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ABSTRACT

Cell-based biosensors (CBBs) have important applications in biosecurity and rapid diagnostics. Current CBB technologies have challenges including cell immobilization on the sensors, high throughput fabrication and portability, and rapid detection of responses to environmental changes. We address these challenges by developing an integrated CBB platform that merges cell printing technology, a lensless charge-coupled device (CCD) imaging system, and custom-developed cell image processing software. Cell printing was used to immobilize cells within hydrogel droplets and pattern these droplets on a microfluidic chip. The CCD was used to detect the morphological response of the immobilized cells to external stimuli (*e.g.*, environmental temperature change) using lensless shadow images. The morphological information can be also detected by sensing a small disturbance in cell alignment, *i.e.*, minor alignment changes of smooth muscles cells on the biosensors. The automatic cell alignment quantification software was used to process the cell images (microscopic image was used as an example) and calculate the cell orientation in seconds. The same images were also manually processed as a control to validate and characterize the integrated platform functionality. The results showed software can measure the cell morphology (*i.e.*, orientation) in an automated way without the need for labeling (*e.g.*, florescent staining). Such an integrated CBB system will allow fabrication of CBBs at high throughput as well as rapidly monitor and measure morphological cellular responses.

Keywords: cell-based biosensor, cell printing, high throughput, CCD

1. INTRODUCTION

Cell-based biosensors (CBBs) are promising tools for environmental monitoring [1-3], biosecurity applications and rapid diagnostics [4-6]. There are several advantages of using CBBs over the use of molecular-based biosensors or living animals [7-12]. A major advantage of using living cells is that the effect of external stimuli can be studied by monitoring response of cells which better mimic physiological situations [2, 13]. This may potentially enhance biosensor specificity and sensitivity [5]. Besides, CBBs can detect unknown compounds and toxins since the behavior of the candidate molecules can be monitored directly through metabolic events and viability in living cells [14-15].

There are several issues associated with current CBB technologies (*e.g.*, manual fabrication and data evaluation) that might limit the extent of their application. A large number of sensing units (*i.e.*, cells) are needed to obtain enough data for statistical analysis (*e.g.*, for insecticide toxin evaluation and detection [16]), and decreasing the cost per test [10], is challenging for current CBBs due to their low throughput capability (*e.g.*, fabrication, slow cell response measurement and processing). Different reporting elements have been used, such as metabolic activity [17], fluorescent probes and reporter genes [18], motility and adhesion [19]. However, most of these are based on averaged responses from a large cell population [20], which may lose some important characteristics, such as spatial and temporal change of cell

morphology [21]. Cell morphology response has been used as an indicator of cell viability and response to environmental stimuli, *e.g.*, pH [22], pathogens or toxic agents [23-26]. For example, in the widely used electrical cell–substrate impedance sensing (ECIS) system [23], the sensing is achieved by measuring changes in the impedance resulting from changes in cellular morphology ('motion' or spreading on a surface) caused by external perturbations. Quantification of cell morphology (*e.g.*, alignment) from microscopic images is challenging. Florescent staining of cells (*e.g.*, DAPI staining of nucleus) can provide better quality images, but is not suitable for long-term monitoring of cell behavior. Also, it is difficult to collect and process the cell response data in a high throughput manner since current CBBs are not suitable for field deployment and automated detection [27].
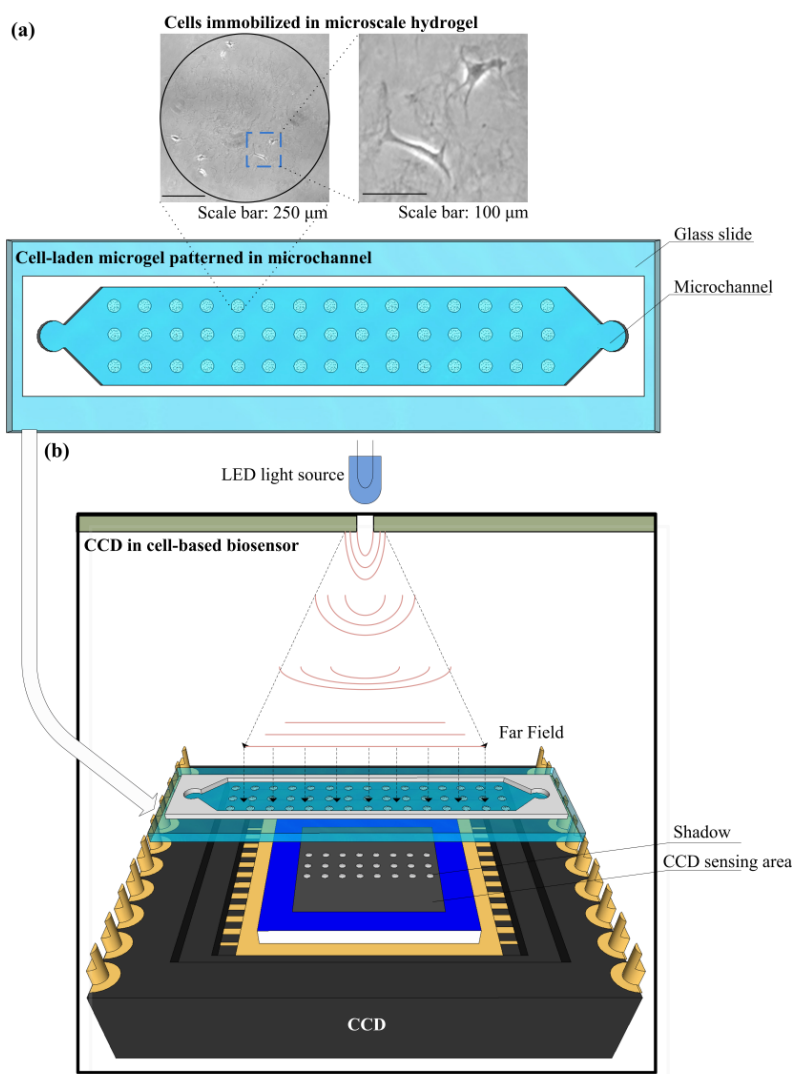

Figure 1. Schematic of the whole-cell biosensor. Cells are immobilized in the patterned hydrogel droplet. The cell response to external stimulus as reflected by cell morphology can be imaged using a CCD sensor.

To address the above limitations of current CBBs, we have previously used cell printing to achieve high throughput immobilization of cells within hydrogel droplets and patterning of these droplets [28]. In this study, we further improved the system by integrating a cell printing technique, a lensless charge-coupled device (CCD), and a custom-developed cell image processing software. We fabricated the microfluidic chip using a laser cutting machine. We immobilized smooth muscle cells (SMCs) in nanoliter volume (~50 nl) collagen droplets at high throughput using cell

printing technology [29-30]. This offers better optical examination since the cell types were grown in a thin hydrogel layer (~20-40 µm). Then, we monitored the cell morphology variation (*i.e.*, cell spread size) using a lensless CCD (Charge Coupled Device)-based fluorescence-free (label free) technology [31]. Also, the morphological information can be also used to look at alignment of spread smooth muscles cells. The change in the spread directionality of cells and their relative positions to each other could also be useful to detect minor changes in the environment, *e.g.*, chemical or temperature [32]. Further, such a method to determine cellular directionality has important applications in tissue engineering to generate tissue constructs that are mechanically functional, since the aligned muscle bundles are shown to better mimic the native tissue structure [33-35]. We quantified the cell alignment using automated image processing software developed in our laboratory using microscope taken images as control samples. The ideal future direction is to obtain the same directionality information from cellular constructs that are obtained through lensless imaging.

2. MATERIALS AND METHODS

2.1 Fabrication of the microfluidic chip

Figure 1a shows a schematic of the microfluidic chip. The chips were designed using a CAD software that runs a laser cutter and fabricated using a thick PMMA inlet/outlet port and a thin microfluidic channel using a laser prototyping technique and double sided adhesive film (DSA), which has been described in detail in our previous work [31]. Basically, a 2 mm thick military specification clear cast acrylic sheet (PMMA, Polymethymetacrylate, 8473K276, McMaster Carr, LA) and 50 µm thick optically clear laminating adhesive sheet (DSA, 3M 8142, St. Paul, MN) were machined using the VersaLASER Platform (Universal Laser Systems Inc., Models VLS2.3, Scottsdale, AZ).

2.2 High throughput patterning of SMC encapsulating collagen droplets

The cells used in this study were primary SMCs isolated from rat bladder tissue [29-30]. After confluence in culture, SMCs were collected and diluted with medium to reach the cell concentration needed (1 million cells/ml). SMC suspensions were mixed with 0.2 mg/ml reconstituted collagen (Type I Rat Tail collagen dissolved in FBS, 10x PBS and SMC medium, and then neutralized with 0.1 M NaOH to pH value of 7.4) at ratio of 1:1 for printing. SMC encapsulating collagen droplets were patterned using a cell printer [29-30] consisting of a 3D stage movement system and a droplet ejection system. The ejector (Solenoid valve ejector, model G100-150300, from TechElan, Mountainside, NJ) deposits cell encapsulating hydrogel droplets onto a receiving plate (*e.g.*, glass slide), **Figure 1a**. We can generate up to 160 cell encapsulating collagen droplets per second with our system. The patterned droplets are the sensing units in the CBB and each droplet acts as an individual monitoring area.

2.3 Monitoring of cell morphology using a CCD sensor

The imaging platform directly images the bottom surface of the CBBs. The CBB was placed on the surface of the CCD image sensor (KODAK, KAI-11002, Rochester, NY), **Figure 1b**. Gray images of the whole CCD area were taken (up to one image per second). The sensor features more than 11 million square pixels (9 µm wide per pixel) across the active sensor array area, 37.25 mm × 25.70 mm. The wide field-of-view CCD allowed us to use commercially available microscope cover slides (24 mm × 35 mm × 0.10 mm). The white light was emitted by a halogen lamp (Micro-Lite, FL3000, Three Rivers, MA) with an annual light guide or LED (Light Emitted Diode, Digi-Key, 160-1704-ND). This point light source works as planar light source if the light source is set up far from an object for simplifying calculation of wave equation with a far field assumption. Light intensity of a cell shadow image is calculated by Rayleigh-Sommerfeld diffraction integral. Fresnel number ($N = \frac{\pi D^2}{4 z \lambda}$) was used to determine whether the optical path was in diffraction (N > 1) or Fraunhofer region (N < 1). Our system operates in the Fraunhofer region (N ≈ 0.4) with the following conditions: cell diameter (D = 20 µm), distance (z = 1.4 mm), and wavelength of light source (λ = 400 ~ 700 nm, white light).

2.4 Quantification of cell alignment using the automated image processing software

We developed a MATLAB program to quantify the cell alignment. The cell images were pre-processed, by applying a Despeckle filter to reduce noise, followed by a band-pass filter to filter out extracellular fibers and debris and correct for uneven lighting. The images were then binarized using Sauvola's adaptive thresholding algorithm. Open (remove one-pixel thin structures) and fill holes operations were applied to the binary image. The angle of orientation of each object in the binary image was calculated.

3. RESULTS AND DISCUSSIONS

3.1 Response of immobilized cells to environmental temperature change

Figure 2a shows cell spread size of SMCs immobilized in printed collagen droplet changes over time (t=0, 2, …, 18 hours) under environmental stimulus (from 37 ^{O}C to 20 ^{O}C), where a decrease in size can be observed as we presented earlier [28]. This can be further verified by the viability tests performed every three hours after placing the cell encapsulating droplets in room temperature. Comparing the cell morphology and corresponding viability at each time point, a relationship following the same trend can be observed. This indicates an indirect link between them although cell morphology is not a true measure of the viability. A CCD sensor (9 µm pixel size) was found to be able to track the morphology changes in the immobilized cells over 2 hours by taking images every 20 minutes. During the process, some cell shadows disappeared and some decreased in size (red arrow and dotted circles), **Figure 2b**.
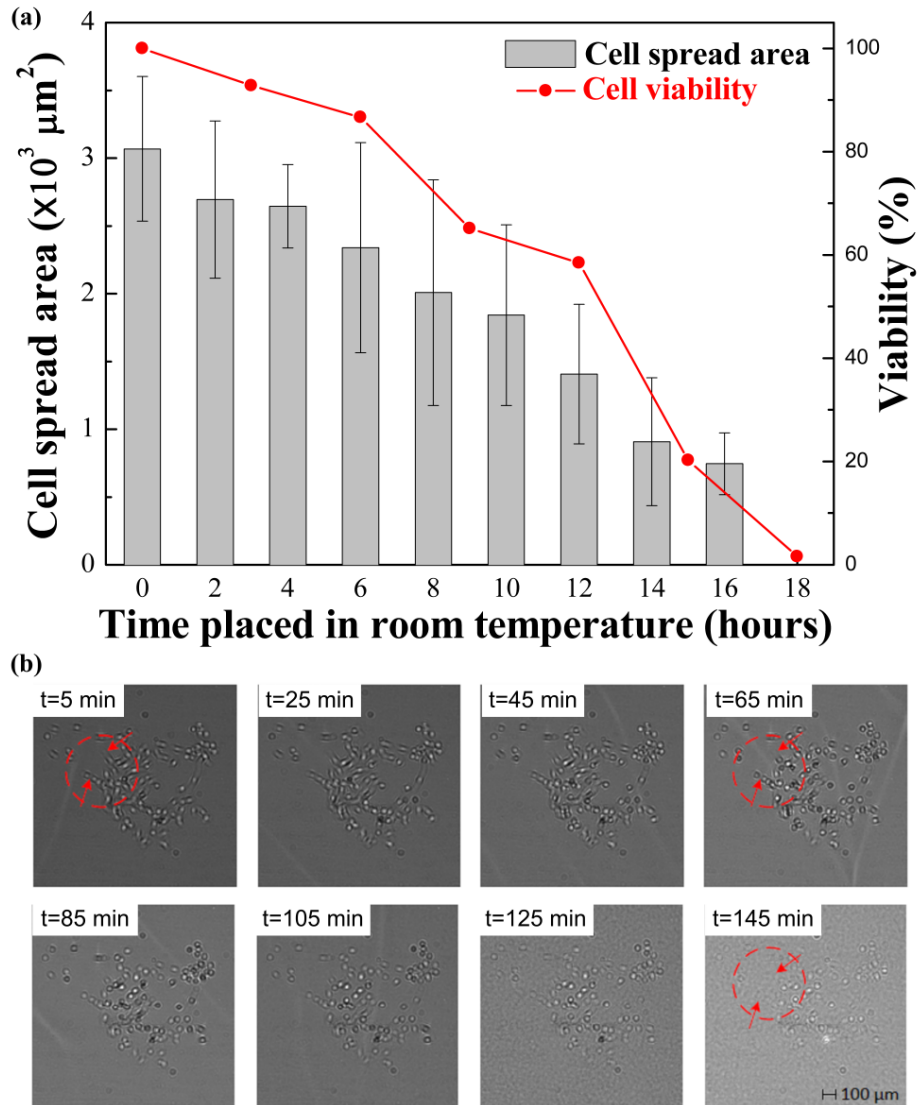

Figure 2. SMC response to environmental temperature change as the temperature decreases from 37 °C to 20 °C [28]. (a) SMC morphology changes with time . A decrease in SMC spread size was observed. Results of cell viability tests agree with the size change. (b) The cell morphology change can be captured using a CCD sensor, *e.g.*, bright field CCD images of SMCs as a function of time in time steps of 20 minutes. Red arrow and dotted circles indicate that SMC morphology changes from a spread shape to a round shape as shadow of cell membrane disappears as the cells die and burst.
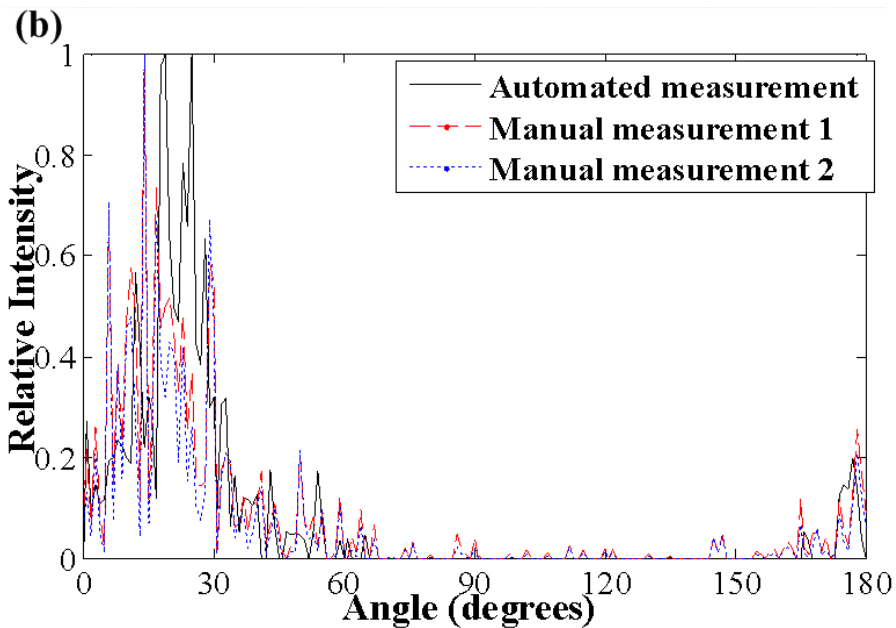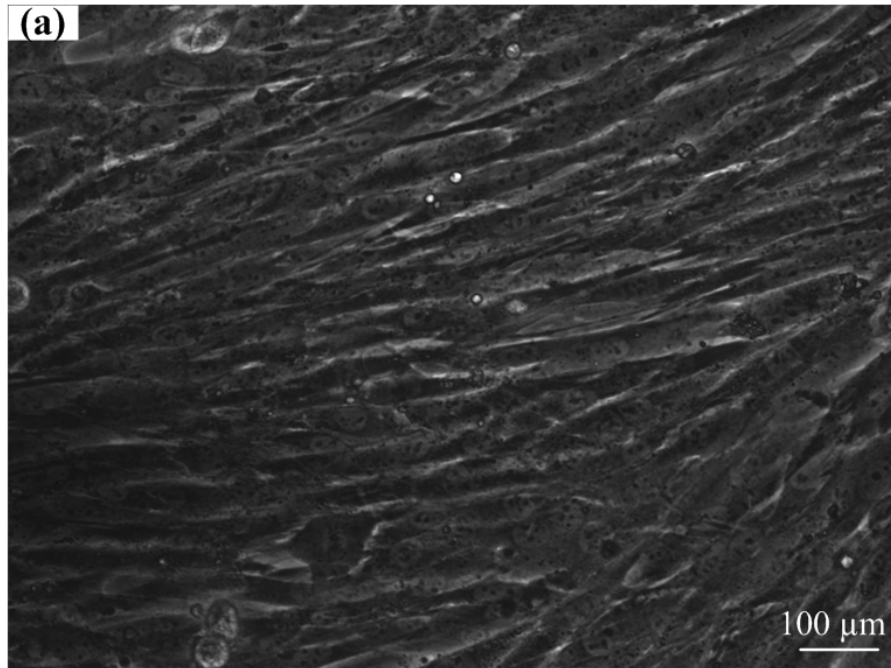
Figure 3. The steps of our alignment quantification method are illustrated using an image of cultured smooth muscle cells. (a) Original image SMCs. (b) The angle of orientation of each object in the binary image is calculated, and the objects are weighted by area to produce an orientation distribution. The alignment obtained using our automated method matched the manual results.

3.2 Automated quantification of cell alignment

The currently methods used to measure cell alignment from microscopic cell images are tedious and prone to inter-observer variability (*e.g.*, manual measurement), and give inaccurate results (*e.g.*, FFT, gradient). Here, we developed a new method to measure cell orientation using microscope images by combining several image processing techniques. The cell image was obtained from printed SMCs in collagen after 7 days culture, **Figure 3a**. A clear cell orientation of

near the horizontal direction (0 degree) can be observed. The imaged was processed using the alignment measurement program as described in Section 2.4 and results were shown in **Figure 3b** (black solid line). Peaks around 15 degrees were obtained. We also manually processed the image by drawing a line along the long axis of each cell and calculating the orientation of the line. We compared the results measured from our methods and manual measurements (read dashed line and blue dotted line), **Figure 3a-b**. The results indicated that our method correctly determines the orientation distributions of cell image and the orientation distributions given by our method are within the range of those given by manually determining orientation.

4. CONCLUSIONS

In this study, we have developed a label-free cell-based biosensor system to address some of the fabrication and sensing challenges associated with current cell based biosensors approaches. Cell printing was used to immobilize cells in hydrogels (*i.e.*, collagen) with maintained biological and physiological sensitivity to environmental stimuli (*e.g.*, environmental temperature change). The series shape changes of SMCs in collagen was detected using a wide area CCD sensor. We also looked into quantified using the image processing software. These results show that our system could lead to fabrication of CBBs in a high throughput manner by rapid patterning of cell encapsulating droplets. Also, this approach could be used to incorporate 3D cell-culture systems in a sensor array. These CBBs will significantly advance functional biosensing of pathogens and toxins in the future.

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