the dye was initially added. PI stain was added and the cells were again imaged 15 min later.

Hoechst 33342 freely enters living or dead cells and binds to the DNA whereupon it fluoresces [30]. A cell undergoing apoptosis shows signs of DNA fragmentation, which can be observed in images of the Hoechst stained cells [31], [32] (Fig. 8). An image of propidium iodide (PI) stained cells was also collected (not shown). PI readily permeates through compromised cellular membranes of nonviable cells and binds to the DNA. The cell membrane of cells undergoing apoptosis remains intact and functions normally [33].



Fig. 9. Sensor response over time for the first experiment. The oxygen concentration of the media in well #6 decreased in oxygen concentration from 6.2 to 0.0 ppm in approximately 9 min resulting in an average respiration rate of 0.23 ppm/min/cell or 5.39 fmol/min/cell.

When using PI and Hoechst together, it is possible to classify cells as either necrotic (accidental cell death caused by injury, inflammation, etc.), apoptotic (programmed cell death), or live. Necrosis in a cell can be identified by no nuclear fragmentation in combination with damage to the cell membrane. Apoptosis, on the other hand, can be identified by the presence of nuclear fragmentation in combination with cell membrane integrity. A cell exhibiting both nuclear fragmentation and membrane damage is classified as late apoptotic [34]. If an image of a cell stained with both Hoechst and PI does not indicate apoptosis or necrosis, the cell is fully intact and is most likely healthy and living [33].

The microwell array used in the first experiment appears to contain a fragmented nucleus (fuzzy image of cellular DNA) in microwell #5, a cluster of three intact nuclei (sharp image of cellular DNA) and two fragmented nuclei in well #6, and one intact nucleus and one fragmented nucleus in microwell #7. No PI penetrated the cell membranes indicating a mixture of apoptotic and live cells, as identified in Fig. 8.

Fig. 9 shows the oxygen consumption results of the first experiment. The oxygen concentration of the media in microwell #6 decreased in oxygen concentration from 6.2 to 0.0 ppm in approximately 9 min resulting in a consumption rate of 0.69 ppm/min for three live cells or an average of 0.23 ppm/min/cell. Each microwell is 50 μ m deep and 150 μ m in diameter resulting in a volume of 0.88 nL assuming a perfect cylinder. Optical profile images have shown that the bottom edges of the microwells are not square but instead curve due to the etching process reducing the volume by approximately 15% to 0.75 nL. Using this volume and the molecular weight of oxygen of 32 g/mol to convert from parts per million, the resulting average consumption rate for the three live cells in microwell #6 is 5.39 fmol/min/cell.

 TABLE I

 Average Oxygen Consumption Rate Comparison

Research Group	Average Oxygen Consumption Rate
Ahmad et al.	3.2 fmol/min/cell
Gardner et al.	4.8-5.7 fmol/min/cell
Molter et al.	5.39 and 5.27 fmol/min/cell

The remaining microwells showed a minimal decrease in oxygen concentration over time. The slight negative slope probably indicates a small leak in all microwell seals. One might expect to see well #7 have a decrease in oxygen, but even though that particular cell seems to be living as indicated by the dyes, it is possible that it has entered an early stage of apoptosis before DNA fragmentation started but after oxygen consumption has ceased. The high variance of sensor #9 is attributed to low emission intensity (low signal-to-noise ratio) due to poor sensor deposition in that particular well.

The microwell array used for the second experiment appeared to contain one intact nucleus in microwell #1, one intact nucleus in microwell #7, and zero cells in the remaining microwells. Again, no PI penetrated the cell membranes indicating that both cells were most likely live cells. The oxygen concentration of the media in microwell #7 decreased from 7.5 to 3.0 ppm over 20 min resulting in a consumption rate of 0.225 ppm/min/cell or 5.27 fmol/min/cell. Microwell #1 reported no significant decrease in oxygen besides the negligible negative slope comparable to the empty wells.

The two proof-of-concept experimental results are now compared with published bulk oxygen consumption rates of the same A549 human epithelial lung cancer cells. Ahmad *et al.* [35] reports a bulk consumption rate of 32 nmol/min/10 million cells, which gives an average respiration rate of 3.2 fmol/min/cell. Gardner *et al.* [36] reports a bulk consumption rate of 48–57 nmol/min/10 million cells giving an average respiration rate of 4.8–5.7 fmol/min/cell.

Table I summarizes the experimental results from Ahmad, Gardner, and the MLSC. The closely matching estimation of the oxygen consumption rate of a single A549 cell compared with published rates gives us confidence that we have reached an important developmental milestone on our path towards robust single-cell investigations.

C. System Constraints

The proof-of-concept experiments presented in this paper highlight a successful architecture for measurement of oxygen metabolism at rates in the fmol/min range. While the automation, image processing, and calibration components of this system are robust, some mechanical issues need to be improved upon to enable the technical reproducibility necessary for vigorous biological experiments.

The three main mechanical bottlenecks, which are close to being solved, are the flexibility of the plastic well that the chip rests on, the lid actuator device, and the material used for the oxygen barrier. The first experiment was forced to end after 14 min because of a broken chip, as seen in Fig. 8(a). The flexibility of the bottom of the wells in the plastic 24-well plate permits the chip to bend and break. A new single-well plate is being developed with a rigid quartz window for the chip to rest on to prevent this from happening.

A new lid actuator is being designed to press the oxygen barrier down onto the microwell array with more control and perpendicularity. A load cell placed in the force axis will allow for real-time measurements of the force being applied for accurate force control and repeatability. In addition, several other oxygen barrier materials are currently being tested to identify a more reliable lid needed to further increase experiment efficiency.

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