Journal of Bioscience and Bioengineering
VOL. 121 No. 1, 117–123, 2016

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Comparisons of cell culture medium using distribution of morphological features in microdevice

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Received 3 November 2014; accepted 19 May 2015
Available online 3 July 2015

As the number of available cell types grows, it becomes necessary to develop more effective ways to optimize the cell-culture medium for each cell line and culture condition. However, because of the vast number of parameters that must be decided, such as the combination of components, optimization is both laborious and costly. Microdevices are a cost-effective way to perform such evaluations because they use only a small volume of media and enable high-throughput analyses. However, assays performed in microdevices are themselves minimized, and each assay unit (well/chamber) commonly contains an insufficient number of cells for comprehensive evaluations such as gene-expression or flow-cytometry analyses. To address this issue, we introduced image-based analysis in conjunction with microdevice assays; this approach allows quantification of every cell in each assay unit. To quantitatively profile differences in cellular behaviors in a microdevice under different culture media conditions, we developed a non-staining image-based analysis method that utilizes cellular morphology. Our approach combines the structural advantages of microdevices, which can increase the stability of images, and the quantitative advantages of an image-based cell evaluation technique that utilizes time-course population change in several morphological features. Our results demonstrate that cellular changes due to small alterations in the concentration of serum in medium or differences in the basal medium can be profiled using only microscopic images.

[**Key words:** Microchamber; Microdevice; Cell morphology; Image-based analysis; Culture medium]

Based on recent advances in animal-cell culture technologies, cell-based assays have been expanded to numerous applications (1–4). In particular, due to the expanding number of human cell types, cell lines, and clones, cell-based phenotypic assays have become an important choice to partially replace animal testing of chemical compounds and drugs (5,6).

Micro-electro mechanical systems (MEMS) technology has led the cell-assay field with micro/nanoliter-sized assay devices that scale down classical cell-culture devices, ultimately to the single-cell scale (7–9). Such micro/nano-scale assay devices have opened a new standard that minimizes consumptions of cells and reagents, and greatly enhance the flexibility and variety of screening. In addition, the precisely controlled liquid handling in micro/nano devices are also powerful feature for homogeneous cell seeding, stable cell culture, and subsequent analysis (10).

One of the most important applications for such micro/nano-scale devices in cell-based assays and regenerative medicine is the optimization of cell-culture media (11). Many cell types are now available for use in these fields, due in part to achievements in stem-cell research such as induced pluripotent stem (iPS) cells (12); however, the development of optimized cell-culture media lags behind. The selection and optimization of cell-culture media still requires laborious and time-consuming processes. Because there are an enormous number of possible combinations for design of culture media (such as selections of additives, their concentrations, and the timing of their addition), parallel assays using micro/nano-scale devices hold great promise for exhaustive evaluation and optimization of the multitude of options. Proteins such as growth factors and cytokines, which are added to cell culture media at low concentrations, are often extremely costly to produce; therefore, micro/nano-scale devices could both reduce their usage and control the delivery of infinitesimal amounts of these substances.

However, although there is a growing demand for culture-media optimization aimed at advancing cell-culture research, quantitative comparisons of slightly different culture media remain a challenge. One of the main reasons is that differences in culture media can result in wide range of cellular responses. The presence or absence of essential factors results in clear differences in cellular phenotypes such as growth, senescence, and differentiation. However, differences in the basal medium difference or the concentrations of small additives result in cellular phenotypic differences that tend to be very small, and can often be masked by similar growth rates. In other words, the lack of a rapid and effective evaluation method that can detect the subtle changes resulting from slight differences in cell-culture media poses a challenge. Such subtle differences in cellular responses are difficult to be measured by single/several biomarker measurements, therefore more comprehensive cellular assays, such as total gene-expression analysis, is now greatly expected for the evaluation. In such case to measure exhaustive target

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.05.011

molecules in cells, certain number of cells is required in order to assure the stability of their measurements. However, sufficient number of cells for morphology measurements is difficult to be collected from micro/nano-devices. For example, total gene-expression analysis on microarrays commonly requires nearly 1 μg of total RNA, it would exceed the capacity of micro/nano-devices since the number of cells is usually roughly 10^4 to 10^5 cells in each assay unit (well/chamber). Therefore, although sensitive assays for cell evaluation do exist, there are very few effective methods for quantitatively profiling the subtle differences that arise in examinations of cell-culture media in micro/nano-chambers.

In our previous reports, we developed a method for image-based cellular morphology analysis, intended for quantitative evaluation of cellular conditions (mixed ratio of different cells, damaged by irregular passage technique, differentiated/undifferentiated state of stem cells) (13). By converting large numbers of cells (400–5000 cells per condition) into multiple morphological parameters, we have successfully evaluated cellular status using only phase-contrast microscopic images of cells (13–15). It should be noted that our image-based analysis approach has two advantages relative to previously reported methods. First, our analysis uses only non-stained cellular images. In contrast to widely used methods for high-content analysis works that multi-disciplinary approach of using fluorescence images (4,16,17), our image-based analysis method was developed to quantitatively measure non-stained cells, in order to permit evaluation of intact cells intended for use in clinical applications (14,15). Second, our analysis can measure all cells in the culture, and summarize all the measured morphology data as histogram representing a population of cells. For example, with 100 cells, a histogram of length with 100 measured length data can be produced. If there are 5 morphological features measured for 100 cells, 5 histograms of 5 morphological features can be produced using 100 measured data for each. The information of such multiple histograms can be used as high dimensional multiple features to describe a group of cells, and commonly reflect the heterogeneity of cells. In our analysis, we designate such histogram of morphological feature as feature distribution or distribution of morphological features. Therefore, our analysis does not simply compress the morphological features into depleted information (such as an average of a well/chamber), but utilizes the information of morphological feature distribution as heterogeneous character of numbers of cells in a well/chamber. The use of such cellular data that can reflect their heterogeneity facilitates identification of tiny variations in cellular morphological features. The benefits of this analysis approach are actually enhanced by micro/nano technologies, which result in several desirable advantages: (i) Homogeneous cell seeding. Because the bias caused by differences in cell density among culture microwell/chambers is one of the most critical drawbacks that disrupt image-based analysis, uniform cell seeding that can be performed in a micro/nano-device significantly improves the stability of image-based analysis. (ii) Optical homogeneity of the field of view (FOV). The meniscus formed in typical culture microwells (Fig. S1A) deteriorates the quality of cellular images due to disordered focus positions in a well. In a micro/nano device, the culture medium is confined within a precisely designed microchamber, resulting in nearly no light interference and more distinct images (Fig. S1B), in turn leading to superior analytical sensitivity and accuracy.

In this work, we combined image-based profiling of heterogeneous cells with microdevices to achieve effective high-throughput evaluation of cell culture conditions by cell morphology in the aim of establishing sensitive platform for optimum culture media investigation. We have previously demonstrated that cells can be homogeneously introduced into nanoliter-scale microchambers using controlled forces derived from surface tension (18,19). In one of the previous studies, cells in microchambers were transfected on spots of reverse-transfection reagents, and their migration was evaluated in order to identify migration-related genes (20,21). The other technology used in this work is image-based cellular morphology analysis, which we have applied to quantification of cellular status (13–15). By measuring the heterogeneous distribution of cells over a time course, we found that the subtle cellular response to the culture media can be detected and profiled as a map.

MATERIALS AND METHODS

Cell culture Normal human dermal fibroblast (NHDF) cells (<6 passages) were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Life Technologies) and 1% penicillin–streptomycin (Life Technologies) at 37°C in the presence of 5% CO_2. Subculture of NHDFs was performed with 0.025% trypsin solution (Life Technologies) in 10-cm culture dishes (Greiner Bio-One, Frickenhausen, Germany).

Device fabrication The microdevice consisted of a polydimethylsiloxane (PDMS) substrate and a glass substrate (Fig. 1). The PDMS structure (Fig. 1A) was fabricated through replica molding using a template formed with thick-film SU-8 photoresist (MicroChem Corp., Westborough, MA, USA). The PDMS substrate was bonded to a cover glass after oxygen-plasma treatment. The microdevice contained two microchannels and eight microchambers. The height of the chambers and microchannels was 250 μm, and the chamber volume was ~440 nL. The principles of operation, procedures, and its resulting cellular homogeneity data about the cell seeding have been reported (22). The number of microchambers could be readily increased to 50 or 96 (Fig. 1B).

Cell culture assay in microchambers Harvested NHDF cells were separated and suspended in four different types of culture medium: condition 1, DMEM + 2% fetal bovine serum (FBS); condition 2, DMEM + 7% FBS; condition 3, HuMedia

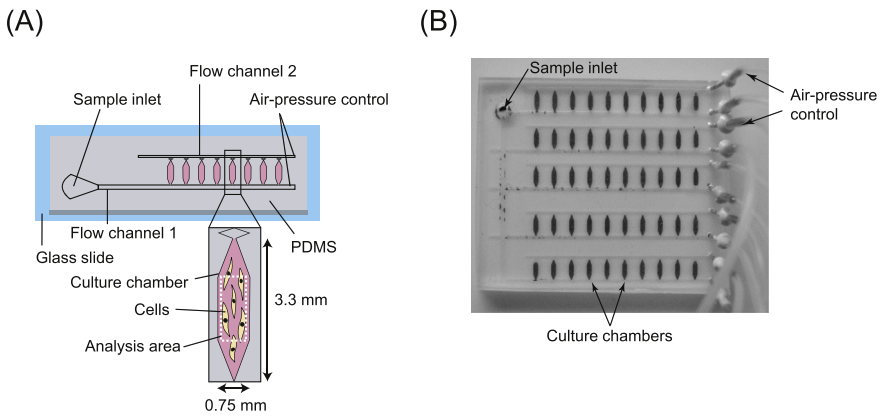

FIG. 1. Image of microdevice for image-based analysis–based selection of culture media. (A) Schematic of a microdevice containing eight microchambers, with a magnified view of the chamber. (B) A representative overview of microdevice containing 50 microchambers. A blue solution was introduced into the chambers to improve visualization.