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A High-Throughput Photodynamic Therapy Screening Platform with On-Chip Control of Multiple Microenvironmental Factors

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Abstract

We present a novel high-throughput microfluidic platform that enables the evaluation of the anticancer efficacy of photodynamic therapy (PDT) drugs over multiple microenvironmental factors. PDT is uniquely complex, originating from its dependence on three separate but essential elements: drug (or also called photosensitizer), oxygen, and light. Thus, obtaining a reliable evaluation of PDT efficacy is highly challenging, requiring considerable effort and time to evaluate all three interdependent parameters. In this paper, we report a high-throughput efficacy screening platform that we implemented by developing microfluidic components that individually control basic PDT elements (photosensitizer concentrations, oxygen levels, and light fluence), and then integrating them into a single triple-layer device. The integrated microfluidic chip consists of an array of small compartment, each corresponding to a specific combination of these three variables. This allows for more than 1,000 different conditions being tested in parallel. Cancer cells are cultured within the device, exposed to different PDT conditions, and then monitored for their viability using live/dead fluorescence staining. The entire screening assay takes only 1 hour, and the collected PDT outcomes (cell viability) for combinatorial screening are analysed and reported as traditional dose-response curves or 3D bubble charts using a custom software. As a proof of concept, methylene blue is adopted as a photosensitizer and its drug efficacy on C6 glioma cells has been successfully evaluated for total 324 PDT conditions using the fabricated chip. This platform can facilitate not only the development of new photosensitizers but also the optimization of current PDT protocols.

Introduction

Photodynamic therapy (PDT) has recently emerged as a promising therapy modality for minimally invasive cancer treatment by selectively activating cytotoxicity of drugs in the target region of the body. The efficacy of PDT relies on three basic elements: drug (photosensitizer), oxygen, and light.^{1,2} The photosensitizer is administered to the patients, where it spreads throughout the body and/or accumulated in the tumor regions. Laser light with a wavelength that corresponds to the photosensitizer's maximum absorption range irradiates the cancerous region, resulting in excitation of photosensitizers only in the target area. The energetically excited photosensitizers transfer its energy to adjacent oxygen

molecules, generating high energy oxygen (singlet state oxygen) which in turn generates several cytotoxic reactive oxygen species. These species have a very short diffusion path. Thus the highly localized elevation of oxidative stress results in damage that is spatially confined to the cancer region being illuminated. PDT is either adopted as a palliative therapy or as the primary therapy, and has specific advantages over conventional radiotherapy, chemotherapy and surgical operations. Due to the highly localized effects, it is minimally invasive and has minimal dark toxicity. Additionally, it is a cost-effective treatment with a short recovery period.^{3–6} Besides the several photosensitizers already approved for clinical use in America, Asia and Europe, many candidates are currently under development, and the drive to improve the effectiveness of PDT and to expand its use to additional cancer treatments has become a major research focus.^{7–12}

Due to the complicated mechanism of PDT and its special dependence on oxygen and light, it is challenging to accurately evaluate photosensitizer's efficacy with controlled oxygen levels and light fluences (illumination doses). Oxygen deprivation is a common phenomenon in solid tumors and believed to increase resistance to PDT treatments. In conventional drug tests, the oxygen level can be reduced by controlled ventilation, which requires preparation of special gas mixtures and takes a long time to equilibrate.^{13–16} Effective light (photon) fluence at the tumor site directly affects singlet oxygen production, which in turn mediates the final damage on the cancerous cells. In the case of skin cancer, light can be directly delivered to the site, but often the light must pass through other tissue structures in typical PDT treatments of subcutaneous tumors. In conventional PDT efficacy tests, illumination is controlled by adjusting the light source itself, which usually requires a complicated optical system or manual operations.^{17–19} Moreover, the aforementioned conventional methods lack the capability of high throughput and consume lots of time, cost and labor.

Several technologies for high-throughput PDT efficacy tests have been developed to accelerate new photosensitizer discovery and optimization. 96-well microplates have been widely used to run drug tests under different treatment conditions (for example, drug concentration) in parallel and increase the throughput. Meerovich *et al.* reported a PDT light source composed of an arc xenon lamp and a bundle of optical fibers, each of which fits into a well of the 96-well microplate and provides a special fluence rate during PDT tests.²⁰ Butler *et al.* reported a similar PDT light source composed of 96-well LED arrays, by which each well's illumination wavelength and intensity can be independently controlled.²¹ Microfluidic technology is also being introduced into this application area, benefiting from its small device size, small sample and reagent consumption, better control in spatial and temporal domains. Jedrych *et al.* reported a PDMS/glass microfluidic system including two identical microstructures for testing two cell lines and photosensitizers in parallel and a concentration gradient generator for testing three photosensitizer concentrations simultaneously.²² Recently, Cho *et al.* reported a droplet-based microfluidic platform which can generate an enormous number of droplets encapsulating cells and photosensitizers for PDT efficacy tests.²³

Although these existing systems have made advances to meet the goal of high-throughput PDT efficacy assay, there are still a need to improve the performance and overcome some challenges to expand its usage in practical photosensitizer development. First, most of current systems still demonstrated a limited throughput of photosensitizer efficacy screening. Further expansion based on the multi-well microplates would dramatically increase labor consumption and require the assistance of automatic robotics, which is bulky and expensive. Droplet microfluidics provides an attractive option for quickly generating a large number of testing samples, but it is challenging to provide dynamic controls for device operation and perform the test of multiple conditions simultaneously. Second, the previous

systems screened only one or two microenvironmental factors at a time with the rest fixed; however, actually multiple factors are interactively affecting PDT efficacy and their compounding influences should also be looked into for photosensitizer development. Third, most systems partially rely on traditional methods/components for controlling PDT conditions, resulting in bulky setups, tedious interconnections, and limited temporal and spatial resolutions.

To overcome these challenges, we propose a highly compact microfluidic platform, based upon multi-layer soft lithography (MSL), which can provide a wide range of on-chip microenvironmental controls of photosensitizer concentrations, oxygen levels, and light fluence rates. Parameter gradients are automatically generated on-chip and readily applied to testing cells in a micron-scale resolution. The PDT illumination is provided by a small red LED light source of a quarter-size. High-throughput PDT efficacy tests for hundreds or even more than a thousand microenvironmental conditions are completed in a single run within an hour using a 24 mm by 30 mm chip. After test, fluorescence LIVE/DEAD staining of cells is performed with microscope imaging, and the resulting PDT efficacy responses to various photosensitizer concentrations, oxygen levels, and fluence can be acquired from single experiment.

As a proof of concept, high-throughput PDT efficacy tests on C6 glioma cells were conducted under various combinational conditions more than 320. This generic platform can be easily extended to a larger array and be used for rapid profiling of photosensitizer efficacy at high-throughput, including the development of new photosensitizer candidates as well as the optimization of conditions and operating procedures for the existing photosensitizers in clinical PDT treatment.

Materials and methods

Device design and fabrication

Figure 1 shows the schematic view of the proposed PDT screening chip. It is composed of three layers: (1) a gas layer (top) for spatial control of oxygen levels; (2) a cell layer (middle) for target cell culture and reagent exchange including drug delivery; (3) a filter layer (bottom) for spatial control of fluence rate, as illustrated in Fig. 1(a). The cell layer has 9 cell culture channels and employs a gradient generator to feed a specific drug concentration to each cell culture channel. Utilizing a gas permeable membrane, it is possible to control oxygen concentrations in the cell culture channels by following corresponding gas mixtures through gas channels adjacent to the cell layer.^{24–26} In the gas layer, we integrated a similar gas-mixing channel network to generate 9 different concentrations of oxygen flowing into the parallel gas diffusion channels with each channel at a different oxygen concentration. Through a polydimethylsiloxane (PDMS) gas permeable membrane, the spatial distribution of oxygen levels in the gas layer can be readily mirrored to the cell layer through diffusion and equilibration in aqueous solution. Channels of the gas layer are overlapped in perpendicular direction with those of the cell layer to generate a full combination of drug concentrations and oxygen levels. The filter layer works as an optical shadow mask. Through the repeated gray scale patterns, uniform illumination turns into multiple distinctive light intensities. For example, in Fig. 1 (b) 16 levels of fluence are generated from a single illumination source by an array of 4×4 grey scale patterns. Each combination of oxygen levels and drug levels will be further combined with 16 levels of fluence. Therefore, the total number of treatment conditions realized in the single chip would be equal to the number of possible combinations of drug, oxygen and fluence levels, which is $9 \times 9 \times 16 = 1,296$ conditions, as illustrated in Fig. 1(b). One of the features of the proposed platform is modularity in design. Each layer can provide the control of one of three PDT elements (photosensitizer, oxygen, and light). Therefore, each layer can work as a

standalone device to screen only single factor. By combining with other layers, we can easily expand the number of screening factors in a modular way (shown in Fig. S1).

Microfluidic devices are fabricated using a customized PDMS multi-layer soft lithography process. Replication molds are fabricated with SU8 photoresist on a silicone substrate. SU8 2010 (MicroChem Co., Newton, MA) or SU8 2050 (MicroChem Co.) are spin-coated single or multiple times to reach a target thickness and patterned with standard photolithography. In the gas layer, microchannels have a height of 33 μm . In the cell layer, cell culture channels have a height of 100 μm . In the filter layer, three different channel heights (15 μm , 33 μm and 51 μm) are formed to generate grey levels. (Details of this liquid filter operation are shown in Fig. 6 and will be explained in the next section.) SU8 replication molds are coated with a self-assembly monolayer (SAM) of Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (448931, Sigma-Aldrich Co., St Louis, MO) by vacuuming for 1 hour in a desiccator. Mixture of PDMS prepolymer (Sylgard 184, Dow Corning Co., Midland, MI) at a 10:1 ratio (base: curing reagent) is either poured onto the mold to form a 3 mm thick PDMS layer, or spin-coated at 1,000 rpm to form a thin PDMS layer of 50 μm . This thin layer is used for a permeable membrane between the gas and cell layers. The PDMS layers are cured on a hotplate at 105 $^{\circ}\text{C}$ for 1 hour. Bonding surfaces are cleaned with 70% v/v Isopropyl Alcohol (IPA) (A459-4, 353002, Fisher Scientific, Hanover Park, IL) and DI water, dried with air, and treated with oxygen plasma (200 Torr, 50 W, 30 s) before each PDMS-PDMS or PDMS-glass bonding. After adhered together, the bonded structures are baked on a hotplate at 95 $^{\circ}\text{C}$ for 30 mins to reach a permanent bonding. The fabrication procedures are shown in Fig. 2(a), including PDMS replication and assembly of the triple layers. The fabricated chip utilizes a 100 μm thick glass slide as a base substrate, which is sandwiched between the cell and filter layers, providing rigid support for cell culture. The core area, where the active PDT efficacy assays are performed for distinctive combinations of drug, oxygen and fluence levels, is around 5 mm by 5 mm as shown in Fig. 2(b).

Device modelling and characterization of each layer

Implementation of the photosensitizer gradient is relatively straightforward. A series of different concentrations can be easily generated through a solution-mixing channel network from two solution inputs (one with photosensitizer-containing cell culture medium and the other with cell culture medium only). We used methylene blue for a photosensitizer and could generate 9 different concentrations from the fabricated gradient generator driven by gravity flow. To characterize the gradient generator, we used a fluorescent dye, fluorescein 5(6)-isothiocyanate (FITC) (F3651, Sigma-Aldrich Co.), which has a similar molecular mass (389) to methylene blue (320) and could properly anticipate its behaviour in the fabricated gradient generator. Solutions of 1,000 ppm FITC in Dulbecco's Modified Eagle Medium (DMEM) (Gibco 11995, Invitrogen Co., Carlsbad, CA) and a pure DMEM were simultaneously introduced from the two inlets under a gravity pressure of $\sim 10^4$ Pa. Fluorescence images were taken after 10 min, allowing FITC distribution to reach the steady state within the entire channels. An inverted fluorescence microscope (ECLIPSE TE2000-U, Nikon Instruments, Inc., Melville, NY) equipped with a cooled digital CCD camera (CoolSNAP ES, Photometrics, Tucson, AZ) and a corresponding filter set (FITC HYQ, Nikon Instruments, Inc.) was used. The microfluidic chip was mounted on a microscope stage and images were taken by focusing on the microfluidic channels containing FITC solutions. The FITC concentration in each channel was estimated from the fluorescence imaging. Fluorescence images were processed in two steps of compensation. First, the background noise (corresponding to images with no fluorescence emission) was subtracted from the captured image. Secondly, the image was normalized to the reference image acquired from a uniform fluorescent background. This process compensates for non-uniformity in excitation illumination and emission imaging from the CCD camera. The gray

value of fluorescence images was measured with ImageJ software. Based on linear increase of fluorescence intensity with concentration, FITC concentration in each channel can be calculated by:

$$C_i = \frac{C_{\max} - C_0}{I_{\max} - I_0} \times (I_i - I_0) + C_0 \quad (1)$$

where $C_{\max}$ is the maximum FITC concentration, C_0 is the minimum FITC concentration, $I_{\max}$ and I_0 are corresponding fluorescence intensities measured in the channels, and I_i is the fluorescence intensity measured at target channel. The methylene blue concentration in each channel was estimated based on the result in FITC concentration changes and the initial methylene blue input concentrations. The results are described in the next section.

The oxygen gradient generation is analysed and optimized by finite element models (FEM) simulations. Fig. 3(a) shows the characterization setup. Gas flows are controlled by two gas tank supplies with the regulated pressures. Depending on channel shape, flow resistance of each segment can be calculated by:

$$R = \frac{8\eta L}{\pi a^4} \quad (2)$$

or

$$R = \frac{12\eta L}{H^3(W - 0.63H)} \quad (3)$$

where η is viscosity, L is channel length, a is cylindrical channel radius, and W and H are rectangle channel width and height, respectively.^{27,28} Since R_t is much smaller (about 5,000 times) than $R_g + R_c$, we simply applied the boundary condition within the microfluidic channels in the following FEM simulations. A 2D FEM model was constructed using COMSOL Multiphysics (Ver.3.5a, COMSOL Inc., Burlington, MA) by importing a mask file of the gas layer, as shown in Fig. 3(b). Gas velocity vector fields were simulated in the incompressible Navier-Stokes subdomain, with an initial dynamic viscosity value of 1.8×10^{-5} Pa·s. No-slip boundary conditions were applied at the channel surfaces. Convection and diffusion subdomains were added for calculating oxygen concentration, with an isotropic diffusion coefficient of 2.19×10^{-5} m²/s. Considering the different viscosity of oxygen and nitrogen, dynamic viscosity was modified as $1.8 \times 10^{-5} \cdot (1 + 0.16 \cdot [O_2]/9.69 \times 10^{-6})$, where $[O_2]$ is local oxygen concentration in mol/m³. Both subdomains were coupled together to calculate the final oxygen distribution under different input gas pressures. An optimized input pressure of 5 psi is determined to guarantee sufficient mixing within the gradient generator and provide discrete oxygen concentrations with a dynamic range from 0.1% to 21%, as shown in Fig. 3(b). Another 2D FEM analysis was performed to simulate oxygen diffusion through the PDMS membrane and reaching equilibrium in the cell layer. The whole model was set up to include both gas and cell layers. Oxygen concentration in each gas channel was preset based on the previous FEM simulation result. The bottom and top boundary conditions were assumed as insulation since glass is not gas permeable. Oxygen concentration in the cell layer was simulated in the convection and diffusion domain, with a diffusion coefficient of 1.3×10^{-9} m²/s in PDMS and 2×10^{-9} m²/s in cell culture medium, respectively. Oxygen levels in cell culture channels were estimated for emulating actual PDT tests. To confirm the simulation result, oxygen concentration in the actual chip was measured by using an oxygen sensitive fluorescent dye, Tris(2,2'-bipyridyl)dichlororuthenium hexahydrate (RTDP) (544981, Sigma-Aldrich). The cell layer was prefilled with the solution of 1,000 ppm RTDP in DMEM. Compressed air and N₂ were

introduced into the gas layer through the two inlets with the same pressure of 5 psi. Fluorescence images were captured and processed in a similar way to the FITC fluorescence imaging except for a different set of filters (Chlorophyll, Chroma Technology Corp, Bellows Falls, VT) for fitting the excitation/emission spectrum of RTDP. Stern-Volmer equation was utilized to calculate the oxygen concentration depending on the RTDP fluorescence change, in a same way as reported before.^{24, 26} The results are summarized in the next section.

The illumination gradient is characterized by measuring the illumination intensities through the liquid filter layer from an LED light source. The initial illumination intensity without filter layer was measured with the same glass substrate when illuminated with the same LED light source (LXHL-ND94, Philips Lumileds, San Jose, CA). The gray levels of the filter layer was determined by comparing the illumination intensity profile with and without the filter. The LED illumination light source and the liquid filter chip were mounted on the microscope stage, with the same distance as used in actual PDT tests. The first image taken without the filter layer was used for normalization. All the images were processed with background subtraction before gray level calculation. We used an illuminance meter (T-10, Konica Minolta, Ramsey, NJ) for characterizing the filter, and ImageJ software for processing the images. Results are described in the next section.

Cell culture and PDT assay protocol

First, the fabricated chip is sterilized with IPA and kept in a vacuum desiccator for 1 hour. Then the filter layer is prefilled with a light-absorbing dye solution by applying a negative pressure at the outlet. We used methylene blue (M9140, Sigma-Aldrich Co.) of 50 mg/ml as a dye because its light-absorbing characteristics are well known. After residual air bubbles are varnished, the filter layer surface was covered with a 100 μ m-thick glass cover, which adheres to PDMS and prevents evaporation of the dye from the filter layer. The cell layer is filled with cell culture medium in a similar way. After all air bubbles varnished in the cell layer, the chip is ready for cell loading and drug tests.

We used C6 glioma cells (CCL-107, ATCC, Manassas, VA) in our experiments. C6 cells were loaded and cultured in cell culture medium composed of DMEM with 10% v/v fetal bovine serum (FBS) (Gibco 16140, Invitrogen) and 1% Penicillin-Streptomycin (Gibco 15140, Invitrogen). These cells were passaged by dissociation with 0.05% Trypsin-EDTA (Gibco 25300, Invitrogen). Trypsinized cells were centrifuged at 1000 rpm for 5 min at room temperature and then suspended in fresh culture medium to a final concentration of 2.0×10^6 /ml. Cell concentration was calculated by counting the cell numbers with a haemocytometer (3500, Hausser Scientific, Horsham, PA). 100 μ l of suspended cells was loaded in the cell layer from the outlet to the cell culture channels by applying a negative pressure at the inlets. After that, residual cells in the outlet reservoir was removed using a pipette and fresh cell culture medium was added. The device was left in an incubator overnight, allowing the cells to attach on the glass substrate and grow into a confluent monolayer.

Methylene blue was adopted as a photosensitizer to apply on C6 glioma cells for high-throughput PDT efficiency assays along with various oxygen levels and fluence gradients. The whole procedure takes around 60 minutes, including the final viability assay (death/live staining of cells). The assay starts with loading the photosensitizer (methylene blue or MB) to the chip. In order to generate a concentration gradient, we introduce a diluted MB (DMEM containing 10 μ M methylene blue) and pure DMEM to the two inlets, respectively, under a gravity pressure of $\sim 10^4$ Pa. These two solutions are used to generate 9 different concentration levels from 0 to 10 μ M by a gradient generator. After 10 minutes, a sufficient amount of time to reach the steady-state of generating methylene blue gradients introduced

to the cell culture channels, gravity pressure is reduced and maintained at ~0 Pa. As a result, a constant methylene blue concentration is maintained for C6 cells in each cell culture channel. After that, the compressed air and nitrogen are introduced to the gas layer with a pressure of 5 psi, generating an oxygen gradient from 1.3% (hypoxia range) to 19.1% (ambient air range). After another 10 minutes (for stabilization of oxygen gradient generation), light is illuminated from a red LED light source (wavelength at 637 nm) for 30 minutes. This exposure time results in the maximum light dose of 42.8 J/cm² for the area with highest transparency; this is comparable to the doses used in previously reported in-vitro tests and clinical applications.^{29–31} After the illumination, DMEM with 10 μ M Calcein-AM and 10 μ M Ethidium homodimer-1 from the LIVE/DEAD Viability/Cototoxicity Kit (L3224, Invitrogen) are introduced into the cell layer for LIVE(green)/DEAD(red) fluorescence staining. After 10 min, the cell viability results are examined under the same fluorescence microscope and recorded for analysis.

Results and discussion

Device performance verification

First, we verified the gradient generator in the cell layer by utilizing food dye solutions of two colors. Fig. 4(a) shows the gradient generation of 9 different concentrations with color coding. As can be seen in the figure, the flows of different colors split and rejoin in the vertical channels, mix in the serpentine channels, and finally feed into each cell culture channel with a distinctive concentration. Fig. 4(b) shows the quantitative measurement result using FITC fluorescence intensity change in the corresponding chip position along the dashed line (red color) in Fig. 4(a). As can be seen in the figure, a linear fluorescence intensity gradient can be established for 9 cell culture channels, and the fluorescence intensity is quite uniform within the channel. After stopping the gravity flow, the gradient generator cannot provide gradients any more and photosensitizers would start diffusing into other channels and eventually reach a uniform concentration in the entire cell layer channels. However, diffusion is so slow that it will not affect the pre-loaded concentration of photosensitizers before finishing the PDT efficacy tests. The concentrations in the active test channel areas can be maintained constant for more than 40 min, which is sufficient for completing the PDT assays (10 min for stabilizing oxygen gradients and 30 min for light illumination), as shown in Fig. S2.

We also characterized the oxygen gradient generated in the cell layer by using an oxygen sensitive dye and fluorescence imaging. As shown in Fig. 1 (a–b), the oxygen gradient levels generated in the gas layer will impose the oxygen concentration to the corresponding cell layer channels underneath by diffusion. This is possible a very thin PDMS membrane (50 μ m) is used between the gas and cell layers. Fig. 5(a) shows the FEM simulation of oxygen concentration for the cross-section along the cell layer channel. As can be seen in the figure, the oxygen gradient generated in the gas layer (9 different levels) can be readily imposed onto the cell layer after it reaches equilibrium. We tested the response time of the oxygen concentration change in the cell layer (Fig. S3). We monitored the time domain response of RTDP (an oxygen sensitive fluorescent dye) fluorescence changes upon the abrupt change of oxygen levels in solution. Fluorescence intensity at the same position of a cell culture channel was recorded every 10 s after switching the gas from the air to nitrogen. It took around 60 s to reach the steady-state of oxygen concentration change in the cell layer, and the transition time was measured only around 30 s, which is similar to the result reported by the other group.²⁴ Fig. 5(b) shows the estimated oxygen levels beneath the gas layer (9 different oxygen levels) measured from the RTDP fluorescence intensities in the corresponding channel, compared with the FEM simulation result. To facilitate the comparison, oxygen levels are calibrated (21% corresponding to 9.69×10^{-9} mol/m³), and the channel numbers are aligned to the corresponding position. As can be seen in the figure,

a linear oxygen gradient ranging from 1.3% (hypoxia range) to 19.1% (ambient air) can be established and imposed onto the cell layer channel, confirming our simulation result.

Finally, we characterized the performance of the liquid filter array. We can generate a 2D array of gray patterns by overlapping the two linear liquid filters that are filled with a light-absorbing dye solution (methylene blue in our work), as shown in Fig. 6 (a). By loading the light-absorbing dye (methylene blue) into the stacked microfluidic layers with different channel heights, we can make a novel gray-scale mask to generate illumination intensity gradient patterns for high-throughput PDT efficacy assays. Each PDMS layer is filled with methylene blue, which has an absorption peak wavelength of 637 nm. It provides four absorption levels from the different channel heights (by providing different absorption light paths). After passing the first microfluidic filter, four levels of intensities are generated from uniform illumination. After passing the second microfluidic filter which is orthogonally aligned with the first one, the intensity levels can be further developed into 16 levels, spatially distributed in a form of 4 by 4 grids. Fig. 6(b) shows the illumination intensity distribution generated from the filter layer loaded with two different concentrations of methylene blue solutions, 25 mg/ml and 5 mg/ml, respectively. As can be seen in the figure, 16 levels of illumination intensities could be repeatedly generated in a 4 by 4 grid array from the uniform light source. Intensity profile along the white dash line is plotted with ImageJ software (Fig. 6(b) bottom). For the entire PDT assay area of 5mm by 5mm, the gray scale patterns repeat within a deviation of $\pm 5\%$. One possible problem of the liquid filter using the dye is photobleaching. In fact, methylene blue could slowly experience photobleaching under strong and continuous PDT illumination (Fig. S4). However, this can be easily compensated for final fluence calculation in the assay, once characterized. Or it can be solved by providing a continuous flow of fresh methylene blue to the liquid filter. While illumination intensity of the static liquid filter gradually increased by 10% due to photobleaching after 40 min illumination, that of the filter layer with refreshing solution maintained a constant illumination over time (Fig. S4).

Actual PDT assays for C6 glioma cells were carried out to confirm that the parameter variation of each layer can be translated to cell viability. The results confirmed that cell viability agrees well with the profile of gradient of each parameter (Fig. S5).

High-throughput PDT efficacy assays

We performed the high-throughput PDT efficiency assays on C6 glioma cells with the protocol described in the previous section (screening all three parameters simultaneously). Fig. 7(a) shows the assay result under 324 different conditions (9 different MB concentrations, 9 oxygen levels and 4 fluence levels). Using a custom written Matlab script, the cell viability was calculated, based on green/red (live/dead) fluorescence ratios and the result was plotted in a 3D chart, as shown in Fig. 7(b). One straightforward observation from this result is that the PDT efficiency shows a general positive correlation with all three factors: the higher the concentrations are, the higher the efficacy is (more cell deaths). From a large number of PDT conditions in one assay, we can acquire the whole spectrum of efficacy responses for all three PDT elements. This will allow us to correlate any combination of factors in a given subset of parameters and easily screen compounding effects of interdependency. Another point worthy of notice is that any limiting factors in PDT efficacy can be identified. For instance, under a certain lower limit of methylene blue concentrations cells may exhibit similar high viability no matter what oxygen levels and/or fluence levels are. Also, we can optimize the PDT efficacy to achieve comprehensive enhancement of effects from the selection of three elements. This can be due to the complexity of PDT mechanism that these factors interact together to generate reactive oxygen species, which is the primary effector of PDT. Lacking in any of them would limit the resulting therapy efficacy. Considering the challenge of this complexity of PDT and the

difficulty in predicting its biological effectiveness in clinical treatments, high-throughput combinatorial assays to rapidly screen all the essential factors is crucial. The assay results in a 3D plot could be useful to provide direct empirical guidance for setting up an effective protocol for the given target cancer, optimized for its local tissue oxygen level and possible drug/light delivery efficiency. Additionally, the large amount of information, based on the three essential factors, could help set up complex PDT dosimetry, which is an ongoing problem for realizing its true potential in cancer treatment.

The value of high-throughput capability has been well recognized and similar PDT efficacy evaluation results have been demonstrated by other groups using their own unique platforms. They reported similar characteristics of PDT efficacy to our results, although they used different photosensitizers on different cells. Meerovich *et al.* demonstrated the phototoxicity results of photosensitizers "Photosence" and dibiotinylated "Photosence" on OAT-75 cells, using a 96-well microplate-based platform.²⁰ 60 inner wells of a 96-well microplate were used for parallel cell culture and PDT treatment. Cell viability gradually decreased from 100% to around 0% following an increase of light power from 0 to 9.5 mW. Also samples with higher drug concentration (11.2 mM) had a faster viability drop than those with lower drug concentration (3.75 mM), which parallels our results. Butler *et al.* reported results from another 96-well microplate-based platform.²¹ 96 wells are fully utilized for parallel cell culture and PDT tests. Tetra methyl rhodamine (TMR)'s phototoxicity on HEK293S cells was evaluated and both light dose-response and drug dose-response are demonstrated. With TMR at 1 μ M, the resulting phototoxicity increased around 4 times as the illumination time goes up from 0 to 3 hours at a constant same illumination level. Also with a fixed 2 hour illumination, the resulting phototoxicity increased around 21 times as the TMR concentration goes up from 0 to 1000 nM. Jedrych *et al.* demonstrated the parallel phototoxicity evaluation results using a microfluidic system.²² Three concentrations (0 mM, 0.375 mM, 0.75 mM) of 5-aminolevulinic acid (ALA) were tested in parallel on A549 cells. And the cell lethality goes up from 3% in lowest concentration to 96% in highest concentration. Cho *et al.* reported the high-throughput phototoxicity results utilizing another microfluidic platform.²³ Phototoxicity of toluidine blue O (TBO) was evaluated against *Escherichia coli* (*E. coli*). With a TBO concentration at 1 μ M, the cell death rate goes up from 16% at no light to 93% at high light dose (637.2 J/cm²). With a constant light dose of 637.2 J/cm², cell death rate goes up from 15% at low TBO concentration (1 μ M) to 96% at high concentration (6 μ M). It also shows samples with extra oxygenated oil supply are having a higher death rate than those without supply, implying the significance of oxygen as we present in this paper. All the previous work showed similar trend and characteristics of photosensitizer efficacy; however, none of them demonstrated the full capacity of characterizing three different elements independently in a single integrated device as we have demonstrated.

Conclusions

We have demonstrated the integrated control of three basic microenvironmental factors in a microfluidic system for comprehensive evaluation of photosensitizer efficacy. We implemented a high-throughput PDT assay chip by stacking three layers in a glass substrate: gas layer, cell layer and liquid filter layer. The core size of the chip is 5mm by 5mm and more than 1,000 combinatorial conditions of PDT assays can be simultaneously performed. In the fabricated chip, parameter gradients are automatically generated on chip and applied to cells under PDT treatments. Cell viability was evaluated to profile the efficacy of PDT assays for various treatment parameters, including photosensitizer concentrations, oxygen levels and illumination levels. Using the fabricated device, we demonstrated high-throughput PDT assays on C6 glioma cells for over 324 different conditions in a single chip within an hour. Further expansion of throughput to over 1,000 or even 10,000 assays would

be rather straightforward, as the minimum unit of each test condition occupies only an area of $80\mu\text{m} \times 80\mu\text{m}$. Our approach provides high throughput with reduced sample consumption, thereby enabling cost effective analysis for comprehensive drug efficacy profiling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

(a) Cross-sectional view of the proposed microfluidic chip composed of three layers that control three elements (photosensitizer, oxygen, and light) for high-throughput PDT efficacy tests. Photosensitizer concentration is maintained constant in each cell layer, while continuous gas exchange from the gas layer maintains constant oxygen levels. Illumination through the filter layer from the bottom generates a series of different fluence. (b) Schematic view of chip composition which includes three layers: top gas layer, middle cell layer, and bottom filter layer. In the gas layer, different oxygen levels are generated from a gas-mixture gradient generator, and fed into parallel micro-channels, which are perpendicular to the cell culture channels located in the cell layer. The cell layer has parallel channels where target

cells are loaded and cultured. Photosensitizers are introduced to each channel with a series of different concentrations through a diffuser and gradient generator. The filter layer is beneath the cell layer with repeated patterns on a 100um thick glass substrate. The repeated patterns generate up to 16 different grayscales for each unit of photosensitizer and oxygen-level combinations, as shown in the enlarged figure.

Fig. 2.

(a) Sequential assembly process of a triple layer chip. The 3mm thick gas layer is peeled off from a SU8 replication mold and gas inlets are formed. Then it is aligned and bonded onto the cell layer after oxygen plasma treatment, and peeled off together. After punching reagent inlets in the cell layer, the two combined layers are bonded onto a glass substrate of 100um thick. The filter layer is prepared in a similar way and bonded to the other side of the glass substrate, realizing final assembly. (b) A fabricated device loaded with different food dyes for visualization: gas layer (yellow), cell layer (red) and filter layer (green). Scale bar: 5 mm.

Fig. 3.

(a) Schematic of oxygen gradient generation in the gas layer. Regulated gas supplies of nitrogen and compressed air are connected to the gas layer inlets via 0.02 inch ID Tygon tubing. Gas layer outlet is open to atmosphere. R_t , R_g and R_c represent flow resistances of the segment of Tygon tubing, the gradient generator and gas diffusion channels, respectively. (b) FEM simulation results of oxygen concentrations in the gas layer generated from different input gas pressures.

Fig. 4. (a) FEM simulation of oxygen concentration diffused from the gas layer to the cell layer in equilibrium. Oxygen can easily diffuse through a thin PDMS (50 μm) membrane and dissolve in media in the cell layer, providing almost the same concentration. Scale bar: 400 μm. For visualization, the vertical dimension is enlarged by 7.8 times. (b) Comparing the FEM simulation result (along the dash line shown in (a)) and the measured value of oxygen concentration distributed along the cell culture channels. Oxygen levels are adequately calibrated (21% corresponding to 9.69×10^{-9} mol/m³), and channel numbers are shown to the corresponding position.

Fig. 5. (a) Image of the microfluidic network for generating photosensitizer gradient (demonstrated with food color dyes). Flow direction is from left to right. Scale bar: 400 μm . (b) Measured FITC concentrations of 9 channels in the cell layer (via fluorescence imaging of FITC solution loaded into the channels by the gradient generator).

Fig. 6.

(a) Schematic diagram of the filter layer for high-throughput screening. An enlarged view of single 4×4 grid unit illustrates how to generate 16 levels of illumination gradient. Light passes through two liquid filter layers with absorption patterns. Arrow length indicates the corresponding illumination level. (b) Measured illumination intensity distribution of the whole test area. Illumination profile along the white dash line is plotted with ImageJ software.

Fig. 7.

(a) Fluorescent Image of C6 cell viability using fluorescent LIVE (green)/DEAD (red) staining after PDT treatment. During the PDT screening, 9 horizontal channels are filled with 9 different concentrations of methylene blue from the lowest (top) to the highest (bottom), and 9 oxygen levels are applied in 9 vertical columns from the lowest (left) to the highest (right). Within each overlap area of horizontal and vertical channels, a gray scale filter provides 4 levels of fluence during the same PDT exposure duration, as shown in the enlarged picture. A comprehensive combinatorial combination of three parameters (methylene blue, oxygen and fluence gradient) allows for screening total 324 PDT conditions ($9 \times 9 \times 4 = 324$) in a single experiment. (b) 3D bubble chart of C6 cell viability.

Viability percentage was calculated by using an automated fluorescence image analysis program written in Matlab. Viability of each combination of methylene blue, oxygen and fluence is displayed by a corresponding bubble with a different color code mapping the viability scale shown in the right.