Journal of Biotechnology 151 (2011) 186–193

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

High-throughput 3-D cell-based proliferation and cytotoxicity assays for drug screening and bioprocess development

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ARTICLE INFO

Article history:
Received 5 April 2010
Received in revised form 7 September 2010
Accepted 17 November 2010
Available online 27 November 2010

Keywords:
High throughput screening
Three-dimensional cell culture
Microplate
Green fluorescent protein
Cytotoxicity
Drug discovery

ABSTRACT

We have designed, built and tested a three-dimensional (3-D) cell culture system on modified microplates for high-throughput, real-time, proliferation and cytotoxicity assays. In this 3-D culture system, cells expressing the enhanced green fluorescent protein (EGFP) were cultured in nonwoven polyethylene terephthalate (PET) fibrous scaffolds. Compared to 2-D cultures in conventional microplates, 3-D cultures gave more than 10-fold higher fluorescence signals with significantly increased signal-to-noise ratio (SNR), thus extending the application of conventional fluorescence microplate readers for online monitoring of culture fluorescence. The 3-D system was successfully used to demonstrate the effects of fetal bovine serum, fibronectin coating of PET fibers, and cytotoxicity of dexamethasone on recombinant murine embryonic stem D3 cells. The dosage effects of 5-fluorouracil and gemcitabine on high-density colon cancer HT-29 cells were also tested. These studies demonstrated that the 3-D culture microplate system with EGFP expressing cells can be used as a high-throughput system in drug discovery and bioprocess development.

1. Introduction

In drug discovery, cell-based assays are increasingly used for drug target validation and ADMET (absorption, distribution, metabolism, elimination and toxicity) studies because cells can provide more representative responses to drugs than simple molecular assays and are easier to use in a high-throughput format than animals (El-Ali et al., 2006; Yang et al., 2008). There are, however, intrinsic drawbacks associated with conventional *in vitro* cellular tests using two-dimensional (2-D) cultures, which lack three-dimensional (3-D) scaffolds to support cell growth and proper tissue functions, and cannot mimic *in vivo* microenvironments (Behravesh et al., 2005; Derda et al., 2009; Griffith and Swartz, 2006; Li et al., 2001; Smitskamp-Wilms et al., 1998; Sutherland, 1988).

The success of cell proliferation and cytotoxicity assays depends upon the quantifications of cell number and viability (Bruchez et al., 1998; Cusan et al., 2002; Slater, 2001; Yang et al., 2002). Conventional cell quantification methods are invasive, and usually require the addition of reagents. Cell cultures must be disrupted for measurement, so they can only provide endpoint data. In order to generate dynamic data that can reveal the toxic action of a drug, multiple cultures are necessary for a time-course study, which is labor intensive and can cause large data variations and errors. Although online measurements of dissolved oxygen, pH, and conductivity changes during cell cultures offer non-invasive methods for monitoring cell (growth) activities (Banerjee and Bhunia, 2009; Banerjee et al., 2010), these indirect methods often do not provide accurate quantification of cell number and proliferation rate, which are the basis for the determination of cytotoxicity of drugs. On the other hand, conventional *in vitro* assays for cell number counting require expensive robotic arms in order to realize automation for high-throughput screening (HTS) (Ashcroft and Lopez, 2000; Beske and Goldbard, 2002).

Optical detection methods are usually based on either measuring the light intensity or imaging the cells. Cell- and tissue-based assays using mechanism-responsive optical signals are increasing in popularity owing to their sensitivity, specificity and relevance to human physiology/pathology (Roda et al., 2004). The reporter gene technique, which couples the expression of an easily quantifiable protein with the control of a regulatory DNA sequence or promoter, can be tailored to detect and quantify cell mass and specific cellular events and functions (Daunert et al., 2000). Green fluorescent protein (GFP) is widely used as a reporter in cell-based assays (Durick and Negulescu, 2001). Because they do not require importing substrates into cells, assays based on GFP are amenable for automation and real-time, non-invasive assessment of both chronic and acute cellular events (Chalfie et al., 1994; Gribbon

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doi:10.1016/j.jbiotec.2010.11.012

and Sewing, 2003). Fluorescence signals were first used to provide online assessment of the density of Chinese hamster ovary (CHO) cells with stable GFP expression in a 96-well microplate using a fluorometer (Hunt et al., 1999). This method is fast, non-invasive and more robust than Trypan blue exclusion and particle counting analysis. However, specific fluorescence signals generated from autofluorescent cells may not be high enough for cell quantification with a fluorometer for *in situ* measurements, especially when the assay responses are affected by changes in the culture environment, including pH and dissolved oxygen, and the continuing releases of cell debris and autofluorescent compounds into the culture medium (Girard et al., 2001). Signals resulting from these non-specific effects could occur at a wavelength close to that of GFP and effectively mask cellular GFP signals. Therefore, it is difficult for cells to elicit distinguishable fluorescence signals readable with a fluorometer when this GFP reporter method is applied to cell-based assays.

To overcome aforementioned problems, we have developed 3-D GFP cell cultures on modified multiwell plates for cytotoxicity and proliferation assays. In general, it is difficult to increase the cell-specific fluorescence (i.e., average fluorescence per cell) in established cell lines; however, with cells cultured in a 3-D fibrous scaffold, the cell number per unit projected area can be greatly increased, resulting in much higher cellular fluorescence signals and signal-to-noise ratio (SNR), thus allowing non-invasive online quantification of cell number that can provide real-time dynamic data in a high-throughput manner. The principle and design of this novel 3-D high-throughput cell culture platform and its applications in drug screening and bioprocess development were demonstrated in this study and the results are presented in this paper.

2. Materials and methods

2.1. Cultures and media

Murine embryonic stem (ES) D3 cells (ATCC CRL-1934) and human colon cancer HT-29 cells (ATCC HTB-38) stably expressing the enhanced green fluorescent protein (EGFP) were obtained by transfection with the plasmid PEGFP-N3 (Clontech, Palo Alto, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 10 days of culturing in media containing geneticin (Invitrogen), green colonies were selected and further expanded in geneticin-containing media, followed with continuously subculturing in the absence of geneticin for over 20 passages, resulting in stable cell lines with more than 97% of cells being EGFP-positive. The expressions of EGFP reporter gene and geneticin resistance gene were under the control of human cytomegalovirus (CMV) promoter, a strong constitutive promoter that controls expression relatively independent of environmental effects.

Unless otherwise noted, D3 and D3-GFP cells were maintained on gelatin pre-coated T-flasks containing Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 M monothioglycerol (Sigma–Aldrich) and 1000 U/ml leukemia inhibitory factor (Millipore, Billerica, MA). Both D3-GFP and its parental strain maintained a high stage-specific embryonic antigen (SSEA-1) expression level (>95%) in this medium. HT-29 and HT-29-GFP cells were cultured in DMEM (high glucose 4.5 mg/mL, Invitrogen) supplemented with 10% FBS. These cultures were incubated in a humidified cell culture incubator at 37 °C with 5% CO_2.

2.2. 3-D cultures in microwells

The microbioreactors were made from commercial multiwell plates (BD Optilux™ Black/clear bottom, San Jose, CA) modified with a computer numerical controlled (CNC) machine (Sherline 2010, Vista, CA). There were 6 microbioreactors on a 96-well plate and 40 microbioreactors on a 384-well plate (see Fig. 1a). Each square chamber, as one microbioreactor, on the plate consisted of a center well and eight surrounding wells with their original walls removed. A polyethylene terephthalate (PET) fibrous disk with a thickness of 1 mm as the scaffold was fitted in the center well to allow cells to grow in 3-D (Fig. 1b). Each microbioreactor had a working (liquid) volume of 3 ml (96-well) or 1 ml (384-well) on the modified plate. Unless otherwise noted, seeding of PET scaffolds was carried out on commercial 96-well plates. Briefly, PET scaffolds were first soaked in 10 μg/ml fibronectin for 12 h and then in the growth medium for another 12 h. After removing the medium, each PET scaffold with a diameter of 6.2 mm in a well was seeded with 25,000 cells (ES-GFP) or 40,000 cells (HT-29-GFP) in 25 μl medium from the top using a pipette, while each PET scaffold with a diameter of 3.9 mm was seeded with 16,000 cells (HT-29-GFP) in 11 μl medium. Cells were well distributed inside PET scaffolds by gently pipetting up and down 3 times. Cells were given 6 h to attach to the scaffolds before additional media (180 μl) were added to completely soak the scaffolds, and then incubated at 37 °C for another 18 h. After seeding, each cell-containing scaffold was transferred into the center well of one microbioreactor on the modified multiwell plate for culture kinetics studies. Multiple plates were stacked onto a rotational shaker (Belly Button Shaker, Stovall, Greensboro, NC), at a rotational speed of 60 rpm for 96-well plates and 90 rpm for 384-well plates. The agitation ensured good mass transfer into the 3-D scaffold region. All cell growth occurred in the 3-D scaffold in the central well; there were no or few cells suspended in the medium or attached to the surface of the surrounding wells. To measure the culture fluorescence, the plate was transferred to a fluorescent plate reader (Cytofluor Series 4000, PerSeptive Biosystems, Framingham, MA for 96-well plates; GENios Pro, Tecan, Durham NC for 384-well plates) at 37 °C, with bottom reading as the measurement mode. Fig. 1c illustrates how the 3-D culture in the microbioreactor can provide a higher fluorescence signal by increasing the cell number/density and reducing the background autofluorescence.

2.3. Measurements of cell number and fluorescence intensity

Cell numbers were determined by the Trypan blue exclusion method or using a Coulter counter. Cells harvested from T-flasks were counted and different concentrations of cells in 200 μl PBS solution were dispensed in a 96-well microplate. The fluorescence intensity from cells on 2-D surface was then measured with a fluorescence microplate reader (Cytofluor Series 4000, PerSeptive Biosystems). To determine the correlation between the fluorescence intensity and the cell number in 3-D fibrous scaffold, various amounts of cells in 25 μl of media were seeded into fibronectin-coated scaffolds (diameter: 6.2 mm) in a 96-well plate. After 6 h, 180 μl of media were added to each well to completely soak the scaffold, and the plate was then subjected to fluorescence reading. The actual fluorescence signal given by the cells was estimated by subtracting the background fluorescence from the scaffold and the medium.

2.4. Growth kinetics in 2-D cultures

For comparison, 2-D cultures of non-GFP D3 (wt) and D3-GFP cells were carried out in conventional multiwell plates. Each well on the 96-well plate was inoculated with 5000 cells. The