Light-addressable measurements of cellular oxygen consumption rates in microwell arrays based on phase-based phosphorescence lifetime detection

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A digital light modulation system that utilizes a modified commercial digital micromirror device (DMD) projector, which is equipped with a UV light-emitting diode as a light modulation source, has been developed to spatially direct excited light toward a microwell array device to detect the oxygen consumption rate (OCR) of single cells via phase-based phosphorescence lifetime detection. The microwell array device is composed of a combination of two components: an array of glass microwells containing Pt(II) octaethylporphine (PtOEP) as the oxygen-sensitive luminescent layer and a microfluidic module with pneumatically actuated glass lids set above the microwells to controllably seal the microwells of interest. By controlling the illumination pattern on the DMD, the modulated excitation light can be spatially projected to only excite the sealed microwell for cellular OCR measurements. The OCR of baby hamster kidney-21 fibroblast cells cultivated on the PtOEP layer within a sealed microwell has been successfully measured at $104 \pm 2.96 \text{ amol s}^{-1} \text{ cell}^{-1}$. Repeatable and consistent measurements indicate that the oxygen measurements did not adversely affect the physiological state of the measured cells. The OCR of the cells exhibited a good linear relationship with the diameter of the microwells, ranging from 400 to 1000 μm and containing approximately 480 to 1200 cells within a microwell. In addition, the OCR variation of single cells in situ infected by Dengue virus with a different multiplicity of infection was also successfully measured in real-time. This proposed platform provides the potential for a wide range of biological applications in cell-based biosensing, toxicology, and drug discovery. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4772604>]

I. INTRODUCTION

In cellular assays and bioreactors, the rapid determination of cell viability is frequently accomplished by monitoring cellular metabolic activity via oxygen consumption. Monitoring cellular oxygen consumption provides useful information when studying critical biochemical pathways, including mitochondrial function, apoptosis, metabolic alterations caused by various stimuli or diseases, and toxicological responses to various compounds.^{1,2} To measure the oxygen consumption rates (OCR) of single cells, some methodologies have been developed either using amperometric electrochemical sensing or luminescent optical sensing. Amperometric sensing is typically manifested as external Clark-type electrode sensors, which are capable of monitoring cellular oxygen consumption at the single-cell level.^{3,4} However, these sensors are

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susceptible to membrane fouling, significant drift, analyte depletion, and the difficulty in positioning the electrode sensors in very close proximity to the cell. Additionally, amperometric sensing does not quantitatively yield the number of oxygen molecules consumed, but rather, the flux near the single cells.

Alternatively, luminescent optical sensing, which is based on the ability of molecular oxygen to dynamically quench oxygen-sensitive photoluminescent dyes, offers a fast linear response and immunity to drift from the consumption of O_2 .^{5–16} Quantification of oxygen concentrations with oxygen-sensitive photoluminescent dyes is measured by either the degree of luminescent intensity quenching^{5–9} or the luminescent lifetime.^{10–16} Detecting the luminescent lifetime to quantify oxygen concentrations has been proven to have a higher sensitivity due to the inherent stability of the signal. Thorsen *et al.* proposed a low-cost platform based on phase-domain lifetime detection using a modulated light-emitting diode (LED)-based optical excitation and a detection system for real-time sensing of aerobic and anaerobic bacteria.¹⁶ However, this platform is unable to direct the modulated light to only excite the region of interest to detect the local O_2 concentration. A movable stage is needed to control either the position of the microfluidic device or the modulated LED light source. Instead, a well-developed digital micromirror device (DMD) is a suitable excitation light source to exploit the spatial light modulation ability of the DMD techniques. Meldrum *et al.* proposed a digital light modulation microscope that utilizes a DMD equipped with an epifluorescence microscope to modulate the excitation light in the spatial and temporal domains to detect the phosphorescence lifetime.¹⁷ This system can address light to each microsensor element and utilize the rapid switching capability of the DMD to generate high-speed light pulses ($\sim 15 \mu s$) to excite phosphorescence to detect the local O_2 concentration through time-domain lifetime measurements. However, the time-domain lifetime measurement inevitably requires high-speed (~ 10 MHz) data acquisition hardware to directly measure the luminescent decay time.

To quantitatively measure the OCR of single cells, instead of the flux, the isolation of single cells in a closed chamber is necessary during O_2 measurement. Multiwell plates or microwell devices have been developed for noninvasively measuring the OCR based on luminescent optical sensing and by diffusionally isolating single cells in a small chamber volume. Microplate-based respirometry was performed by creating a small, temporary chamber volume around the cells within a well by lowering a piston-like probe into the well to amplify the changes of oxygen consumption.¹⁸ This measurement volume is not completely isolated from the environment because the piston does not fully enclose the temporary microchamber and the polystyrene plate material is not impermeable to O_2 . Atmospheric O_2 leaks into the measurement volume, leading to an underestimation of the biological oxygen consumption rate. As a result, a correction method is needed to compensate for this underestimation for the measured O_2 in this open or semi-closed respirometer chambers. Instead, Molter *et al.* proposed a microwell device for measuring oxygen consumption rates where a glass lid attached to the end of a piston is manually pressed onto a single microwell array to diffusionally isolate the single cells in an array of ~ 80 pl microwells using luminescent oxygen sensors.^{13,14} The lid blocks any diffusion of oxygen into or out of the microwell containing the cells. However, the manual mechanical actuator required to diffusionally isolate the microwells is massive, complicated, and not conducive for miniaturization into a lab-on-a-chip device. The manual mechanical actuator is also not well-suited for multiple, high-throughput measurements. Additionally, the lid is not able to controllably seal the respective microwell of interest.

In this study, we proposed a digital light modulation system that utilizes a modified commercial DMD projector equipped with a UV LED as a light modulation source to modulate the excitation light in the spatial and temporal domains. Phase-based phosphorescence lifetime detection was selected as the preferred detection scheme for cellular OCR measurements. The phosphorescence lifetime was calculated by measuring the phase shift between the reference (modulated excitation light) and corresponding phosphorescent signals to directly measure the luminescent decay time without the need for high-speed data acquisition hardware or complicated and expensive facilities. An array of glass microwells was deposited with Pt(II) octaethylporphine (PtOEP) as the oxygen-sensitive luminescent layer. Single cells were cultivated on the

PtOEP layer within a sealed microwell by pneumatically actuated glass lids set above the microwells to controllably seal the microwells of interest. The automated, pneumatically actuated glass lids allow for multiple, long-term measurements and are able to controllably seal the respective microwell of interest. By controlling the illumination pattern on the DMD, the modulated excitation light can be spatially projected to only excite the sealed microwell for cellular OCR measurements. To examine the potential of our proposed system for rapid determination of cellular metabolic activity via monitoring the OCR, the OCR variation of the single cells in situ infected by Dengue virus with a different multiplicity of infection (MOI) was also successfully measured in real-time.

II. MATERIALS AND METHODS

A. Principle of operation

The microwell array device consists of a combination of two components: an array of glass microwells deposited with PtOEP as the oxygen-sensitive luminescent layer and a microfluidic module with pneumatically actuated lids set above the microwells to controllably seal the microwells of interest, as shown in Fig. 1. A glass chip with 2×2 arrays of etched microwells is seeded with a suitable solution of cells yielding a cell population monolayer adhered to the PtOEP layer within each microwell as a proof of concept. After cell seeding, a microfluidic module with pneumatically actuated lids was set above the microwells to controllably seal the microwells of interest. High pressure air is used to press a rounded glass lid attached to the end of a piston onto a single microwell to seal the microwell of interest (Fig. 1). The lid blocks any diffusion of oxygen into or out of the microwell containing the cells. 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. The sealed microwell forms a small temporary chamber volume around the cells, which enables the rapid, real-time measurement of changes in the oxygen concentration. An air-pressure system, as described in our previous work,¹⁹ was used to pneumatically actuate the glass lids to controllably seal the microwells of interest. A regulated compressed-air source was connected to multiple three-way solenoid valves (Lee Inc., United States), where each valve is controlled by a

FIG. 1. A schematic of a microwell array device to detect oxygen consumption rates of single cells via phase-based phosphorescence lifetime detection. The microwell array device consists of a combination of components: an array of glass microwells deposited with PtOEP as the oxygen-sensitive luminescent layer and a microfluidic module with pneumatically actuated lids set above the microwells to controllably seal the microwells of interest. The phase-based phosphorescence lifetime detection system utilizes a modified commercial DMD projector, which is equipped with a UV LED as a light modulation source. This system was developed to spatially direct excitation light toward a microwell array device to detect local O_2 . The long-term cellular OCR measurement was repeated over time by a periodic three-stage operation for replenishing the fresh medium into the microwell, entrapment of the chamber volume, and measurement of the oxygen concentration.

custom LabVIEW program (National Instruments, Inc.) to switch rapidly between atmospheric and input pressure. After switching the three-way solenoid valve to atmospheric pressure, the glass lids were raised within 1 s to open the microwell to replenish the fresh surrounding medium into the microwell.

The OCR measurement of the cells cultivated on the PtOEP layer of the sealed microwells was performed by tracking the oxygen concentration of the cell medium over time via phase-based phosphorescence lifetime detection. To this end, we set up a digital light modulation system utilizing a modified commercial DMD projector equipped with a UV LED as a light modulation source to modulate the excitation light in the spatial and temporal domains toward a microwell array device to detect the OCR via phase-based phosphorescence lifetime detection. The phosphorescence lifetime was calculated by measuring the phase shift between the reference (modulated excitation light) and the corresponding phosphorescent signals to directly measure the luminescent decay time without the need for high-speed data acquisition hardware or complicated and expensive facilities. The relationship between the oxygen concentration, phase shift (θ) and the lifetime (τ) is described in Sec. II C. By controlling the illumination pattern on the DMD, the modulated excitation light can be spatially projected to only excite the sealed microwell for cellular OCR measurements.

To enable repeatable and long-term OCR measurements, each OCR measurement of the single cells was performed with a three-stage operation. In the first stage, denoted as O-stage, we raised the lid to unseal the microwell for a few minutes by releasing the air pressure in the microfluidic module to replenish the fresh surrounding medium into the microwell to re-equilibrate and restore cells to normal status. In the second stage, denoted as S-stage, we lowered the lid to seal the microwell by applying the air pressure and waiting for a few minutes for the cells to stabilize before performing the OCR measurement. In the third stage, denoted as M-stage, we performed the OCR measurement for a preset period via phase-based phosphorescence lifetime detection. The long-term cellular OCR measurement was repeated over time by the periodic three-stage operation of replenishing fresh medium into the microwell, entrapment of the chamber volume, and measurement of oxygen concentration. The time for each stage consisted of 30 s for O-stage, 150 s for S-stage, and 180 s for M-stage, unless otherwise stated.

B. Fabrication of the microwell array device

The microwell array device consists of a combination of two components: an array of glass microwells with PtOEP deposited in them and a microfluidic module with pneumatically actuated lids set above the microwells to controllably seal the microwells of interest. Figure 2 shows the exploded drawing and images of the microwell array device. The microwell array device was assembled using three layers (layers 1 to 3) of PDMS structures to serve as a microfluidic module and one layer (layer 4) of a glass substrate with 2×2 microwells within a polydimethylsiloxane (PDMS) microchamber. The glass microwells were chosen because their low diffusivity to oxygen increases the sensor stability and sensitivity. A 2×2 glass microwell of 1 mm in diameter and 50 μm deep was etched into soda-lime glass substrates, as shown in Figs. 2(b) and 2(c), using a fast, low-cost, and reliable process.²⁰ A thin layer of AZ 4620 positive photoresist (PR) was used for buffered oxide etching (BOE) of the soda-lime glass. A novel two-step baking process prolongs the survival time of the PR mask in the etchant, which avoids serious peeling problems associated with the PR. The glass chip was repeatedly dipped into a 1 M hydrochloride solution to remove any precipitated particles generated during the etching process to improve the surface roughness of the microwells. In this study, we fabricated 2×2 glass microwells with 0.4, 0.6, 0.8, and 1 mm diameters, 50 μm deep, and spaced 0.4, 0.6, or 1 mm apart.

Platinum octaethylporphyrin (PtOEP, $\lambda_{\text{ex}} = 381 \text{ nm}$, $\lambda_{\text{em}} = 646 \text{ nm}$, from Aldrich, USA) was used as the oxygen-sensitive luminescent layer and deposited into the microwells to monitor the oxygen concentration within each microwell. PtOEP displays strong room-temperature phosphorescence with high quantum yields and long lifetimes (ca. 100 μs).⁵ First, a 7 wt. % solution

FIG. 2. (a) The expanded drawing and (b) the image of the microwell array device, which was assembled by three layers (layers 1 to 3) of PDMS structures serving as a microfluidic module and one layer (layer 4) of a glass substrate with 2×2 microwells within a PDMS microchamber. (c) The SEM image of the glass microwell with a 1 mm diameter and $50 \mu\text{m}$ depth, which was etched into the soda-lime glass substrate.

of polystyrene (PS, average MW of 280 000, from Aldrich, USA) dissolved in toluene and containing PtOEP at a concentration of 180 mm was prepared and spin-coated on the pre-etching glass substrate at 1000 rpm for 30 s. The spin-coated films were dried at room temperature and stored in the dark prior to use. The PtOEP film on the surface of the glass substrate outside the microwells can be easily scraped and removed with a scalpel. The PtOEP film thickness on the bottom surface of the microwells was approximately $1 \mu\text{m}$. Before cell seeding, the PtOEP films within the microwells were treated with O_2 plasma through a shadow mask to increase hydrophilicity of the PtOEP layers. This process can prompt the attachment of cells onto the PtOEP surface and not on the surface of the glass substrates.

The microfluidic module with pneumatically actuated glass lids was assembled using the 3 layers (layer 1 to 3) of PDMS structures, as shown in Figs. 2(a) and 2(b). The PDMS structure with microfluidic channels in layer 1, which provide an entrance for fluid and high pressure air, was fabricated using standard soft lithography. An SU-8 mold (MicroChem, USA) was made on a silicon wafer using standard lithography techniques. The microchannels were $100 \mu\text{m}$ in height and $200 \mu\text{m}$ in width. Layer 2 was composed of a PDMS slab of 6 mm in thickness and was punched with six $500\text{-}\mu\text{m}$ diameter holes, corresponding to the six holes in layer 1. Layers 1 and 2 were precisely aligned and then permanently bonded after the surface treatment with oxygen plasma. Hence, the aqueous solution (denoted with a blue dashed line) and air pressure (denoted with a red dashed line) can be introduced from layer 1 to layer 2 via six holes. The PDMS structure with 2×2 microposts of 5 mm in diameter used in layer 3 was fabricated and cast from the SU-8 mold using standard soft lithography. The PDMS pre-polymer was first spun and cured onto the SU-8 master at 1000 rpm for 30 s to form a PDMS membrane $100 \mu\text{m}$ thick. Before removing the structure from the mold, the slab of PDMS structures containing layers 1 and 2 was precisely aligned and bonded onto layer 3 after the surface treatment with oxygen plasma. After carefully removing the structure from the mold, glass lids of 8 mm in diameter were manually adhered to the microposts using PDMS pre-polymer as the glue, as shown in Fig. 2(b). Finally, the glass substrate with 2×2 microwells (layer 4) was adhered to the microfluidic module using double-side adhesives for ease of operation. The glass substrate with 2×2 microwells was disposable, but the microfluidic module was able to be reused for each measurement.

C. DMD-based light modulation system for phase-based lifetime detection

A schematic of the digital light modulation system for phase-based phosphorescence lifetime detection is shown in Fig. 3(a). Detailed descriptions of the facility setup, data acquisition, and the phase-based phosphorescence lifetime detection have been reported in our previous work.²¹ The digital light modulation system utilizes a modified commercial DMD projector (BENQ PB6240) equipped with a 15° narrow-angle UV LED (390 nm, 3 W, EDISON OPTO)

FIG. 3. (a) A schematic of the digital light modulation system utilizing a modified commercial DMD projector equipped with a UV LED as the light modulation source to spatially direct the excitation light toward the sealed microwell of interest to detect local oxygen via phase-based phosphorescence lifetime detection. (L1: built-in condensing lens; L2: projection lens; L3, L4: focus lenses; BS: beam splitter; RS: reference signal; DS: detection signal; APD: amplified photo-detector; and PMT: photo-multiplier tube) (b) The phosphorescent images of the 2×2 microwells that were excited by sequentially addressing the modulated UV light to each microwell to detect local oxygen via phase-based phosphorescence lifetime detection. The inserts on the lower-left of all the images indicate the pattern on the DMD (the white areas represent the switched-on micro-mirrors).

as the light modulation source to spatially direct the excitation light toward the sealed microwell of interest to detect local oxygen via phase-based phosphorescence lifetime detection. The structured light patterns of the DMD were controlled by a computer. The reference signal (RS) was recorded by measuring the light intensity of the modulated excitation light after passing through the DMD using an amplified photo-detector (ET-2030A, Electro-Optics Technology, Inc.) via a 50/50 beam splitter (BS). The detection signal (DS) was recorded by simultaneously measuring the light intensity of the corresponding phosphorescence with a photo-multiplier tube (PMT-R928, Hamamatsu) and a cooled CCD camera (CoolSNAP HQ², Photometrics) in real time along with the reference LED signal. The reference LED signal (RS) and the phosphorescent detection signal (DS) were both recorded on a computer via a USB DAQ card (USB-6251, National Instruments) and then followed by a signal conditioning process. The excitation LED was modulated at 5 kHz, which enabled phase-based lifetime detection and low-frequency filtering to be performed with the signal conditioning circuitry to subtract any dc interference signal that resulted from ambient lighting. The whole projected area on the glass substrate is about 6 mm × 5 mm through 10× objective lens in our developed DMD-based light modulation system. To achieve acceptable phosphorescent signals for cellular OCR measurements, the minimum excitation area is about 0.2 mm × 0.2 mm by controlling the illumination pattern on the DMD to only excite the sealed microwell for cellular OCR measurements.

The phosphorescence lifetime was calculated by measuring the phase shift (θ) between the reference LED signal (modulated excitation light) and the phosphorescent detection signal to calculate the luminescent lifetime (τ) via phase-based phosphorescence lifetime detection. The relationship between the phase shift (θ) and the lifetime (τ) can be approximated by the following¹⁵

$$\tan(\theta) = 2\pi\nu\tau, \quad (1)$$

where ν is the modulation frequency. In this study, a modulation frequency of $\nu = 5$ kHz was used to measure the luminescent decay time for phase-based phosphorescence lifetime detection. The phase shift (θ) between the reference and detected signals was determined by digital lock-in analysis, as described in our previous work.²¹

Although the field of view of the DMD-based light modulation system covers multiple microwells, the modulated excitation light can be spatially projected to only excite the microwells of interest by controlling the illumination pattern on the DMD. Figure 3(b) shows phosphorescent images of the 2 × 2 microwells with a feature size of 600 μm in diameter that was excited by sequentially addressing modulated UV light to each microwell to detect local oxygen via phase-based phosphorescence lifetime detection. The inserts on the lower-left of all the images indicate the pattern on the DMD (the white areas represent the switched-on micro-mirrors). The upper left image in Fig. 3(b) shows a phosphorescent image of the 2 × 2 microwells when the illumination pattern was spatially projected to simultaneously excite the four individual PtOEP sensing layers within the microwells. The other images in Fig. 3(b) sequentially show the phosphorescent images when the illumination pattern is spatially projected to only excite the microwells of interest by controlling the illumination pattern on the DMD. The results demonstrated the ability of the proposed DMD-based light modulation system to independently measure the local O₂ concentration by sequentially addressing an individual microwell. The non-uniform intensity distribution in Fig. 3(b) is derived from the heterogeneity in the thickness or distribution of the dye-supporting matrix. However, we adopted the phase-based phosphorescence lifetime detection to quantify oxygen concentrations. The luminescent lifetime is an intrinsic property of the oxygen-sensitive luminescent dye and is therefore not susceptible to variations due to the intensity of the incident light or the heterogeneity in the thickness or distribution of the dye-supporting matrix.

D. Stern–Volmer calibration curve

Deionized (DI) water was placed into a sealed reservoir and bubbled with N₂ and O₂ gas for a minimum of 10 min to provide 0% and 100% oxygen references. The dissolved oxygen

(DO) concentration in the oxygen-saturated water was calculated to be approximately 20 ppm, which is equivalent to 20 mg/l in an aqueous solution. The oxygen level in the reservoir was continuously monitored by Clarke microelectrode sensors (DO-5510, LUTRON). The pure solutions of oxygenated and deoxygenated water and calibrated mixture were withdrawn from the reservoir by using a gas-tight glass syringe. The solutions were then introduced into the device, the glass lid was pushed down to seal the microwell, and calibration tests were performed immediately. Calibration tests on the phase shift (θ) versus the dissolved oxygen concentration were performed by introducing the device with DO concentrations of 0, 4, 8, 15, and 20 mg/l. Figure 4(a) shows the typical time domain of the reference LED signal (modulated excitation light) and the corresponding phosphorescent detection signal. A phase shift (θ) was clearly observed between the reference LED signal and the corresponding detection signal.

For the 5 kHz excitation and emission signals, the phase shift (θ) was determined by the digital lock-in detection in the LabVIEW module and then used to calculate the luminescent lifetime (τ), according to Eq. (1). The luminescent lifetime (τ) related to the DO concentration is described by the Stern–Volmer relationship as follows:

FIG. 4. (a) The typical time domain of the reference LED signal (modulated excitation light) and the corresponding phosphorescent detection signal. (b) Variation of the luminescent lifetime (τ) and the corresponding Stern–Volmer calibration curve of the normalized lifetime (τ_0/τ) as a function of the DO concentration, which was measured by a modulated excitation light at 5 kHz.

$$\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + K_{SV}^S [O_2], \quad (2)$$

where I is the intensity, τ is the lifetime, K_{SV}^S is the Stern–Volmer constant for the solution, I_0 and τ_0 are the reference values in the absence of oxygen, and $[O_2]$ is the oxygen concentration in solution. Figure 4(b) shows the variation of the luminescent lifetime (τ) and the corresponding Stern–Volmer calibration curve of the normalized lifetime (τ_0/τ) as a function of the DO concentration measured at a modulated excitation light at 5 kHz. In our Stern–Volmer calibration curve, a nearly linear relationship was observed between the normalized lifetime (τ_0/τ) and the DO concentration. The ratio I_0/I_{100} or τ_0/τ_{100} in conjunction with the Stern–Volmer constant is commonly used as an indicator of the sensitivity of the sensing film.⁶ The sensitivity (τ_0/τ_{100}) was 8.06 in our Stern–Volmer calibration curve; sensors with I_0/I_{100} or τ_0/τ_{100} ratios larger than 3.0 are considered to be suitable for use in oxygen-sensing devices.

E. Cell culture and Dengue virus type II (DV2)

Baby hamster kidney-21 fibroblast cells (BHK-21) were chosen to demonstrate the cellular OCR measurement and potential biological applications in this study, due to its higher oxygen consumption and susceptibility to be infected by Dengue virus type II (DV2). The BHK-21 cells were cultured in 75-cm² tissue culture Petri dishes at 37 °C in a humidified atmosphere of 5% CO₂/95% air. The BHK-21 cells were incubated in RPMI (Medium 1640 (1X) liquid), which was supplemented with 5% bovine calf serum, 200 U/ml of penicillin, and 200 µg/ml of streptomycin. The cells were grown to preconfluence and passaged by trypsinization in a 0.5% trypsin/0.01% EDTA solution in phosphate buffered saline (PBS) for 5 min at 37 °C. The cell suspension was diluted (1:4) with BHK-21 culture medium and centrifuged at 800 rpm for 5 min. After aspiration of the supernatant, the cells were reconstituted in a fresh BHK-21 culture medium and counted using a hemocytometer. The viability of the BHK-21 cells was typically better than 95%, as determined by trypan blue exclusion. The chemicals used in the cell cultures were purchased from GIBCO Inc.

A local isolate (PL046) of Dengue virus type II (DV2) was supplied by the Institute of Preventive Medicine, Nan Kung, Taipei, Taiwan (Republic of China). The PL046 strains of Dengue virus type II (DV2) were maintained in suckling mouse brains for preparation of virus stocks and further experiments at the Laboratory Animal Facility, College of Life Sciences, National Taiwan Ocean University, Keelung, Taiwan.²²

III. EXPERIMENTAL RESULTS AND DISCUSSION

A. Confirmation of the sealed microwells

To verify the impermeability of the sealed microwells from the surroundings, we first introduced the 0% O₂ water and sealed the left-side microwell using the actuated lid, as shown in Fig. 5. The normal water (ambient oxygen concentration) was then introduced into the chamber without sealing the right-side microwell. The normal water served as the surrounding water to observe any change in the oxygen of the sealed microwells. The image in Fig. 5(a) indicates that the left-side sealed microwell with 0% O₂ water showed a higher phosphorescent intensity than that of the right-side microwell with normal water. By controlling the illumination pattern on the DMD, we can spatially direct the modulated excitation light to, respectively, excite the sealed or unsealed microwell for local oxygen concentration detection. As shown in Fig. 5(b), the luminescent lifetime (τ) for the sealed microwell with 0% O₂ water remained roughly constant at $\tau = 48.5 \pm 0.29$ µs over 12 min, but the luminescent lifetime (τ) for the unsealed microwell with an ambient oxygen concentration was $\tau = 16.7 \pm 0.19$ µs. This result verified the impermeability of the sealed microwells from the surroundings because no change was observed in the oxygen concentration of the sealed microwells.

FIG. 5. (a) A testing schematic and phosphorescent image of the sealed and unsealed microwells for impermeability confirmation by first introducing 0% O₂ water and sealing the left-side of the microwell and then introducing normal water (ambient oxygen concentration) into the chamber without sealing the right-side microwell. (b) The time variation of the luminescent lifetime (τ) for the sealed microwell with 0% O₂ water and unsealed microwells with ambient oxygen concentration.

B. OCR of single cells

To demonstrate the utility of the microwell array device, the BHK-21 cells were used to experimentally determine the OCR of single cells. Before cell seeding, 2×2 glass microwells of $600 \mu\text{m}$ in diameter were well cleaned and treated with O₂ plasma through a shadow mask to increase hydrophilicity of the PtOEP layer within the microwells. This process can prompt

the attachment of cells onto the PtOEP surface and not on the surface of the glass substrates. A volume of 1 ml of the BHK-21 cell suspension with approximately 10 000 living cells in Dulbecco's modified eagle medium (DMEM) without antibiotics was loaded in the microwells. The cells were incubated at 37 °C, in 5% CO₂ for 1 h to allow them to settle and attach to the bottom of the microwells. PtOEP cytotoxicity for cell viability was determined using a live/dead assay (Invitrogen, CA) containing calcein acetoxymethyl (AM) (live cells, green) and ethidium homodimer (dead cells, red). A typical image of a microwell containing live/dead stained BHK-21 cells obtained with a fluorescent microscope is shown in Fig. 6(a). The BHK-21 cells were successfully seeded onto the PtOEP layer and cultivated with normal growth. The result indicated that the PtOEP layer exhibited low toxicity and can be used to directly contact cells for short-term cell culture.

After seeding and culturing cells within the glass microwells, the microfluidic module was mounted onto the glass microwells and the cell media was introduced for the OCR measurements. A pneumatically actuated glass lid set above the 2 × 2 glass microwells was used to controllably seal the microwells for repeatable OCR measurements. The modulated excitation light was spatially projected to only excite one of the 2 × 2 glass microwells by controlling the illumination pattern on the DMD. Phase-based phosphorescence lifetime detection was performed to measure the aqueous oxygen concentration over time, as shown in Fig. 6(b). At the start of the oxygen consumption measurement, we raised the lid for 5 min to replenish the fresh surrounding medium into the microwell to re-equilibrate cells to normal status (O-stage). During the O stage, the aqueous oxygen concentration was maintained at an approximately constant value of $221 \pm 0.1 \mu\text{M}$ over time. The result is that the decreased oxygen, which was consumed by the BHK-21 cells within the microwells, was rapidly replenished by the surrounding medium, resulting in a roughly constant oxygen concentration. The slight variation in oxygen concentration consumed by cells was not measurable in an open large chamber with the small quantity of cells within the microwell.

The lowering of the lid to create a small, sealed, and temporary chamber volume in the microwell was performed during the S-stage. After waiting for a few minutes for the cells to stabilize, we performed the phase-based phosphorescence lifetime detection for 180 s to measure the aqueous oxygen concentration (M-stage). The aqueous oxygen concentration gradually decreased with time during the M-stage. The slight variation in oxygen concentration consumed by the cells was successfully measured by diffusionally isolating single cells in a small, temporary chamber volume around the cells to amplify the changes in [O₂] during the measurement. Using the microfluidic module to controllably seal the microwells, we successively performed three measurements by periodic sealing of the 2 × 2 glass microwells. Each measurement period included the three-stage operation for replenishing the fresh medium into the microwell (O-stage), entrapment of the chamber volume (S-stage), and measurement of the oxygen concentration (M-stage).

To calculate transient changes in the oxygen consumption rate (OCR(t), $-\text{d}[\text{O}_2]/\text{dt}$), time-based differentiation was used to calculate $-\text{d}[\text{O}_2]/\text{dt}$ from the measured [O₂] time lapse data shown in Fig. 6(b). The measured [O₂] time lapse data were filtered using a 7 point wide, second order polynomial, first order derivative Savitzky-Golay kernel (0.107, 0.071, 0.036, 0, -0.036, -0.071, -0.107).¹⁸ This method provides additional smoothing of the calculated differentiated signal to alleviate the amplification of data noises during the calculation of the $-\text{d}[\text{O}_2]/\text{dt}$ data. Figure 6(c) shows the oxygen consumption rate of the single cells over time (OCR(t)) within the microwells for the successive three measurements by periodic sealing of the 2 × 2 glass microwells. For the unsealed microwells (O-stage), the OCR(t) maintains an approximate zero value of about $0.03 \pm 0.025 \text{ pmol/min/well}$ due to the invariant oxygen concentration, as shown in Fig. 6(b). The OCR(t) of the single cells was not able to be measured for the unsealed microwells. In contrast, the OCR(t) were 13.5 ± 0.24 , 13.8 ± 0.38 , and $12.8 \pm 0.25 \text{ pmol/min/well}$ for the successive three measurements by periodic sealing of the microwells. The mean oxygen consumption rate calculated for each measurement period did not decay significantly over the repeated measurements, indicating that the respiration of the BHK-21 cells cultivated on the PtOEP layer within a sealed microwell is constant over time. Repeatable and consistent measurements indicated that the oxygen measurements did not adversely

FIG. 6. (a) Typical images of a microwell containing live/dead stained BHK-21 cells. The time variation of the oxygen concentration $[O_2]$ (b) and the oxygen consumption rate of single cells ($OCR(t)$, $-d[O_2]/dt$) (c) within the microwells for the successive three measurements by periodic sealing of the 2×2 glass microwells.

affect the physiological state of the cells measured. The results also indicated that the glass microwells provide low diffusivity of oxygen from the atmosphere and walls into the chamber and increased the stability and sensitivity of the OCR measurements. The findings also confirm the impermeability of the sealed microwells using the pneumatically actuated glass lids set above the microwells through our proposed microfluidic module. No correction method is needed to compensate for the underestimation of the OCR due to O_2 diffusion from the atmosphere and walls into the chamber for open or semiclosed respirometer chambers.

C. Correlation of the OCR with cell number

To verify the correlation of the OCR with the cell number, we performed the OCR measurements on the BHK-21 cells cultured within microwells with different diameters ranging from 400 to 1000 μm . The BHK-21 cells have the tendency to form a cell attachment monolayer after 1 h of incubation and flushing away suspending cells.²² A decrease in the microwell diameter results in a decrease in the number of attached cells within the microwells. Figure 7 shows the dependence of the OCR (pmol min^{-1}) of the single cells and a single-cell OCR ($\text{amol s}^{-1} \text{cell}^{-1}$) with the diameter of the microwells. With an increase in cell number, the rate of oxygen consumption by the cells would increase correspondingly. Indeed, the OCR of the BHK-21 cells exhibited a good linear relationship with the diameter of the microwells ranging from 400 to 1000 μm , which contained approximately 480 to 1200 cells within a microwell. To calculate the single-cell OCR ($\text{amol s}^{-1} \text{cell}^{-1}$) within the different diameters of the microwells, we divided the OCR by the cell number within a microwell. The single-cell OCR showed a roughly constant value of approximately $104 \pm 2.96 \text{amol s}^{-1} \text{cell}^{-1}$ for the BHK-21 cells, which is close to the value of $83 \pm 0.02 \text{amol s}^{-1} \text{cell}^{-1}$ for the BHK-21 cells previously validated with a Clark electrode.^{23,24} This result is significant and suggests that our proposed system can be used to compare single-cell respiration rates to a large number of single cells in multiple arrays.

D. OCR variation of the BHK-21 cells infected by Dengue virus

To examine the potential of our proposed system in the rapid determination of cellular metabolic activity caused by diseases or toxicological compounds via monitoring the OCR,

FIG. 7. The dependence of the OCR (pmol min^{-1}) of the single cells and a single-cell OCR ($\text{amol s}^{-1} \text{cell}^{-1}$) with the diameters of the microwells.

simple tests were performed using BHK-21 cells in situ infected by Dengue virus with a different MOI. The MOI is a ratio defined by the number of infectious virus particles deposited in a well divided by the number of target cells present in that well. BHK-21 cells were chosen due to their high susceptibility to be infected by Dengue virus.²² After the BHK-21 cells were cultured on the PtOEP layer within the 2×2 glass microwells of 1 mm in diameter, Dengue virus (MOI = 0.1, 0.5, and 1) was added to the microwells and incubated at 37°C for 1 h with gentle shaking every 15 min during the virus infection process. After incubation, the unabsorbed virus was removed, the cells were subjected to PBS wash, and the microfluidic module was mounted onto the glass microwells and used to introduce cell media for the OCR measurements.

Figure 8 shows the time variation of the normalized oxygen consumption rate ($OCR_{(t)}/OCR_{(t=0)}$, %) of the BHK-21 cells infected by Dengue virus with a different MOI within the microwells by periodic sealing of the microwells. The normalized oxygen consumption rate ($OCR_{(t)}/OCR_{(t=0)}$, %) was measured at a 30-min time interval. For the BHK-21 cells uninfected with Dengue virus (MOI = 0), the $OCR_{(t)}/OCR_{(t=0)}$ did not significantly vary for 13 h, indicating that the cells were at a normal physiological state during long-term and periodic OCR measurements within the sealed microwell. In contrast, for the BHK-21 cells infected with Dengue virus, the $OCR_{(t)}/OCR_{(t=0)}$ dramatically decreased from 100% to 0%, 28%, and 65% for MOI = 1, 0.5, and 0.1 after 8 h post-infection, respectively. Increasing the MOI means an increase in the possibility that the BHK-21 cells may be infected by Dengue virus. The infectious cells cause mitochondrial malfunction and decrease the cellular metabolic activity, which is reflected in the oxygen consumption rate. When more cells were infected by Dengue virus, the $OCR_{(t)}/OCR_{(t=0)}$ decreased over time, accordingly.

We also performed the standard MTT assay for cell viability of the BHK-21 cells incubated in the 96-well plates and infected by Dengue virus with MOI = 0.1, 0.5, and 1. The MTT assay is one of the most popular assays for assessing toxicity and measuring proliferation. However, the results did not show a notable decrease in cell viability for MOI = 1, 0.5, and 0.1 after 8 h post-infection. The cell viability for MOI = 1 decreased from 100% to 80% after 24 h post-

FIG. 8. The time variation of the normalized oxygen consumption rate ($OCR_{(t)}/OCR_{(t=0)}$, %) of the BHK-21 cells infected by Dengue virus with a different MOI within the microwells by periodic sealing of the microwells. The normalized oxygen consumption rate ($OCR_{(t)}/OCR_{(t=0)}$, %) was measured at a time interval of 30 min.

infection (data not shown). The standard MTT assay, which typically requires that the treated and control cells be incubated for 2–4 h with the MTT reagent before the absorbance measurement, can damage cells and cannot measure the cell viability to assess toxicity in real-time. MTT assay generally reflects the number of viable cells, which are used to measure cytotoxicity (loss of viable cells) of potential medicinal agents and toxic materials. MTT assay cannot reflect metabolic dysfunction of mitochondrion for the damaged cells. Even if these cells are damaged, MTT assay still reflects the cells alive, showing high cell viability. This is why the cell viability does not decrease notably after 8 h infection measured by the MTT assay. The cell viability can show a notable decrease of cell viability because a large number of the infected cells were eventually dead after 24 h infection. In contrast to the standard MTT assay, our proposed system can rapidly determine the cellular metabolic activity that is caused by diseases or toxicological compounds via monitoring of the OCR. Our proposed system could be also used to carry out time-course or dose-response measurements of cellular metabolic activity in rapid and real-time detection.

IV. CONCLUSIONS

The proposed microwell array device, which comprises an array of glass microwells and a microfluidic module with pneumatically actuated glass lids to controllably seal the microwells of interest, enables real-time monitoring of the single cell OCR via phase-based phosphorescence lifetime detection. The digital light modulation system utilizing a modified commercial DMD projector can control illumination patterns toward the microwell array device to monitor oxygen consumption in each microwell. Repeatable and consistent measurements indicate that the oxygen measurements did not adversely affect the physiological state of the cells measured. We also demonstrated variation in the real-time OCR for single cells that were in situ infected by Dengue virus at different MOI. In contrast to the standard MTT assay, our proposed system can rapidly determine the cellular metabolic activity that is caused by diseases or toxicological compounds via monitoring of the OCR. The next step is to incorporate additional capabilities to measure extra-cellular analytes such as the pH, Ca^{2+} , Mg^{2+} , and CO_2 . The proposed platform provides the potential for a wide range of biological applications in cell-based biosensing, toxicology, and drug discovery.

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