Optimization of fluorescent cell-based assays for high-throughput analysis using microchamber array chip formats

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Abstract

This study describes the optimization of fluorescent cell-based assays using microchamber array chip formats as well as using automatic nanoliter volumes of sample dispensing system for high-throughput screening analysis of anticancer drugs. Cell-based assays can be employed efficiently in the screening of potential anticancer drug candidates and bioactive compounds with distinct biological function. Identification and development of cell-based assays adapted to high-throughput screening requirements is important when screening chemicals for their potential anticancer properties. Cell-based screening assays using microchamber array chip formats and automatic nanoliter volumes of sample dispensing system requires an optimization as a prerequisite for parameters including assay liquid volume and number of cells per each chamber, and the total cell-based assay itself. Further, the anticancer effect of mitomycin C was studied as an example against human cervical carcinoma cell line-HeLa 229 using cell-based assay that was optimized on chamber array chip formats and determined the cytotoxicity of mitomycin C by measuring the cell proliferation of HeLa with Calcein-AM fluorescent dye. The cell-based screening assay that was performed using chamber array chip formats was compared with the conventional 96-well plate formats was discussed. The assay described in this study is rapid, simple and inexpensive that is desirable in selecting anticancer candidates.

Keywords: Microchamber array chip; Nanoliter dispensing system; Cell-based assay; HeLa; Anticancer drug screening; Cell proliferation

1. Introduction

Cell-based assays are one of the newest tools being used to broaden and strengthen drug discovery for the identification of new therapeutic agents. Cell-based assays are fast becoming the assay format of choice, especially in target validation, lead identification and optimization. Cell-based assay screening is by nature more complex and automation is more difficult than biochemical screening. These assays hold the promise of increasing productivity in the discovery process and the ability to screen out compound failure earlier in the development process. The last few years have seen an increasing number of cell-based assays being used driving the market to an anticipated $500 million mark by 2005 [1,2]. Cell-based assays play a very important role in the post-genomic era focusing on high-throughput functional genomics and drug discovery. High-throughput screening assays play a pivotal role in the search of novel drugs and potential therapeutics. Over the last decade, a variety of scientific advances include the growing number of potential therapeutic targets emerging from the field of functional genomics and the rapid development of large compound libraries derived from parallel and combinatorial chemical synthetic techniques, driven the need for improved drug discovery screening technology [3,4]. Testing very small samples is very attractive because it requires only minute amounts of both the component being assayed and the components of the assay, both of which may be quite expensive or available in very limited amounts [5]. High-throughput cell-based assays reduces the total cost in screening the specific therapeutic target for a specific disease remarkably reducing the time. High-throughput drug screening methods employed so far, involve use of 96 and 384-well microtiter plates and requires at least minimum of 100 µl-assay mixture and suggests further miniaturized assay formats in order to reduce the total cost of drug screening. Matsubara et al. [6] recently reported the application of microchamber array formats for DNA amplification. So far, there are no reports on using...
miniaturized microchamber array chip formats coupled with automatic nanoliter sample dispensing system for screening of bioactive compounds of choice with a cell-based assay. Combining miniaturized technology with developments in automation, sensitive signal-detection, plate formats, automated compound-delivery and data management results in highly efficient, and cost-effective, integrated miniaturized ultra high-throughput screening systems. The most convenient reactions to test in high-throughput are those involving chromogenic or fluorogenic substrates [3]. The need to screen very large numbers of compounds rapidly, in increasingly automated dispensing systems, and with very small reaction volumes prompted us to carry out this study.

It was of interest to develop a high-throughput cell-based assay using miniaturized microarray chip formats. Here, we report on optimization of fluorescent cell-based assay using high-throughput microchamber array chip formats for screening of anticancer chemicals.

2. Experimental procedures

2.1. Cell line cultivation

HeLa 229, Human cervical carcinoma cell line, was purchased from the Dainippon pharmaceutical company, Japan. The cells were maintained in α-minimal essential medium (Dainippon Pharmaceutical Company, Japan) consisting of 10% (v/v) fetal bovine serum, 1% (w/v) glutamine and 1% (w/v) non-essential amino acid mixture. They were subcultured every 2 days and maintained in an incubator at 37°C with 5% CO₂ atmosphere [7].

2.2. Microchamber array chip fabrication

The microchamber array chip for high-throughput cell-based screening of anticancer compounds was fabricated using micromachining techniques including photolithography, and anisotropic wet etching on the optically polished side of a silicon (100) wafer (ShinEtsu, Tokyo). The chip substrate was designed to be 2.54 cm × 7.62 cm for compatibility with dispensing system employed. The fabrication of procedure describing detailed schematic steps of the silicon chamber array chip is shown in Fig. 1A. Before proceeding to microfabrication, silicon wafer was washed thoroughly with acetone and then immersed in a 60% (v/v) hydrogen fluoride solution and finally allowed to dry at room temperature. Subsequently, oxidation of silicon wafer surface to silicon oxide (SiO₂) was achieved by wet thermal oxidation at 1000°C for 8 h. A photo mask with 1248 chambers having 24 × 52 pattern was then printed on the surface, after being coated with positive photoresist (OFPR-800, Tokyo Ohka Kogyo, Kanagawa) layer, by a photolithographic process. The soaking of chip substrate in a NM3-3 solution for 5 min removes the light exposed OFPR portions and followed by treatment with HF/NH₄F (v/v) 10:60 volumes solution removed the exposed oxide layer and then remaining portions of photoresist by treating with acetone. Then, chip substrate was anisotropically etched with 25% (w/v) tetramethyl ammonium hydroxide (TMAH) in an aqueous solution to a depth of 250 μm for 8 h at 80°C. A layer of SiO₂ was grown on the surfaces including the etched chamber walls and then positive photoresist (OFPR-8000, Tokyo Ohka Kogyo, Kanagawa) was spin coated and further photolithography was performed in order to leave the SiO₂ layer inside the microchamber walls. The oxidized layer and photoresist were removed by using HF/NH₄F (v/v) 10:60 volumes of solution and acetone, respectively. The microchamber feature was observed by a color laser 3D profile microscope VK-8500 (KEYENCE, Osaka) and scanning electron microscope (HITACHI, Tokyo). Each chamber was parallelepiped with dimensions of 550 × 550 × 250 μm³ and accommodates 50 nl (Fig. 1B). The total number of chambers on each chip is of 1248.

2.3. Nanoliter liquid volume dispensing system and microarray scanners

Dispensing of assay reagents was performed by using MicroSys 250™, manufactured by Cartesian Technologies, Inc., Irvine, CA. Fifty nanoliters volume of 2 μM fluorescein isothiocyanate (FITC; Wako Pure Chemicals, Japan) solution was dispensed precisely through a ceramic tip with nozzle having diameter of 100 μm. This system can be able to complete less than in 5 min to distribute assay mixture in the continuous fashion demonstrates its speediness and precision.

The CRBIO IIe FITC is laser-based fluorescence scanner with built-in software system was purchased from Hitachi software, Japan and it provides highly precise and accurate microarray scans and analyses. The CRBIO IIe FITC with the resolution of up to 5 μm, sensitivity <0.1 fluorescent molecules/μm² fitted with filters having two emissions of 535 and 585 nm. The data can be measured quantitatively in the chamber array formats using DNA SIS (R) Array version 2.1 software.

2.4. Cell proliferation assay on chip

Twenty nanoliters of cell solution (consisted of 250 cells) was dispensed precisely into chambers of a microarray chip by using Nanoliter dispensing system (Cartesian Technologies, USA) and incubated for 2 h in the humidified chamber at 37°C with 5% (v/v) CO₂ atmosphere to allow the cells to adhere to the chamber wall surface, followed by 20 nl of Mitomycin C (Wako Pure Chemicals, Japan) dissolved in ethanol at 250 μM concentrations was distributed into microchambers of the chip (550 × 550 × 250 μm³) by a nanoliter dispensing system while maintaining the humidity over 90% inside the dispensing system which allows cells in its native activity
inside the chambers and also to avoid dryness [8]. The chip containing the cells were then incubated in a chamber at 37 °C with 5% (v/v) CO₂ atmosphere for 18 h. At the end of incubation, chip was processed with 1 μM Calcein-AM (Dojindo, Japan) [9]. The chip was processed and developed with 1 μM Calcein-AM that was dissolved in 50 μM phosphate buffered saline (PBS) buffer (pH 7.4) and allowed for 30 min at the room temperature while protecting the chip from the light by covering with the aluminum foil. The cells were then washed three times with PBS buffer and then treated with 1:1 volumes of acetone and methanol (Wako Pure Chemicals, Japan) for 1 min for drying the cells. The chip was then scanned with CRBIO IIe FITC microarray scanner (Hitachi Software, Japan) and fluorescence read out.

Fig. 1. Schematic illustration of microchamber array chip fabrication procedure (A) and nanoliter volume dispensing system (B).
was recorded with a filter having excitation and emissions of 473 and 532 nm, respectively. The cell viability was measured from the intensity of the fluorescence signal emitted by the living cells and quantified the cell death using DNA SIS (R) Array Version 2.1 software.

2.5. Relative standard deviation

Precision of the nanoliter volumes of sample dispensing system was determined by measuring the mean and standard deviation of a set of volumes (usually greater than 10 measurements). The quantification of fluorescence signals from the experiments was calculated by conducting the four independent experiments and the mean were taken for calculation. A common method for stating precision of microdispensing technologies for drug discovery applications evaluated in terms of relative standard deviation (RSD) is calculated as follows [10]. Standard deviation (SD), a measure of variability, is the square root of the average of the squares of deviation about the mean of a set of data.

\[
SD = \sqrt{\frac{(x_1 - \bar{x})^2 + (x_2 - \bar{x})^2 + (x_3 - \bar{x})^2 + \cdots}{n - 1}}
\]

The RSD is often more convenient and is expressed in percentage and is obtained by multiplying the standard deviation by 100 and dividing this product by the mean.

\[
\text{RSD}(\%) = 100 \times \frac{\text{Standard Deviation}}{\text{mean}}
\]

3. Results and discussion

3.1. Optimization of assay volume per each chamber by the nanoliter dispensing system

The optimization of assay liquid volume per each chamber of the chip format by the dispensing system is shown in the form of histogram (Fig. 2). Fifty nanoliters volume of 2 \( \mu \)M FITC solution was dispensed precisely into 192 chambers of the microarray chip by using automatic nanoliters of sample dispensing system. The fluorescence intensity profile of the 192 chambers was shown in Fig. 2 and the average fluorescence intensity was found to be \( 10.6 \times 10^7 \) arbitrary units (a.u). For the sake of rapidity of the assay, only 192 chambers were selected in this experiment for the optimization of assay liquid volume. The shape of the fluorescent profile of the histogram seems to be similar to that of 1536-well assay format [11].

The optimization of cell number per chamber is represented in Fig. 3A and B. Cell number optimization per each chamber was carried out with different dilutions (11, 23, 46, 92 and 184 cells/50 nl) of cells and were distributed into five parts on a single chamber array chip format and fluorescent signals were measured by using CRBIO Ile FITC scanner. It was found that the fluorescence intensity is linearly proportional to the number of cells that are present in the chambers and this linearity remains constant as demonstrated in Fig. 3B. The experimental results revealed that over 100 numbers of cells per each chamber is recommended for the cell-based assay using chamber array formats. Because the background fluorescence interference from the empty chambers with the experimental fluorescence intensity was almost nil
otherwise it might get interfered with the background fluorescence of the empty chambers.

3.3. Optimization of cell-based assay using microchamber array chip formats

Optimization of cell-based assay was carried out with implementing the optimized parameters of assay liquid volume and the cell number that are obtained in this study. Cell-based assay was characterized by dispensing 50 nl of cell solution that consisted of 250 HeLa cells into 192 chambers of the chip format and incubated for 2 h at 37 °C for cell adhesion to the chamber walls. Fig. 4 represents the fluorescent profile that was generated from the individual chambers and the average fluorescent intensity was recorded to be $12 \times 10^5$ a.u. The experiment was carried out independently four times and the average RSD found to be $16 \pm 3.7\%$. While the same assay that was carried out on conventional 96-well plate format showed the RSD of $7.7 \pm 1.3\%$ (Table 1). The difference in the RSD of the chip format was contributed probably from the cell number. The RSD of cell-based assay using chip formats was two times over 96-well plate format and this difference can be explained from the experiment that was carried out with beads, as a model to the cell (Section 3.2), contributed 12% RSD, the remaining difference possibly from

Fig. 3. Optimization of cell number per each chamber. Different numbers of HeLa 229 cells were dispensed and fluorescent signals were depicted. For details are given in Section 3.2.
the differences in the cell shape and biological condition of each individual cell that resulted in varying levels of fluorescence intensity. The chip format facilitates the cell-based assays with very minute volumes of the assay sample (50 nl), which is 2000 times lower to the conventional 96-well plate formats together with the automatic nanoliter dispensing system increase the rapidity of the screening procedures.

3.4. Determination of anticancer effect of mitomycin C using cell-based assay on microchamber assay formats

The anticancer effect of mitomycin C against HeLa 229 cells using fluorescent cell-based assay on the microchamber format is demonstrated in Fig. 5. It was found that the mitomycin C was cytotoxic in the inhibition of cell-proliferation of HeLa 229 and the anticancer effect was determined to be 73% (Fig. 5) and this means 27% of the total cells was remained alive for the cytotoxic effect of mitomycin C. It is assumed that the hydrophilic surface of the microchamber played a role on the cell adhesion of

Table 1
Comparison of cell-based assays performance in the microchamber array cell chip and 96-well plate formats

| Assay formats     | Relative standard deviation (%) | Average (%) RSD |
|-------------------|---------------------------------|-----------------|
|                   | Number of experiments           |                 |
| Cell chip         | 20                              | 16 ± 3.7        |
| 96-well plate     | 7.9                             | 7.7 ± 1.3       |

Fig. 5. Cytotoxic effect of Mitomycin C on the HeLa 229 cell proliferation using microchamber array chip formats. Number of cells per chamber 250; concentration of mitomycin C 250 μM; assay was carried out in the incubator for 18 h and the details are given in Section 2.4.

HeLa cells and this provided better way of action by mitomycin C on HeLa cells and the cytotoxic effect of mitomycin C was comparable with the 96-well plate formats. Takara et al. [12] reported that mitomycin C showed the cytotoxic effect on different kinds of cell lines studied. These experimental results clearly revealed that the cell-based assay that was optimised using microchamber array chip formats could be applied for high-throughput screening of potential anticancer candidates.

4. Conclusion

The cell-based assays that are characterized on the microchamber array chip formats in this study could be employed in the screening of therapeutic targets and other bioactive molecules against different varieties of cells in a high-throughput manner. As there are no reports on screening of bioactive compounds using the cell-based assay on microchamber array formats of this dimension, and hence direct comparison with other reports seemed to be difficult. Commercially available high-throughput screenings so far mainly using 96 and 384-well plate formats adopting solution-based assays. But in this paper, we report the direct effect of mitomycin C on HeLa cells was measured by cell proliferation assay using microchamber array chip formats, and as this assay needs longer incubation times ranging few hours, high-throughput screening could be achieved by running the experiments parallel with several chips. The chip formats that are described could perform various assays with very tiny volumes (nanoliters level) in a short period and this application can be extended to the samples that are available in low quantities having very short life period. Apart from that, these formats can also be applied successfully for the analysis of specific DNA amplification and protein–protein interactions, etc.
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