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Effect of Cell Density on Reproducibility in a Cell-Based Biosensor Using a Microwell Array

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Abstract

Combinations of living cell-based biosensors and microdevices are attractive tools for real-time monitoring of gene expression profiling in a small population of cells involving small amount of analytes. However, due to the heterogeneous responsiveness of cells, cell-based biosensors have poor reproducibility and a low signal-to-noise (S/N) ratio. Previously, we constructed a "sensor cell", a GFP reporter cell line containing an engineered Heart Shock Protein 70B' promoter generated by stably transfecting mouse NIH/3T3 cells. In this study, we manipulated the cell density to overcome the lower signal and poor reproducibility using the sensor cells. We found that a cell density of 2 x 10^5 cells/cm^2 provides good responsiveness of sensor cells that appears to be related to the G0/G1 phase of cell cycle. However, higher cell densities had a negative effect for on sensor performance. We also designed microdomains to regulate cell density. The GFP-positive rate of cells grown on domains at 2 x 10^5 cells/cm^2 density was approximately 1.5 times higher than that of control cells. Our results suggest that cell density is an important factor for the design of cell-based biosensors with microdevices.

Keywords: Cell-based biosensor; Microdevice; Cell array; Responsibility; Cell density

Introduction

During initial investigations of an undefined substance that may be diffused by chemical or biological terrorism, identification of the substance is of course important. However, determination of the level of toxicity of the substance is also important. Living cells may be used for the detection of toxic effects of substances [1]. In this regard, living cell-based biosensors have been developed to determine the effects of extracellular chemical or/and physical stimuli through cellular function. Recently, various types of cell-based biosensors have been developed [2-6]. We have also developed sensor cells that can undergo changes in gene expression in response to extracellular stimuli [7-11]. In addition, cell-based biosensors are often combined with microdevices [12,13], for instance cell-based microarrays [14-17], and microfluidic devices [18,19]. These biosensors can detect real-time cellular events from small populations of cells and small amounts of analyte.

However, due to the heterogeneous response of living cells, cell-based biosensors have poor reproducibility and low signal-to-noise (S/N) ratios for individual cells. This is a major problem for analysis using small populations of cells, especially for cell-based biosensors with microdevices [20]. Previously, the authors have tried to obtain higher signals and better reproducibility from living cell-based biosensors, e.g. using genetic engineering and cellular engineering [21,22] and employing the data analysis methods, principal component analysis (PCA) and Hierarchical Cluster Analysis (HCA) [23]. Moreover, cell homogenization with cell cycle synchronization in the G0/G1 phase was found to enhance the sensitivity of cell-based biosensors [24], and non-invasive cell cycle synchronization devices with cell size-separation features have been developed [25]. In general, only 40-50% of cells respond to cadmium chloride used for heart shock protein 70B' (HSP70B') promoter-reporter gene transfected sensor cells, which could be detected protein, denatured cellular stress, such as heavy metals ion [7-9]. However, approximately 80% of cells respond when synchronized in the G0/G1 phase using serum starvation treatment. These results suggest that cell cycle synchronization is an important tool to enhance the responsiveness of cell-based biosensors and facilitate good reproducibility.

In the present study, we report a new approach to enhance sensor responsiveness by controlling cell density. As is well known, cell density is intimately related to cell cycle status in G0/G1 phase. Our investigation centered on determining whether control of cell density can influence the responsiveness of sensor cells. This approach promises not only to improve the reproducibility and S/N ratio of cell-based biosensors but also to provide a design guide for microdevices. To this end, we first determined the cell density effect upon the responsiveness of sensor cells, and next we designed a microdomain for control of cell density. The results indicate that cell density is an important factor for cellular responsiveness and that consideration must be given to cell density when designing cell-based biosensor microdevices.

Materials and Methods

Chemicals

g-line positive photoresist (OFPR-800LB, 34cp) and developer solvent (2.38% tetramethylammonium hydroxide solution, NMD-3) were obtained from Tokyo Ohka Kogyo Co., Ltd. (Kanagawa, Japan). 3-Methacryloxypropyltrimethoxysilane (MPTMS) was purchased from Shin-Etsu Chemical Co. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified phosphate buffer saline (DPBS), and RNase were purchased from Sigma (St. Louis, MO. USA). Cadmium chloride, G418, ammonium persulphate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED) and acrylamide were purchased from Wako Pure Chemical Ltd. (Osaka, Japan).

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Cell culture

We used cadmium chloride induced-cytotoxicity sensing cells, stable cells with the HSP70B' promoter upstream a GFP reporter gene. Cells were cultured in DMEM supplemented with 10% Fetal bovine serum (FBS) and 800 μg/mL G418 on Tissue culture grade polystyrene dishes (TCPS). Cultured cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Effect of cell density on sensor response

Cadmium chloride was dissolved in deionized water and diluted in the culture medium immediately before exposure to the cells. The cells were evaluated for the GFP-positive rate using a flow cytometer (FACSCaliber; BD Bioscience, Bedford, MA, USA). Histogram plots of GFP-positive cells were created by WinMDI software. The threshold point was determined by analyzing approximately 99.5% Cd^{2+} non-exposed cells that were used to normalize background autofluorescence). The GFP positive rate was calculated from the Cd^{2+} test data with reference to the threshold point.

Preparation of the micropatterned substrate

The micropatterned substrate was fabricated using a previously described maskless photolithography method using a liquid crystal display projector [26]. Briefly, glass coverslips (Matsunami Glass Ind., Osaka, Japan) were cleaned by oxygen plasma treatment, and were silanized by MPTMS. Silanized coverslips were covered with positive photoresist using a spin coater (ACT-300D, Active Co., Ltd., Saitama, Japan). The surface patterning of positive photoresist on the coverslip was performed by irradiation of visible light through the patterned images on the liquid crystal display projector. After light irradiation, the coverslip was immersed in developer solvent to dissolve the irradiated areas of the positive photoresist. To form the micropatterns, the coverslips were immersed in 20% acrylamide and 0.1% APS and TEMED mixture solution for 2 h at 4°C. Finally, the photoresist was lifted off with acetone.

Sensor response of cells cultured on a micropattern

The micropatterned substrate was incubated in 5 μg/mL fibronectin for 1 h at 37°C. After incubation, the substrate was washed with PBS. The sensor cells were seeded at 1×10^5 cells on the substrate and incubated for 24 h. Then, the cells were exposed to cadmium chloride for 8 h. After exposure, domains showing a GFP fluorescent signal were counted by fluorescent microscopy (Olympus). Domains were counted and averaged over 100 domains for each size.

Results and Discussion

We first examined the effect of cell density on sensor cell responsiveness. The sensor cells were seeded at densities of 2×10^4 cells/cm2, 2×10^5 cells/cm^2, and 4×10^5 cells/cm^2 on TCPS. After 24 h cultivation, the cells were examined for cell cycle distribution. Figure 1 shows the cell cycle distribution of sensor cells cultured at different densities as determined by flow cytometric analysis. The relative populations of cells in different phases of the cell cycle at each seeding density were calculated from their DNA contents based on PI staining. The distribution, at a seeding density of 2×10^4 cells/cm^2, over the three phases of the cell cycle (G0/G1, S, and G2/M) was 56.06%, 40.50%, and 3.44%, respectively. At the seeding density of 2×10^5 cells/cm^2, 71.32%, 22.34%, and 6.32% of cells were in the G0/G1, S, and G2/M phases, respectively. The proportion of cells in the S phase increased depending on the seeding density. Furthermore, at the seeding density of 4×10^5 cells/cm^2, 82.57%, 13.68%, and 3.75% of cells were in the G0/G1, S, and G2/M phases, respectively.

In our previous study, we constructed cytotoxic stress-sensing cells containing a GFP reporter gene downstream of an engineered HSP70B' promoter and generated by stably transfecting mouse NIH/3T3 cells [8,9]. The cells can detect cytoplasmic protein denaturation-induced cytotoxicity, such as heavy metal-induced cytotoxity. We described therein that cell cycle synchronization of the sensor cells in the G0/G1 phase improved the sensor response [24]. Hence, the responsiveness of sensor cells was investigated by counting the GFP positive cells. The HSP sensor cells were exposed to 8 μg/mL cadmium chloride for 8 h on TCPS and GFP-positive cells were measured by flow cytometric analysis. Figure 2 shows the GFP-positive rate among sensor cells cultured at the three different seeding densities. About 50 % of cells showed GFP fluorescence at a seeding density of 2×10^4 cells/cm^2. On the other hand, 80 % of cells showed GFP fluorescence at a seeding density of 2×10^5 cells/cm^2. The percentage of cells in the G0/G1 phase was 56.0% and 71.32% for densities of 2×10^4 cells/cm^2 and 2×10^5 cells/cm^2, respectively. This strongly suggests that the sensor cell population in the G0/G1 phase positively regulate the response of HSP sensor cells. However, although over 80% of cells were in the G0/G1 phase at the seeding density at 4×10^5 cells/cm^2, the percentage

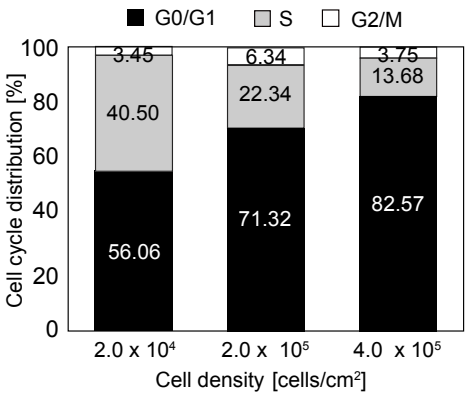

Figure 1: Cell cycle distribution of cells cultured on TCPS for 24 h at densities of 2×10^4 cells/cm^2, 2×10^5 cells/cm^2, and 4×10^5 cells/cm^2. Black, gray, and white represents G0/G1, S and G2/M phases of cell cycle, respectively. Cell cycle phase was determined by flow cytometric analysis with PI staining of DNA. Data represent the average of three experiments.

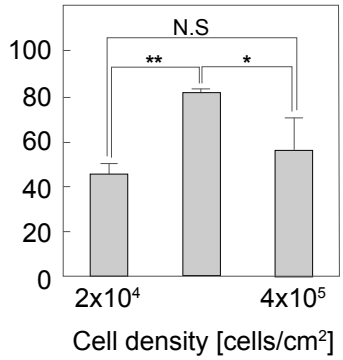

Figure 2: GFP-positive rate of HSP sensor cells. The cells were cultured on TCPS for 24 h and then exposed to 8 μg/mL $CdCl_2$ for 8 h. The GFP-positive cells were counted by flow cytometric analysis. Data represent the mean ± SD of three experiments with ** and * indicating $P < 0.01$ and $P < 0.05$.

of GFP-fluorescent cells was 60%, and was not significantly different compared the rate at 2×10^4 cells/cm^2. Commonly used methods for cell cycle synchronization in the G0/G1 phase (e.g. serum starvation and growing to full confluency) often reduce cell survival and increase DNA fragmentation [27]. Therefore, culturing cells at a density of 4×10^5 cells/cm^2 might have negative effects upon the cells and sensor responsiveness. From these results, we conclude that the optimal cell density for improving sensor responsiveness is 2×10^5 cells/cm^2.

In order to improve the sensitivity and reproducibility of microarrays with cell-based biosensors, we strived to produce the cell density of 2×10^5 cells/cm2 on the microdomain. To regulate the cell density on the microdomain, we prepared a micropatterned substrate comprising 30×30 μm^2 or 50×50 μm^2 domains to generate islands coated with fibronectin and separated by non-adhesive regions coated with acrylamide gel as shown in Figure 3a. The cells had restricted protein adsorption (Figure 3b) and cell attachment areas (Figure 3c) within the domain. Histograms for cell numbers occupying each size domain are shown in Figure 4. The cells were cultured on the fibronectin-coated domains over night. Nearly 90% of the domains contained single cells at 30×30 μm^2. On the other hand, 3 to 6 cells were clustered on 50×50 μm^2 domains. The cell densities on these domains were calculated to be at 1.1×10^5 cells/cm^2 and 1.2 to 2.4×10^5 cells/cm^2 for 30×30μm^2 and 50×50 μm^2 domains, respectively. Thus, cell density could be regulated based on the size of the domain.

Figure 5 shows fluorescence microscopic images of sensor cells attached to 30×30 μm^2 (a), and 50×50 μm^2 (b) domains. The cells were exposed to 8 μg/mL of cadmium chloride for 8 h. The GFP-positive cell rate on the 50×50 μm^2 domains was greater than that on the 30×30 μm^2 domains. Figure 6 shows the percentage of GFP-positive cells among the cells on 30×30 μm^2 and 50×50 μm^2 domains when they were exposed to 8 μg/ml of cadmium chloride at 37°C for 8 h. The GFP-positive rate of cells exposed to cadmium chloride was 50% and 70% for 30×30 μm^2 and 50×50 μm^2 domains, respectively. Among the cells

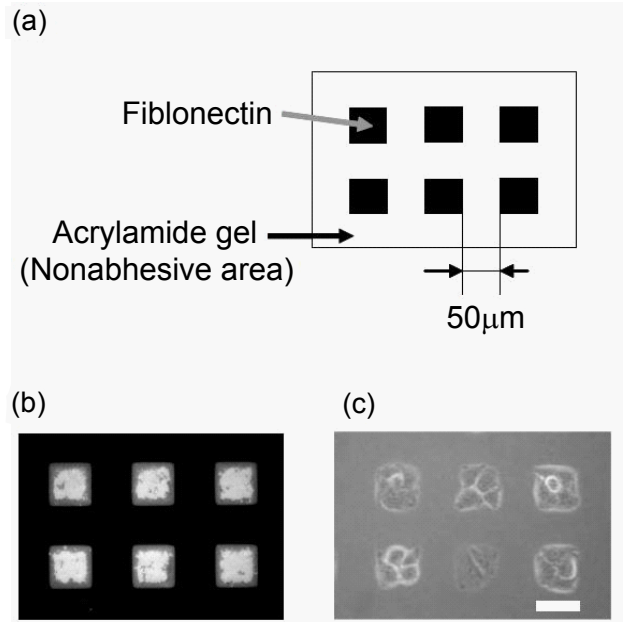

Figure 3: Schematic illustration of a cell array. The design of a cell array (a), Adsorption of rhodamine-conjugated fibronectin on the domain (b), and phase contrast image of cells on the domain (c). Bar = 50 μm.

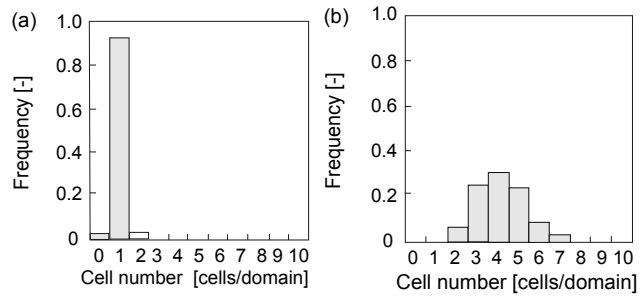

Figure 4: Histogram of numbers of adherent cells on a 30×30-μm^2 domain (a), and 50×50-μm^2 domain (b). The cells were counted and averaged over 100 domains for each size. Unattached cells on the surface were removed by medium change before taking photographs.

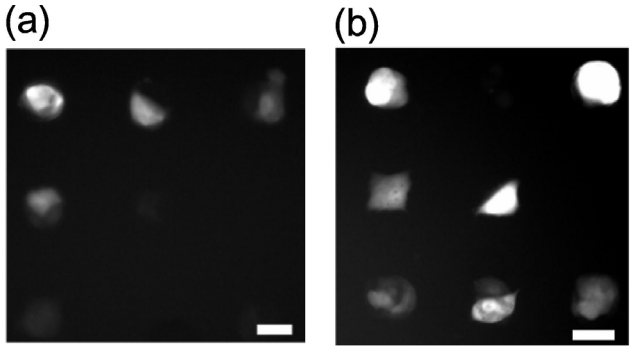

Figure 5: Fluorescent image of response of sensor cells on the 30×30-μm^2 domain (a), and 50×50-μm^2 domain (b). Cells were exposed 8μg/mL of CdCl2 for 8 h. Bar = 30μm (a) and 50μm (b).

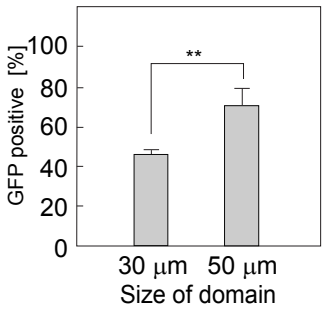

Figure 6: Percentage of GFP-positive cells detectable in domains of each size. Sensor cells cultured on the domains were exposed to 8 μg/mL $CdCl_2$ for 8 h. After exposure, the domain GFP fluorescent signal was counted. Data represent the mean ± SD of three experiments with * indicating $P < 0.05$.

on the 50×50 μm^2 domains, the GFP-positive ratio was greater because cell density was higher than on the 30×30 μm^2 domains.

In summary, in order to enhance and reproduce the responsiveness of the sensor cells, cell density was controlled on the substrate. We found that cell density is directly associated with the sensor-cell response. A cell density of 2×10^5 cells/cm^2 provided good responsiveness of the sensor cells, but higher cell densities had a negative effect on sensor responsiveness. From these results, we challenged that optimization of microdomain design for cell-based biosensor. An optimal cell

density of approximately 2×10^5 cells/cm^2 was identified based on optimizing the size of the domain. This approach promises to yield good responsiveness and reproducibility in the design of cell-based biosensors with microdevices.

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