3-D CELL-BASED HIGH-THROUGHPUT SCREENING FOR DRUG DISCOVERY AND CELL CULTURE PROCESS DEVELOPMENT

DISSERTATION

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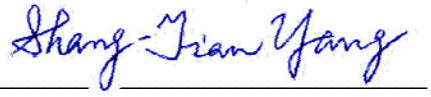

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ABSTRACT

High-throughput screening (HTS) is widely used in the pharmaceutical industry for target identification and hit/lead selection. However, conventional HTS often generates a large number of poorly qualified leads that must go through expensive and time-consuming animal experiments. The development of a new HTS platform with more relevant *in-vivo* biological information that can reduce the number of leads into animal experiments is thus critical to the drug discovery process. Based on three-dimensional (3-D) cultures of GFP-expressing mammalian cells, a novel microbiorector array capable of online monitoring of biological activities was developed for high-throughput drug screening. The 3-D microbioreactor array can afford parallel, automated, and long-term (over one month) cell bioactivity assays. It can also increase signal to noise ratio (SNR) by at least one order of magnitude as compared to the conventional 2-D culture system and at the same time remove most of the detection interference caused by cell activities. It used inexpensive materials and proven tissue engineering principles, and can be used for fast cell culture media development and cytotoxicity assays for drug screening.

Toxicity of embryotoxic reference chemicals and anti-cancer drugs was measured in the 3-D multicellular models and the predicted toxicity was compared to that from monolayer cultures. It showed that the 3-D system was a more realistic pharmacotoxicological test

system than 2-D monolayer cultures. With the 3-D system, acquired tissue resistance in the treatment of bulky tumor tissues could be revealed in a high-throughput manner. As a bridge over the gap between monolayer cell cultures and animal models, this 3-D system can improve the drug discovery process when being applied in toxicity and efficacy tests prior to animal experiments.

Besides high-throughput toxicity screening, autofluorescence detection of 3-D tissue cultures could also be extended to immobilized cell culture process development. Butyrate treatment was used as a case study to demonstrate the performance of the new system. The microbioreactor array developed was used for high-throughput cell process development to improve monoclonal antibody (MAb) production in a fibrous bed bioreactor (FBB) using CHO cells. A novel online fluorescence probe was developed and used in spinner flasks and a lab-scale perfusion fibrous bed bioreactor to non-invasively quantify cell growth and MAb productivity. The results from this study showed that GFP fluorescence could indicate recombinant protein production and thus provide a fast, reliable and robust platform for cell culture process development to optimize target protein productivity without cell counting or protein analysis.

Dedicated to my family

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FIELDS OF STUDY

Major Field: Biochemistry

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CHAPTER 1

INTRODUCTION

1.1. Drug Discovery

Drug discovery is a process that identifies active substances with desired biological effects. It usually involves target validation, identification of hits and selection of leads by high-throughput screening, and lead optimization by medicinal chemistry. It also includes animal models as preclinical evaluation to study compound efficacy and ADMET (administration, distribution, metabolism, elimination and toxicity). Drug discovery to market availability is a long and costly journey with an average cost of $800 million and 7 years [1]. The initial cost from screening assays to investigational new drug filing normally takes 1-3 years with a relatively low cost $2-4 million. The first step of drug discovery is compound HT screening (generally > 50,000 compounds) using different assays. In this step, as a surrogate for clinical trial, these screening assays require high sensitivity and specificity mimicking their *in vivo* environment. In addition, robustness, reproducibility, simplicity and automation are also highly desired. Current screening assays generally use 96-well or 384-well plates, but cell-based assays are still difficult to realize in a 384-well manner because of lots of manipulations involved. Dynamic assays and high-content screening provide temporal and spatial resolutions but

generally require additional labor and time. After the primary screening, hits are evaluated and selected for leads in the secondary screening. This step is done first using cell viability and proliferation assays, followed by animal experiments to analyze pharmacokinetics, pharmacodynamics, and ADME. The last step of drug discovery is lead optimization using synthetic/medicinal chemistry to optimize chemical structures of leads in order to improve their efficacy, specificity and ADMET properties before clinical trials [2].

1.2. 2-D and 3-D Cell-based Assays in Drug Screening and Discovery

After initial screening, compounds selected (hits) are evaluated for many criteria, including efficacy and toxicity to identify leads. In this step, cell viability and proliferation assays in cell culture models are usually applied. Currently, pharmaceutical firms spend a large amount of money on the compound efficacy and cytotoxicity test [1]. However, there is still a 78% failure rate for all drugs, which may be devastating to developing companies. Effective compounds *in vitro* may be non-effective *in vivo* for many reasons, including differences between *in vitro* and *in vivo* target biology, interrelated biochemical mechanism, metabolism, poor penetration into solid tissues, etc. Currently, almost all cell-based assays or biosensors are developed in 2-D culture systems, although conventional 2-D cultures usually suffer from contact inhibition and a loss of native cell morphology and functionality [3; 4]. In comparison with 2-D cultures, 3-D cell models create a more realistic representation of real human tissues, which is critical to many important cell functions, including morphogenesis, cell metabolism, gene expression, differentiation and cell-cell interactions. Our group used fibrous materials,

such as non-woven polyethylene terephthalate (PET), to maintain tissue native morphology, because they can provide high specific surface areas, mechanical properties and void volumes [5-8]. Discrepancies in predicted drug treatment effectiveness in 2-D and 3-D cultures implicate the advantage of using 3-D culture systems [9-11]. For cell-based sensing, particularly in studying cytotoxicity and drug discovery, maintaining cells in their native functional state in a proper 3-D environment would improve predictions and have the potential to reduce clinical trial failures. Colon cancer cells exposed to gemcitabine showed that 3-D multilayer cells were over 1000 times more resistant to gemcitabine than cells in 2-D cultures [11]. These results coincided with the lack of *in vivo* efficacy of this drug for colon cancer in clinical trials. Therefore, although designing 3-D models is much more complicated than designing the 2-D counterparts, cell- and tissue-based assays with a 3-D model are superior and are the assays of choice for HTS of drug cytotoxicity [4].

1.3. Online Biosensor in Bioprocess Development

One of the most complex systems in the world is the biological system such as proteins and whole cells. Because precise prediction of a biological system is extremely difficult, virtually all biological studies are mainly based on detection. This complexity is further increased when bioprocesses using these systems are the interests, so the measurement of parameters during the process becomes more critical [12].

Process development is a crucial step in bring a therapeutic biologics into market. The goal of process development is to optimize the process by quantifying critical parameters. It is preferred to have on-line monitoring for those important parameters especially including factors in closed control loops. Compared to off-line measurement, online biosensors also reduce intensive time and labor in addition to the high risk of contamination during sampling [13].

1.4. Objectives

1. To introduce 3-D tissue culture into high-throughput drug discovery and screening. A high-throughput format is required in drug development to screen large numbers of candidates. Lack of *in vivo* function and mechanism makes monolayer cell-based assays not a satisfactory screening for high-quality leads. This objective was to develop, apply and validate novel *ex vivo* screening models with 3-D tissue cultures in drug discovery to provide a better predictive value.
2. To develop novel platforms for immobilized cell culture process development. Fibrous Bed Bioreactor (FBB) provides high porosity, high surface to volume ratio, high permeability, low pressure drop, easy downstream processing, low material cost, and *in vivo* mimicking microenvironment. Perfusion cultures using the FBB are able to allow high density cell culture and give high productivity for a long time. However, immobilized cell growth in fibrous scaffolds could not be well quantified in the middle of the culturing process, making the study of cell seeding and growth in 3-D matrix very difficult. The objective was thus to

develop a new platform for *in situ* monitoring of cell growth for cell culture process development.

1.5. Scopes of Study

An overview of this study is shown in Figure 1.1, including the development of novel platforms and the validation of their applications in high-throughput drug screening and immobilized cell culture process development.

1.5.1. Development and Validation of a 3-D GFP-based Cell Assay System (Chapter 3)

A high-throughput, real-time, bioactivity assay was designed, built and tested based on the 3-D culture of GFP-expressing embryonic stem (ES) cells and colon cancer HT-29 cells. The 3-D culture system increased signal to noise ratio (SNR) by at least one order of magnitude as compared to the conventional 2-D culture system.

1.5.2. Embryocytotoxicy in 2-D and 3-D Cultures (Chapter 4)

The embryocytotoxicy of dexamethasone (DM), diphenylhydantoin (DPH), Penicillin G and 5-FU in different culture models was studied using the established system. The results suggested that the 3D culture system was able to reveal physiological responses of chemicals and enabled toxicological studies in a high-throughput manner.

1.5.3. High-throughput Drug Screening (Chapter 5)

3 commonly used drugs 5-fluorouracil (5-FU), gemcitabine and sodium butyrate were tested on a mouse ES cell line and a colon cancer cell line at both low and high cell

densities (multicellular structures). The system allowed high-throughput study of drug cytoxicity on 3-D multicellular structures representing *in vivo* biology and had a much better predictive value than 2-D monolayer cultures.

1.5.4. Fluorescence Protein in Immobilized Cell Culture Process Development (Chapter 6)

An online sensor system based on GFP fluorescence was developed and used for monitoring cell growth and monoclonal antibody production in immobilized cell cultures. The system provided a rapid, reliable and robust approach for cell culture process development to optimize target protein productivity without sampling for cell counting and protein analysis.

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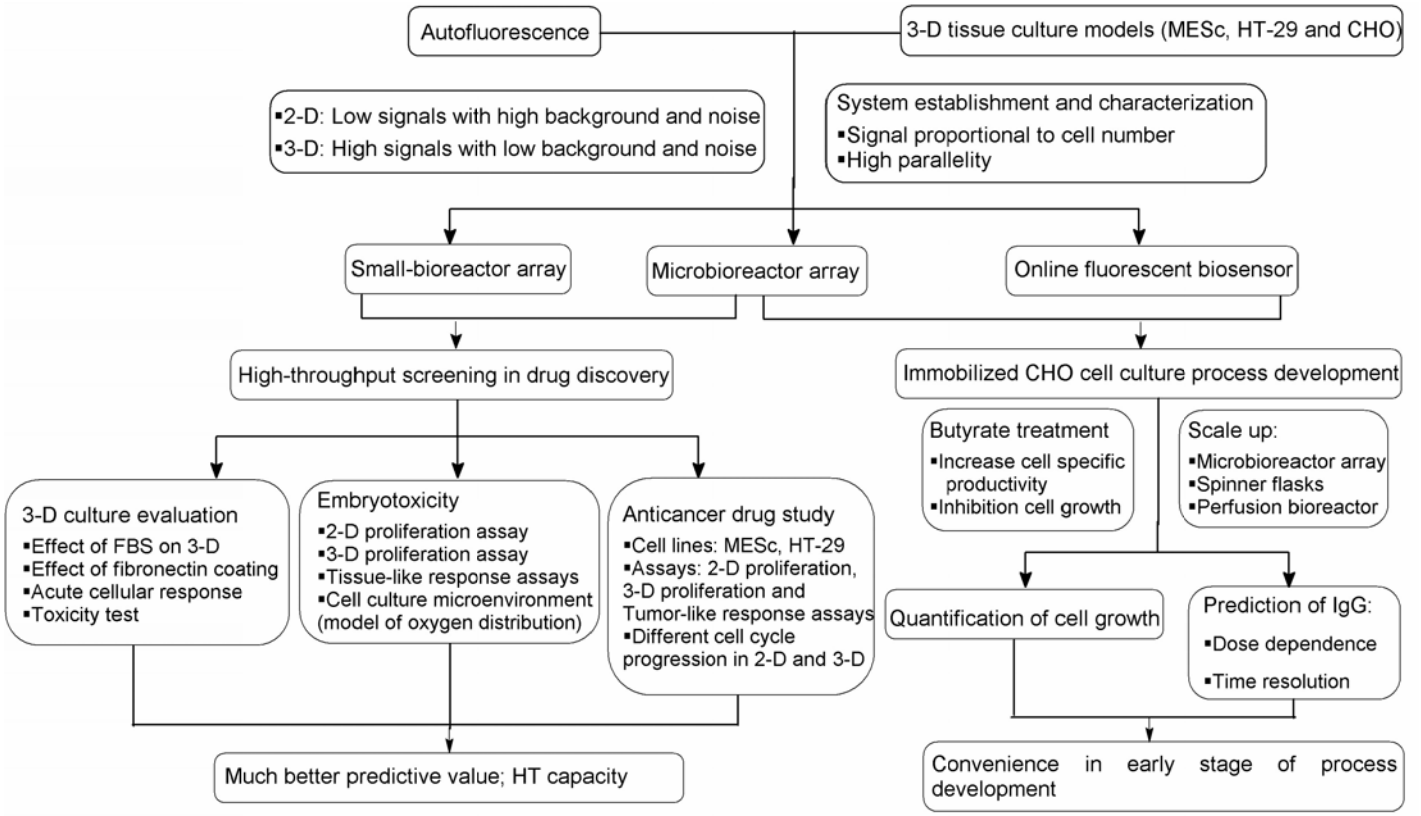

Figure 1.1. Schematic diagram of research areas.

CHAPTER 2

LITERATURE REVIEW

2.1. Cell-Based Assay and Microbioreactor Array

2.1.1 Introduction

High-throughput screening (HTS) attracts lots of attentions currently. Firstly, drug discovery is a process involving biological target identification and validation, the selection of lead compounds for optimization, and clinical trials [1]. High-throughput screening plays an essential role in the initial identification of leads because the pool of chemical compounds and biological targets is rapidly expanding. The tangible revolution of drug discovery with HTS was initiated within the pharmaceutical industry for the sake of increasing efficiency and lowering the cost of the drug discovery process. Secondly, both USA and EU have started programs to evaluate thousands of chemicals toxicologically, such as the U.S. High Production Volume (HPV) Challenge Program and a future European Chemicals Policy (Commission of the European Communities, 2001). More than 10 million animals were estimated to be used in the near future [2]. In addition to their high cost, intensive labor and increasing protection from animal right advocates and governments, animal models are just proxies for human. In this case, alternative

predictive approaches to reduce the number of animal experiments and increase testing efficiency in a high-throughput manner are required. Thirdly, the fast evolution of high-throughput screening (HTS) follows the increasing demands in analyses and assays of the avalanche of genomics and proteomics information.

Years before, people relied on radiometric and absorbance-based assays for detection in the assay experiments. However, HTS requires miniaturization, which the old assays cannot meet. Environmental and safety concerns of radiometric assays are also reasons for using fluorescence assays, which are popular currently.

Biochemical assays are mainly used to collect analytical data instead of functional information in human beings [3]. On the other hand, animal models, which allow for preliminary predictions of the systemic effects of drugs and have contributed greatly to the advancement of drug testing in the past, often exhibit anatomical and physiological differences between species, which can cause data inconsistencies. Animal experiments are also costly and time consuming, and present moral issues. Cell-based assays are easier to use in a high-throughput format than animals, and can uncover important biological issues, such as cellular toxicity or the complex cell biology surrounding the target, that biochemical assays cannot. In this case, cell-based assays are a good compromise between whole organisms and *in vitro* biochemistry systems [4]. They have been widely used in drug target validation and the study of drug ADMET (administration, distribution, metabolism, elimination and toxicity). They can be also applied to basic molecular genetics study, cellular biology, pharmacology, toxicology, and detection of environmental threats in a high-throughput manner [5].

2.1.2 Cell Sources

The most popular cell types for cell-based assays are excitable cells such as neurons and cardiomyocytes. The physiological status of these cells, such as electrical activity, is highly sensitive to external stimuli and can be recorded using non-invasive extracellular electrodes. Non-excitable cell types also play important roles in cell-based assays. Different detection signatures can be provided by different cell types; for example, assays employing hepatocytes can be used to predict the toxicity effects of environmental agents and the metabolism of compounds [6-8]. Both primary hepatocytes and hepatoma cell lines have been used in hepatotoxicity assays, while epithelial cells such as Caco-2 have been intensively used to study the permeability and absorption of compounds across the intestinal epithelial cell barrier *in vivo* [9; 10]. Endothelial cells are also considered important targets for the study of angiogenesis, which can be applied to anticancer chemotherapies [11]. Although not commonly used for toxicity assays, immune cells have been used for the detection of pathogens and superantigens, which can be detected by increased cytokine release in specific immune cells [12].

Established embryonic stem cell (ESC) lines have been used to study embryogenesis and the effects of chemicals, drugs and environmental pollutants. Derived from the inner cell mass of blastocysts, ESCs have three unique characteristics: unlimited undifferentiated proliferation, the capability to develop into all-specialized somatic cell lineages, and ease of genetic modification [13]. Currently, ESCs are the only true immortal stem cells with a normal diploid karyotype, which makes them more suitable for developmental studies than other immortalized cell lines and primary cells. Furthermore, as different somatic

cell lineages such as cardiomyocytes, neurons, hepatocytes and blood cells can be attained from ESCs by established methods, the differentiation of ESCs provides access to unlimited homogeneous somatic cells. This can address many issues associated with the use of primary cells, which is limited and can vary greatly according to the donors. Cytotoxicity based on the ESC test (EST) using established ESC lines has thus been increasingly used in drug screening [14; 15].

2.1.3 Cell Culture Mode

Cell-based assays usually involve culturing cells as single cells, monolayer cells on a two-dimensional (2-D) surface, multilayer or aggregates in a 3-D scaffold, or even as fresh tissues and organs.

2.1.3.1 Single Cell

A multiparameter cell array at single-cell level was developed using optical tweezers and adhesive proteins to localize cells on a miniaturized chip [16]. Intracellular proton and calcium concentrations for each cell type were imaged simultaneously to demonstrate the specific activation of T-lymphocytes. Although single-cell arrays can also be used in HTS for cytotoxicity and drug discovery, they are usually used for endpoint analysis and are thus unsuitable for dynamic measurements.

2.1.3.2 2-D Monolayer

Most current cell-based assays use 2-D monolayer cells cultured in multiwell microtiter plates, which are relatively easy to handle in cell-culture laboratories. The low cost and

high speed of testing agents made them important in the drug discovery process and environmental detection.

2.1.3.3 Engineered 3-D Culture

2.1.3.3.1 Introduction

Although 2-D cell assay demonstrates an extensive application in current studies, 2-D cultures for cytotoxicity assays are inherently prone to errors because of the lack of a 3-D scaffold to support cell growth and proper tissue function. 2-D surfaces and static cultures do not provide a physiologically relevant environment for cells to exhibit the fully authentic responses found *in vivo* [17-20]. Reported research describes the variation of cellular performance observed between 2-D and 3-D cell cultures, including morphology, growth kinetics, growth factor expression and other functional properties [21-25]. 3-D cultures are particularly important for *in vivo*-like differentiation, proliferation, metastasis potential of tumor cells, and development of characteristic heterogeneity within the tumor population [26].

2.1.3.3.2 Engineered Neuron Culture

Typical 2-D cultures can't satisfy the formation of synaptic network from neurons and multiple point contacts with nearby astrocytes. Therefore, not only neuronal viability is compromised, but also the predictive value of neuron is limited. Various polymers, such as acrylamide derivatives, agarose and collagen, have been used to mimick the *in vivo*

extracellular matrix and support 3-D neuron growth and formation of self-assembled networks [27-29].

2.1.3.3.3 Engineered Hepatocyte Culture

Various bioartificial liver systems for replacing failed liver function have been studied with limited success. 3-D matrix can maintain hepatocyte viability and cytochrome oxidase activity. Hepatocytes were cultured on 3-D biodegradable polymer scaffolds under continuous flow conditions and the results indicate that flow provided a significantly higher albumin and dissolved oxygen (DO) concentration, glucose level and a more physiological pH compared to static conditions [30]. Differentiated function of hepatocytes could be enhanced by cultures on a low density of fibronectin or collagen gels [31; 32]. Liver-specific function and differentiated ultrastructure could be discovered by hepatocytes in self-assembled multicellular spheroids [33]. Coculture of hepatocytes and 3T3 in a microfabricated system could induce hepatic functions in hepatocytes [34].

2.1.3.3.4 Engineered Endothelial Cell Culture

Since capillary endothelial cells are embedded in an investing basement membrane and migrate cross an interstitium in bodies, extracellular matrix is thought to play key roles to regulate endothelial cells [35; 36]. Gels of collagen, fibrin or Matrigel have been used to culture endothelial cells for *in vitro* angiogenesis. Cells cultured in these 3-D gel systems not only form tube-like structures [37], but also show responses to different angiogenic and anti- angiogenic factors.

2.1.3.3.5 *Ex vivo* Solid Tumor Culture

In anti-cancer toxicology study, the failure to be faithful to *in vivo* cellular behaviors and predict drug efficacy is often illustrated. This could be mainly due to *in vitro* culture loses sight of specific microenvironment context of cells *in vivo*, which would control the regulation of important cellular behaviors [38] and environmental physical properties. For a chemical with direct toxicity *in vivo*, it must not only be able to kill the targeted cells, but also reach the cells with optimal quantity [39]. First, a possible limitation of toxicological evaluation using 2-D cell cultures is the overestimation of drug toxicity because this model exposes all cells to the same dose as in the medium while in 3-D multi-cellular organization the access to drugs is limited due to mass transfer. Second, the responses of individual cells to chemicals might alter due to many mechanisms such as gene expression perturbation in 3-D microenvironment [40-42]. For example, the high percentage of quiescent cells in solid tumor limits the efficacy of many chemotherapeutic drugs, such as 5-fluorouracial and gemcitabine, which target proliferating cells. Mellor et al. (2005) used serum-free medium to culture quiescent tumor spheroids, which mimicked the quiescent microregions of solid tumors. A quiescence marker $p27^{kip1}$ and a proliferation marker Ki-67 were used to study the cell cycle progression of the quiescent tumor spheroids (Figure 2.1). Finally, a long-term tissue response to external agents can rarely be reached due to the metabolic-waste accumulation and contact inhibition in the static culture. Therefore, although conventional 2-D cell cultures can be used in toxicological tests at a low cost and high speed, they cannot reproduce complex 3-D microenvironment *in vivo* in the long term and thus usually give unsatisfactory and

misleading results in the prediction of *in vivo* responses [38; 40]. In this aspect, 3-D *in vitro* cell models serving as a link between single cells and organs have a more physiologically relevant morphology, resemble cellular environment, and can reproduce *in vivo* cell metabolism, gene expression and differentiation considerably [43]. These resemblances can determine a similar biological behavior of the cells to chemicals that 2-D cell cultures are not able to offer [22; 44-47]. Furthermore, discrepancies in predictions of drug treatment effectiveness between 2-D and 3-D cultures highlight the advantage of using 3-D culture matrices [24]. Although 3-D cell models have significant advantages over conventional 2-D cell models and many different classes of models have been developed, such as tumor multi-celluar spheroids and multi-layer cell cultures, they very difficult to apply in high-throughput toxicological tests.

As a very complicated system, in addition to tumor cells, *in vivo* solid tumors consist of non-tumor stroma, which usually includes vasculature, extracellular matrix, fibroblast and immune cells. The complexities of tumor microenvironments make the study of tumor cell behaviors very difficult [47]. Tumor cell aggregation assays have been used for more than 25 years. Although these assays don't meet the current requirements of HTS in a temporal resolution, few other assays have been developed to replace it. In this aspect, Sasser et al. (2007) developed a 3-D assay system through detecting the signals from a red fluorescent protein to quantitatively and non-invasively monitor embedded 3-D cell growth [48]. This is a very good approach for the future development of HTS assays.

2.1.3.3.6 3-D ESC Differentiation

Recently, one of the most exciting areas of 3-D culture research is the study of embryoid bodies derived from stable ESC lines [43]. ESCs normally require the formation of aggregation structures, termed embryoid bodies (EBs), for *in vitro* differentiation to three primary germ layers and further specialization into somatic cells. These *in vivo* processes can be reconstructed *in vitro* in a 3-D environment. Imamura et al. (2004) stimulated EB differentiation and induce hepatic histogenesis after the formation of EB in polypropylene conical tubes [49]. Differentiated cells formed cordlike structures with liver-specific gene expression, which didn't exist in 2-D monolayer systems.

2.1.3.3.7 3-D Tissue Culture in PET Scaffolds

To mimic native tissues for *in vitro* toxicity studies, the key concept is that there is a strong relationship between tissue structure and function. Therefore, in order to achieve the desired functional attributes in a tissue-engineered construct for cell-based assays, the culture environment must represent the native counterpart. An important component in the tissue-engineered construct that allows for *in vivo*-like culture is the 3-D scaffold that supports cell adhesion, organization, growth and function. Tailoring the properties and culture system of the 3-D scaffold are thus essential in developing a representative *in vitro* tissue model for cytotoxicity assays. Proper matrix materials and configuration are important for high-density cell cultures, maintenance of excreted matrix molecules, cell aggregation, and gas and nutrient supplies [50]. Fibrous materials, such as non-woven polyethylene terephthalate (PET), have been shown to be advantageous because of their

high specific surface areas, excellent mechanical properties, and high void volumes [51]. The cells were able to aggregate naturally, attach along the fibers, and form bridges spanning between fibers in the matrix with their native morphology maintained.

Our group has developed a bioreactor with a 3-D fibrous matrix for culturing mammalian cells and tissues, including human trophoblast, osterosarcoma, colon cancer, and breast cancer cells [51-57]. Matrices of various porosities and pore size distributions were obtained by using a thermal compression technique [51]. It was shown that the porosity and pore size of the matrix indeed affected proliferation, differentiation, gene expression, and tissue function [51; 56; 58]. Detection of fibronectin secretion shows that the cells were able to aggregate naturally. It was established that the perfusion culture system could maintain the functional activities of the cells for a longer period of time compared to other culture systems [56]. Cow luteal cells (primary cells) cultured in a 3-D bioreactor environment could better maintain its normal function (progesterone secretion in response to LH stimulation) for a longer period as compared to cells cultured in the 2-D T-flasks. The ability for cells to maintain their normal function and response to environmental stimuli is critical in the development of cell-based biosensors.

2.1.3.4 Fresh Tissue and Organ

Another concern to better agree with real responses in body is to utilize fresh tissue, organ and even whole organism. Isolated perfused organ is the biological matter with the best *in vitro* physiological properties for different tests, but assays based on organs can't reduce the number of animals and the varieties between organs are large [59]. Tissue

slices are normally prepared from liver, heart, kidney, neural tissue and reproductive organs. Noraberg (2004) recommended to use brain slice culture for formal validation of neurotoxicity testing [60]. Sugai et al. (2004) reported the use of rat spinal cord slice culture into preclinical drug screening for amyotrophic lateral sclerosis [61]. However, although fresh tissues might be more organo-typic than simple cell culture, they are only good for short-term study because the preservation is problematic. Gupta et al. (2006) used frozen-thawed organ culture system to reduce the need to continual access to fresh tissues [62]. Another disadvantage is that because manipulation of tissue and organ culture is complicated, it is difficult to allow high-throughput assays with these cultures.

2.1.4 Detection Methods

2.1.4.1 Traditional End-point Assay

In general, the success of cytotoxicity assays depends upon the quantification of cell number and viability. There are many methods for quantifying cell number, including direct cell counting using a hemocytometer or Coulter counter, and indirect methods that detect an antigen, measure DNA synthesis, or monitor the reducing environment of cells. Cell viability is usually studied via the exclusion, inclusion or release of certain molecules; for example, trypan blue is excluded and neutral red is absorbed by viable cells, and lactate dehydrogenase release occurs only in damaged and dying cells [63]. Two assays based on medium oxygen concentration using ruthenium dye [64] and Alamar Blue [65] have been developed to save the manipulation of sample treatment; however, the sensitivity of ruthenium dye is low and Alamar Blue is unreliable for the

measurement of kinetics. There is also a spectrum of commercially available assays for measuring the intracellular reducing potential of proliferating cells, with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay being the most popular [66-68]. All of these methods are, however, invasive, requiring the addition of reagents. Cell culture must be disrupted for measurement, and multiple cultures are necessary for a time-course study, which is not only labor-intensive but can also result in large variations and errors. Furthermore, because of the invasiveness of these conventional assay techniques they can only provide endpoint data, which has been an intrinsic drawback for *in vitro* toxicity tests. It is preferable to have dynamic data that can reveal the toxic action of drugs. In addition, conventional endpoint assays require expensive robotic arms in order to realize automation for high-throughput screening (HTS). Also, HTS requires miniaturization that cannot be easily accommodated by conventional detection methods. Recent advances in cell biology, electrochemistry, optical instrumentation and nanotechnology have ushered in various new detection methods for HTS. Table 2.1 summarizes some of the commonly used detection methods in cell-based assays for HTS. They can be classified mainly into two categories: electrochemical and optical methods.

2.1.4.2 Electrochemical Methods

2.1.4.2.1 Introduction

Electrochemical methods of detection generally include three different approaches: (i) measurement of the electrical responses of electrogenic cells; (ii) measurement of the

conductivity of mechanical contacts between cells and a substrate surface; and (iii) measurement of the chemical signals (eg, pH) resulting from changes in metabolic products (eg, lactic acid) or substrates (eg, glucose) [69]. These methods can usually provide real-time, dynamic information on cell activities. Toxicity can be detected based on the electrical responses of electrogenic cells and tissues, such as heart muscle and nerve cells. Nerve cells have high-specificity receptors that are sensitive to many neuroactive compounds. Changes in the membrane potential influence the measurable capacity between a microelectrode and axon when nerve cells are grown on a field-effect transistor [70]. Devices using microelectrode arrays, such as multichannel systems, have been developed to monitor extracellular activities for toxicity assays [69].

2.1.4.2.2 Detection of Living Cells

A novel electrical impedance sensor array integrated into the bottom of a microtiter plate has been developed for the quantitative detection of living cells [71]. The presence of the cells at the electrode/solution interface affects the local ionic environment and increases the electrode impedance, which can be calibrated to obtain a 'cell index' that is proportional to the cell number. This method is based on the cell/substrate mechanical contact and is sensitive to the electrode area covered by the cells, which increases according to the total number of cells attached on the surface and their size. The device, called a 'real-time cell electronic sensing system' has been used to assay cytotoxicity, and may also be used to predict acute toxicity (Figure 2.2).

2.1.4.2.3 Detection of Metabolic Products or Substrates

Changes in metabolic products (eg, lactic acid and carbon dioxide) or substrates (eg, glucose and dissolved oxygen [DO]) can be monitored by measuring pH, DO or glucose, using a specific ion or enzyme electrode. A microphysiometer has been developed to measure the acidification rate in the vicinity of cells [72]. The pH value in pH-sensing chambers was used to indicate cellular biochemical responses resulting from the accumulation of lactic acid and carbon dioxide. A miniaturized system for oxygen detection was also reported for the study of heterogeneous pO_2 distributions around tissues [73]; however, this approach has not been widely applied in high-throughput cell-based assays, partially because metabolic activities can be affected by many environmental factors, which are usually difficult to control in a microbioreactor.

2.1.4.2.4 Advantages and Limitations

In general, electrochemical methods of detection are non-invasive and offer an appropriate temporal resolution; however, they cannot provide information on specific cellular activities that are directly related to certain cell functions, biomarkers or signaling pathways, which are important to the better understanding of the cytotoxicity effects and mechanisms of drugs.

2.1.4.3 Optical Methods

2.1.4.3.1 Introduction

Optical methods of detection are usually based on either measuring the intensity of fluorescent, UV or visible light, 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. The reporter gene techniques can be tailored to detect and quantify cell mass and specific cellular events and functions. The general approach is to couple the expression of an easily quantifiable protein with the control of a regulatory DNA sequence or promoter (see Table 2.1 for examples). Reporter proteins that are commonly used in cell-based assays include luciferase, galactosidase and various GFP mutants [4]. Compared with the GFPs, assays based on luciferase or galactosidase require the import of substrates into individual cells and the continuous provision of substrates in order to maintain certain doses during long-term dynamic measurements. As no sample preparation is required, except for initiation, assays using GFPs are superior in their capabilities of automation and real-time, non-invasive assessment of both chronic and acute cellular events [74].

2.1.4.3.2 Fluorescence Protein Technology

Recent advances in fluorescence protein technology and molecular genetics have made non-invasive live-cell kinetics assays using cDNA to encode a fluorescent protein extremely popular. The expression level of fluorescent protein is a function of the

corresponding regulatory element and can be directly detected by fluorescence intensity. This technique has attracted intensive attention because its non-invasiveness, simplicity and speed allow for miniaturization and HTS applications [75]. This technique can be responsive to targeted effects, such as gene expression and activation of signal transduction pathways, and is thus suitable for use in disease-relevant assays. It can also provide dynamic and multiplexing information, which is important because cellular responses to external agents are dynamic with multivariate information [71; 76; 77]. The GFP was first discovered in the jellyfish *Aequorea victoria* (avGFP), in which bioluminescent energy is generated by a proximal luciferase enzyme and turned into green fluorescence by resonance energy transfer. Many mutants of avGFP have since been developed with emission light colors ranging from blue to yellow. More recently, *Discosoma* species red fluorescent protein (dsRed) has become commercially available, and is often multiplexed with EGFP [78].

Whole-cell autofluorescence-based systems can be classified into three different quantification methods: flow cytometry, quantitative fluorescence microscopy and fluorometry. Despite the development of advanced high-throughput flow cytometry, quantification with a flow cytometer requires manual sample preparation and is suboptimal for high-throughput live-cell kinetic assays [79]. Commercial laser scanning imaging systems with fluorescence microscopy and quantitative image analysis, such as the KineticScan high-content screen reader from Cellomics Inc, the Pathway Bioimager from BD Biosciences and the IN Cell Analyzer 3000 from GE Healthcare, can perform live-cell kinetic assays for high-throughput drug screening. Such systems can examine

the context of living cells, quantify intracellular proteins and monitor the trafficking events of proteins fused with fluorescent reporters or some subcellular structures [80]. All of these measures can be translated into fluorescence signals, providing high spatial and temporal resolution; however, such systems are limited to late-phase compound characterization because of their high cost and relatively low capacity for high-throughput design [81]. The accuracy of the quantifications is another concern because they are limited to planar images and the fluorescent intensity from a single sample varies according to different foci, especially for three-dimensional (3-D) cellular structures (such as embryoid bodies) and *in vitro* 3-D tissue models. A fluorometer can provide a simple, accurate and economic platform for real-time quantification of fluorescence signals, but has not been widely used in HTS because of other limitations.

Fluorescence signals were first used to provide on-line assessment of the density of CHO cells involving stable GFP expression in a 96-well microplate using a fluorometer [82]. This method is fast, non-invasive and more robust than trypan blue exclusion and particle counter analysis; however, fluorescence signals generated in such assays are generally too weak to be quantified for *in situ* measurements using a fluorometer. This is largely because of multiple non-specific effects on the assay responses caused by changes in the biological environment, including pH, and the release of cell debris and other autofluorescent components into the culture medium [83]. Signals resulting from these non-specific effects could occur at a wavelength close to that of GFP and effectively mask GFP signals. Therefore, it is difficult for cells to elicit distinguishable fluorescence signals when this GFP reporter method is applied to cell-based assays.

Additionally, different fluorescent reporters can be used in fluorescence resonance energy transfer (FRET), which can report molecular proximity inside living cells [84]. Energy transfer between two close fluorophores can alter emission wavelength, which is dependent upon subcellular distances between the fluorophores. The assay is based on energy transfer between two tandem reporters such as GFP mutants fused with a peptide linker incorporating specific cellular function; for example, when the linker comprised a caspase-3 cleavage site (Asp-Glu-Val-Asp), the activation of caspase-3 in live cells could be studied dynamically [85; 86]. However, although exciting progress has been achieved using GFP-FRET constructs to sense cellular events in live cells, high-throughput application of these constructs suffered from insufficient signals above plate background and biological environmental autofluorescence unless detection is based on microscopy or flowcytometry, which is suboptimal for high-throughput design [87].

2.1.4.3.3 Nanoparticles

Fluorescent nanoparticles have been widely used as labels in cell-based assays. The use of conventional fluorescent molecules for simultaneous excitation and quantification of multi-targets with different emission peaks can be difficult because of their narrow excitation spectrum and broad emission spectrum. In addition, they are photo-unstable and can be quenched by continuous excitation. In contrast, semiconductor nanocrystals provide a better performance in this respect; they are photochemically stable and provide a narrow and adjustable emission. In addition, they can be excited by light of any wavelength shorter than that of the emission peak. Thus, multiplexing information from many emission colors can be obtained using nanoparticles of different sizes with light of

a single wavelength [88-91]. Quantum dots (QDs) are one of the most popular nanomaterials currently applied in biomedical research, mainly because they can be internalized into cells and used as fluorescent labels in cell-based assays [92]; however, QDs can be toxic to cells and are usually used in endpoint analysis.

2.1.4.3.4 Other Approach

Another approach is to induce natural color changes in cells such as chromatophore cells in the skin and scales of fish [93]. This approach can realize continuous, dynamic measurements, but the cell sources and functions of assays are extremely limited. Infrared radiation has also been employed as the detection subject in infrared-sensitive python pit organ using an infrared-sensitive camera [94].

2.1.5 Microbioreactor Array and Its Sampling

Rapid development in molecular cell biology, 3-D culture technologies, micro-fabrication, extracellular electrical recording, optical detection, nanotechnology and stem cell technology has pushed and allowed culturing mammalian cells in microbioreactor arrays for high-throughput cytotoxicity assays and cell culture process development. The attractive future of microbioreactors has brought up a commercial HT system, SimCell microbioreactor array from Bioprocessors, which has the ability to control and operate several hundreds of cultures at the same time. As a high-throughput scale-down model for stirred-tank bioreactors, this advanced system combined microfluidics, gas-permeable membrane and optical measurement technologies to optimize processes and predict the process performance in manufacture. However, multiwell plate-based systems have

become the majority of these miniature systems because their standardization eases the integration with existing automated, robotic liquid handling platforms to give the highest throughput capability [95-97].

Microfluidic perfusion culture is mainly applied in the *in vitro* development of functional livers and vasculature. Powers et al. (2002) fabricated a microbioreactor array for the perfusion culture of liver cells [98]. The 3-D device was constructed on an ion-etched silicon wafer, which provided an array of channels and tissue culture treated surfaces (Figure 2.3). They provided a continuous perfusate to liver cell culture by using a cell-retaining filter and the fluid was maintained at a physiological shear stress. Tissue-like structures were found 2 weeks after inoculation and remained for a long time. Fidkowski et al. (2005) described the microfabrication of capillary networks with PGS. Endothelial cells in the device were cultured with a physiological flow rate and formed tissue-engineered microvasculature [99].

Off-line sampling during the cell culture process is limited owing to the small amount of medium used in microbioreactors, ranging from several μl to several ml, so the expense of HTS capability is quality and quantity of information [95]. In this case, on-line detections are always required. Among the integrated measurement devices, optical sensors are easier for miniaturization and thus more popular than electrochemical sensors [100].

A number of different microbioreactors have used fluorescent dyes to monitor pH and DO in cultures [97; 101-105]. This is considered to be major progress toward a fully

instrumented microliter-scale bioreactor with useful applications that can benchmark with benchtop, liter-scale bioreactors. Kostov et al. (2000) set up an optical sensing system to continuously monitor pH, DO, and optical density in microbioreactors with a 2-mL working volume and their work showed that the profiles of all these parameters were very similar to a bench-scale bioreactor [103]. Zhang et al. (2006) integrated fluorescent sensors for pH and DO measurement into a 150 µl microbioreactor fabricated with PMMA and PDMS [106]. They also used gas-permeable membrane for oxygen diffusion to support high density cell growth. The cell growth in their microbioreactor was benchmarked with bench-scale operations. Another novel detection approach that has been developed involves using a mass spectrometer probe with a membrane inlet [107]; however, in addition to its complex design and high cost, this technique is only useful for monitoring volatile compounds and is not ready for use for high-throughput experiments, limiting its application. Although current microbioreactors can be featured with online pH and DO measurements, it is not feasible to non-invasively monitor protein productivity as a function of time. Betts et al. (2006) suggested that incorporation of biomarkers such as GFP could help to solve this problem because GFP was not harmful to cell activity [95]. However, high and fluctuant background fluorescence and relatively low signals from live cells have limited this application in mammalian cell cultures [83].

2.1.6 Applications

Cell-based assays and related microbreactor arrays have been applied in various fields including safety assessment, high-throughput drug screening with chemical libraries, biodiagnostics, and biological manufacturing [4].

2.1.6.1 Detection of Environmental Unknown Threat Agents

Cell-based biosensors are devices combining intact, living cells with physicochemical detectors in order to estimate the physiological consequences of unknown threats such as pathogens, toxic chemicals, and other agents. It is important for environmental monitoring and homeland security. Molecular interrogation using biological cells could be easily realized in laboratory, but in non-laboratory settings, it is still a challenge. Cell-based biosensors are developed to reduce this discrepancy [108-110]. Cell-based biosensors share the same preference as laboratory cell-based assays to use non-invasive detection approaches for continuous usage, so electrical recordings and live fluorescent measurements are preferred. The main characteristic which a cell-based biosensor has while a laboratory cell-based assay not is portability. The realization of portability is often hampered by the issue of maintenance of the same cell status and biological environment. The reliability would also be reduced by the discrepancy of *in vitro* cultures and *in vivo* microenvironment, and low signal to noise ratio (SNR) due to environmental fluctuations. Advancement in stem cell and 3-D tissue culture technologies could increase the field application of cell-based biosensors in the near future [12].

2.1.6.2 Drug Discovery

High-throughput screening plays an essential role in the initial identification of leads because the pool of chemical compounds and biological targets is rapidly expanding. After initial screening, compounds selected (hits) are evaluated for many criteria, including efficacy and toxicity to identify leads such as absorption potential, blood-brain

barrier, enzyme inhibition, enzyme induction. In this step, cell viability and proliferation assays in cell culture models are usually applied. However, cell-based assays usually suffer from inconsistent culture environments different from their *in vivo* counterparts [41; 42]. So far, few studies focused on the application of 3-D *in vitro* mimicking tissue cultures in high-throughput drug discovery, because 3-D culture models have proved very difficult to use in high-throughput screening.

2.1.6.3 Biological Research

Probably the most attractive application of cell-based assays in basic biological research is newly established transfected cell array (TCA) technology. The human genomes project completed in 2005 has determined all base pairs and genes in human genomes and provided the opportunity to high-throughput study genetic information. TCA was developed to fulfill this purpose. TCA transfected cells with printed DNA or RNA on a solid array in order to overexpress or silence specific genes in spatially distinctive sets of cells. The cellular biology was then studied with different read-out such as live fluorescence or immunostaining [111-114].

2.1.6.4 Bioprocessing

Mammalian cell microbioreactors offer a potential to reduce medium cost and intensity of labor and time for various cell cultivations in process development by highly parallel evaluation of process options. They have been widely applied in the fields of medium development, strain selection and process optimization, increasing industry productivity to bring new drugs to market [95; 100; 115; 116]. It is desired that microbioreactors could

mimic larger scale operations in order to provide useful information which would be further tested in bench-top bioreactors followed by pilot-scale bioreactors.

With all of these developments, it is envisoned that, before long, the use of microbioreactors for bioprocess development and cell-based assays will become routine practice in pharmaceutical industry.

2.2 Embryonic Stem Cells

Derived from the inner cell mass of mouse blastocysts, mouse ESCs could only be maintained by co-culture with embryonic fibroblasts at the beginning. Later it was found that conditioned media from mouse embryonic fibroblast could replace the feeders. Now, cytokine leukemia inhibitory factor (LIF) is commonly used for undifferentiating maintenance of feeder-free mouse ESC culturing.

2.2.1 Molecular Characterization of Undifferentiated Embryonic Stem Cell

Undifferentiated ESCs have a special pattern of gene expression, such as SSEA-1, Oct-4, gp130, alkaline phosphatase and telomerase. These discoveries can be used for maintenance of pure ESC culturing. For example, with the introduction of a neomycin resistance into the *Oct-4* gene, the presence of G418 can kill all differentiating mouse ESCs, which have lost their *Oct-4* expression [117]. Eiges et al. (2001) extended this approach to human ES-like cells by using the expression of green fluorescence protein (GFP) driven by a stem cell-restricted *Rex-1* promoter, which allowed FACS purification to maintain undifferentiating status [118].

2.2.2 ESC Differentiation

2.2.2.1 ESC Differentiation with Embryoid Body Formation

As shown in Figure 2.4, ESCs have the capacity to develop into any specialized cell lineage in the body, including cardiac, neuronal, muscle, epithelial, hematopoietic, pancreatic, hepatic, chondrogenic, osteogenic, and germ cells [119]. *In vitro* differentiation protocols of ESCs for specialized cell lineages such as cardiomyocytes, neurons and pancreatic cells have been established [120]. ESCs normally require forming EBs for *in vitro* differentiation to three primary germ layers and further specialization to somatic cells. Generally, undifferentiated ESCs are cultivated as EBs in handing drops or suspension for 4-9 days, varying from the targeted differentiated cell types and different protocols, before being plated on an adhesive substrate such as gelatin-coated plates for further differentiation. During differentiation, specific genes, proteins, and ion channels are expressed in a similar continuum as the developmental pattern of embryogenesis. These specific genes, proteins and ion channels provide very useful markers for the study of developmental processes.

2.2.2.2 ESC Differentiation with Monolayer Culture

Currently, differentiation with monolayer culture has become an alternative to differentiation in EBs. However, the phenotypes by this novel method require further careful study. Nishikawa et al. (1998) first used differentiation in monolayer cultures to produce specific mesodermal cell types [121]. Tropepe et al. (2001) reported the derivation of primitive neural stem cells with monolayer cultures [122]. This technique

may provide a more promising prospect for high-throughput screening since less manipulation is required [13].

2.2.2.3 Isolation of a Specific Lineage and Monitoring Specific Cellular Events

Because of EB formation as aggregates and simultaneous development to various cell phenotypes during differentiation, the quantity and purity of tissue-restricted lineages limit their application to therapeutic regime. There are three ways to enrich or isolate a population of a differentiated lineage. The first is to plate EB in medium with specific growth factors to induce a specific cell type. The second is called embryonic lineage selection by flow cytometry sorting or drug selection, which uses stable ESC lines with integrated reporter genes such as GFP or drug resistant genes under the control of tissue-restricted promoters [123; 124]. The last approach is to overexpress transcription factors such as MyoD to force differentiation [125; 126]. Among these 3 methods, the second is able to reach the highest purity (>99%) and theoretically can be applied for differentiation of any specific lineage. Table 2.2 is a summary of undifferentiating and tissue-specific promoter/enhancer used for isolation or detection of a specific lineage.

2.2.3 Embryotoxicity

2.2.3.1 Antineoplastic Therapy with Pregnancies

There are about 3500 cases of cancer-complicated pregnancies every year in USA [127]. Dilemmas are always raised for physicians because life-saving antineoplastic therapy for mother poses life-threatening concerns for fetal well-being. All chemotherapeutic drugs

are potentially very dangerous, because they might act on actively proliferating cells, which are more similar to cells in rapidly growing tumor than most of non-proliferating somatic cells. It has been reported that the risk of abortion and malformation will increase if chemotherapy comes up at the beginning of a pregnancy. The potential adversities of these drugs are related to the drug regimen and the timing. However, physicians must rely on retrospective studies from case reports because no clinical trial is likely to be allowed. In this case, the evaluation of chemotherapeutic risk on fetus well-being is difficult to establish. Recently, as the quick advance of embryonic stem cell biology, the systematic study of reproductive outcome of cancer treatment regime has been performed with different assays and embryonic stem cell test (EST) is among the most promising assays for developmental toxicology test [128].

2.2.3.2 Embryonic Stem Cell Test (EST)

Currently, three most promising *in vitro* tests for embryotoxicity and cytotoxicity are being subjected to formal validation in European ring trial: the rat limb bud micromass assay (WEC) test, the rat whole embryo culture assay (MM) test and the embryonic stem cell test (EST).

For in vitro screening systems of embryotoxicity or cytotoxity, the effects of drugs, compounds or environmental factors include two phases, regeneration of pluripotent cells and differentiation of a specialized cell lineage, both of which ESCs meet. In contrast to the WEC and MM tests, the EST has the advantage of using established cell lines without the need to sacrifice pregnant animals [14; 59; 128]. Using two established mouse cell

lines, 3T3 fibroblasts and D3 ES cells, the EST applied three endpoints to test the toxicity of chemical compounds: cytotoxicity of ESCs, cytotoxicity of mouse 3T3 fibroblasts and the effects of differentiation inhibition into cardiomyocytes (Figure 2.5). The concentration of a chemical causing 50% inhibition of cell number at a certain time point (IC_{50}) in conventional 2-D culture was used to define the cytotoxicity of this chemical. After comparison of the potential of chemicals in EST to *in vivo* experiment, it was discovered that this assay is accurate to predict embryotoxicity or cytotoxicity [2; 15; 129].

2.2.3.2.1 General Assay in EST

ESCs form aggregates as EBs and simultaneously develop into various cell phenotypes during differentiation process. An *in vitro* assay with w/t ESCs for the quantitative assessment of toxicity related to a specific lineage is complicated. Normally, eye observation, immunological or molecular methods are required to quantify the effects on a specific cells type, which are time-consuming and therefore not suitable for the design of an automated and high-throughput screening cell-based assay. It is necessary to develop an easy method to quantify specific cell types within EBs.

2.2.3.2.2 Application of Fluorescence Protein in EST

With a stable ESC line expressing GFP under the control of a cardiac alpha-actin gene promoter, Bremer et al. (2001) used FACS analysis following trypsin treatment to determine the effects of 15 chemicals on cardiac differentiation [129]. Their results showed that fluorescence given by GFP inside of cardiomyocytes could provide an

appropriate quantification to determine the toxicity of chemical compounds on developing cardiac cells. Furthermore, this genetically engineering technique also offers an objective measurement, instead of original evaluation by an experienced person. However, FACS is suboptimal for the design of high-throughput screening cell-based assays despite the current development of high throughput flowcytometry (HTFC). An easier way to measure the fluorescence of GFP in EB bodies is the quantitative image analysis without the treatment of EB bodies [130]. Paparella et al. (2002) established a stable D3 cell line with GFP expression regulated by an alpha-fetoprotein enhancer, which was used as a marker for endodermal cell differentiation. The effects of chemicals on the development of endodermal cells were evaluated by microscopic image analysis (Figure 2.6). Although the robustness was enhanced and the measurement could be repeated at different time points in the same plate since this novel method had no requirement to manipulate the EBs, quantification of cells in 3-D aggregates with a microscope which can only achieve planar images would cause issues such as different intensities from the same sample according to various focuses. In this context, the measurement of GFP with fluormetry may provide a more accurate and convenient way to quantify the embryotoxicity and cytotoxicity in real time. Furthermore, the use of other tissue-restricted promoters would extend the analysis on developmental cardiomyocytes to other specialized cell types. Theoretically, every specialized cell type would have its own embryotoxicty-screening assay with a corresponding gene cassette inserted.

2.2.3.3 Other Toxicological Study Using ESCs

In addition to the developmental process of ESCs, another approach to use ESCs in the toxicological field, which is still being validated, is the application of ES-derived specialized lineages for the assessment of toxicity. It is arguable that fully functional cellular types such as cardiomyocytes, hematopoietic cells and hepatocytes from mouse ESCs can play a better role than primary cells in *in vitro* cytoxocity tests due to their sufficient and uniform supply, and superior cellular functions of certain ES-derived progenies [13].

2.3 Colon Cancer and Anti-colon Cancer Drugs

Colon cancer is the third most common cancer and the second leading cancer causing death only next to lung cancer in western countries. Surgical extraction combining chemotherapy, immunotherapy and radiotherapy is the typical treatment of colon cancers [131; 132]. 5-fluorouracil was the most commonly used chemotherapeutic agent against colon cancer during the past few decades and new usages combining 5-FU with other drugs are continually emerging. Currently, advanced colon cancer is treated with 5-FU and folinic acid, but the remission rate is only 23% and response rate is 40-50% [133].

2.3.1 5-FU

Fluorouracil, as an antimetabolite, was first developed in 1957 following the observation that tumor cells utilized more uracil for RNA synthesis than other normal cells. 5-FU was used to treat numerous types of neoplasms including breast, esophagus head and neck

cancer, and the greatest impact of it is in colon cancers. 5-FU can inhibit a target enzyme thymidylate synthase (TS) and cause the misincorporation of fluoronucleotides into RNA and DNA. 5-FU inhibits cell growth with cell cycle accumulation in early S phase [134; 135] and its activity is limited in S-phase [136]. Understanding the mechanism of its cytotoxicity and the resistance of solid tumors to it are critical for the future 5-FU modulation development [137].

2.3.2 Gemcitabine

Gemcitabine, another antimetabolite, is now an established antineoplastic agent in pancreatic cancer and non-small cell lung cancer chemotherapies. It also has a remarkable activity on ovarian cancer, but not colon cancer [138; 139]. In the study of human tumor xenografts, colon cancer xenografts were less sensitive to gemcitabine than ovarian, head and neck tumor xenografts [140-142]. Its chemotherapeutic activity of gemcitabine is mainly due to the incorporation of its metabolite into DNA and RNA and the inhibition of nucleotide-synthesizing enzymes. Therefore, it is another drug to cause S-phase arrest.

2.3.3 Butyrate

Butyric acid, one of the 3 most common short chain fatty acids produced in mammalian colon, has pleiotropic stimulatory effects [143]. Butyric acid has the ability to help abnormal or transformed cells change into a normal status. It can also induce the hyperacetylation of histones, which results in the increase of DNases accessibility to chromosome DNA [144]. Cell cycle arrest at G0/G1 phase is commonly observed in the

presence of butyrate and accompanied with the induction of a cell differentiation and finally apoptosis. Its antineoplastic activity by the induction of differentiation and antiproliferation can be synergized with other therapeutics including 5-FU [145]. Bras-Goncalves et al. (2001) reported that the synergistic efficacy of 5-FU and butyrate could be due to the further reduction of thymidine synthase (TS) and prevention of thymidine salvage [146]. Butyrate is also a popular productivity enhancer in biopharmaceutical companies. Boffa et al. (1981) reported that butyric acid could cause cells to continue synthesize proteins with DNA synthesis stopped [147].

2.4 Mammalian Cell Culture

Although on the current market, biotechnological products from microbial processes are dominated numerically, blooming biopharmaceutical industrys uses more and more animal cell cultures to produce protein products in their desirable ways such as glycosylation, which microbial processes cannot. Mammalian cell cultures require more stringent processes to produce complex products such as monoclonal antibodies, viral vaccines and other therapeutic proteins. Therefore, intensive research has been carried on the development of novel bioreactors, among which fed-batch and perfusion are considered as the most popular in industrial manufacturing with large capacity. Fed-batch processes occupy 90% of biopharmaceutical manufacture because of their decent productivity and easy process manipulation. Compared to Fed-batch process, perfusion culture systems have a much higher throughput of products (approximately 10 times higher than batch or fet-batch). However, in addition to their complexity in process

development, clogging filter during cell retention often cause problems in perfusion systems [148-150].

2.4.1 Fibrou Bed Bioreactor (FBB)

Because of the fragility of cell membrane, mammalian cells are very sensitive to shear stress caused by mechanical agitation and aeration. In both batch and fet-batch bioreactors, a high cell density with conventional suspension is difficult to achieve due to the accumulation of metabolites and depletion of nutrients [150]. Cell immobilization in perfusion bioreactors can overcome both problems by protecting cells from shear damage and continuously refreshing medium. A fibrous bed bioreactor (FBB) using polyethylene terephthalate (PET) as fibrous matrices has been developed for cultivation of various cell lines, such as CHO, hybridoma, human osteosarcoma, human cytotrophoblast, mouse ESCs, and human ESCs [51; 53-56; 151; 152]. Ouyang et al. (2006) established a continuous culturing process to economically expand mouse ESCs [153]. In their two-stage process system, STO cells were used to provide conditioned medium to remove the requirement of LIF. ESCs could expand 193-fold within 15 days. The FBB provides high porosity, high surface to volume ratio, high permeability, low pressure drop, easy downstream processes, low material cost and *in vivo* mimicking microenvironment. When a FBB is used in perfusion culture, clogging filter can be prevented because all cells are immobilized within 3-D scaffolds. Previous results show that a perfusion FBB was able to allow high density cell culture (3×10^{8} cells/ml) and give high productivity for a long time (up to months) [52-55; 151]. Compared to other immobilized cell culture systems, FBBs are more likely to be applied into industrial production because of these

advantages. One reason that it has not been commercialized is partially because cell growth on fibrous scaffolds couldn't be well quantified in the middle of culture, which made the study of cell seeding and growth in 3-D matrix very difficult.

2.4.2 On Line Biosensors in Bioreactors

Besides commonly used online pH, DO and temperature probes, people show great interest in real time detection of viable cell density. There is a common sense that online quantification of viable cell density is one of the focuses in the future development of monitoring and control regime in bioprocessing. It is often necessary to improve bioprocess quality and address optimization by modeling cell population dynamics, because protein productivity has a positive relationship with specific growth rate [154; 155]. Many different approaches have been developed for online growth quantification in bioreactors, but more effort is necessary to find a robust and reliable online detection system, which could be easily adapted to most cultivations. Optical density and medium capacitance are not linearly responsive to cell mass and have low sensitivity due to high noise, although they are probably the easiest to detect. The sensitivity improves with the probe of turbidity, but values are subject to changes in cell viability and aeration. The acoustic system and advanced spectroscopy such as NIR and NMR are still under development and not available for common usage even for academic uses [150; 156]. Newly developed *in situ* microscopes (ISM) have proven suitable for cell mass measurement. Guez et al (2004) used pulsed illumination of the liquid broth and synchronized with a camera frame to avoid blur due to cell motion, followed by principal component analysis to evaluate the robustness of ISM prediction [157]. However, their

application is limited to suspension cultures. To date, none of these methods is suitable for immobilized cell cultures.

Besides viable cell density, attention has been drawn to the real time, non-invasive assessment of cell physiological status and productivity. Due to the requirement of reactions or chromatography, reliable *in situ* probes for direct protein production measurement are virtually impossible. The conventional method consists of collection, dilution and further analysis at different time points, which is time-consuming and prone to contamination. Flow injection analysis (FIA) measuring nutrients and metabolites provides another solution by automatic online sampling and allows on-line quantification of mAbs with integrated HPLC [158; 159]. Blankenstein et al. (1994) connected a FIA to enzyme sensors for glucose, lactate, glutamine, glutamate and ammonia detection. It could monitor a culture up to 15 days and the analysis of all five compounds could be completed in 42 min [158]. However, FIA is still limited by the issues of enzyme instability, interferences with other similar components and risk of contamination during sampling in addition to their complexity and expense [156]. Green fluorescent protein (GFP) is normally used as a non-invasive reporter in mammalian cells without interference with other cell activities. The application of it to reflect promoter activity, recombinant protein production and location has been widely studied [160; 161]. In comparison with synthetic indicators, all manipulation steps and toxicity caused by reagent addition are eliminated in GFP technology, so the approach provides a great potential to monitor dynamic properties of cultures such as cell mass, cell physiology, and even protein productivity [162-166]. Randers-Eichhorn et al. (1997) was the first to

realize online fluorescent detection of GFP in bioreactors with LED excitation [167]. Hisiger and Jolicoeur (2005) applied GFP as a non-invasive indicator of the physiological state of cells to monitor culture progress [163].

2.4.3 Effects of Butyrate on Protein Production

Butyrate has pleiotropic stimulatory effects. It can enhance protein productivity by inducing histone hyperacetylation to increase the accessibility of DNase to chromosome DNA. At the same time, it can also stop DNA synthesis, inhibit cell growth and cause apoptosis. Because of these opposite effects, butyrate is a popular enhancer for protein production in bioprocessing, while the overall effect on productivity varied and is unpredictable with different cell lines and different treatment strategies [144; 168]. Cherlet et al. (2000) reduced the inhibitory effect by adding butyrate after cell growth had reached a significant density in the middle of a batch culture and maintained the culture long enough for its action before the final collection [169]. It was a successful strategy to increase the monoclonal antibody production. Hunt et al. (2001) optimized butyrate treatment by the measurement of GFP expression. Their work showed that fluorescence could help to examine the optimal butyrate concentration, the exposure time, and the time of addition [170]. Jiang and Sharfstein [171] indicated that proteins with a lower expression level would have greater enhancement by butyrate treatment than those with a higher expression level.

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Detection method	Targets	Comments	References
Electrochemical			
Electrical impedance	Mechanical contact between cells and electrode surface	Electrical impedance increases for cell contact with electrode surface owing to reduced conductivity	[69; 71; 172-174]
Electrical response	Electrogenic cells	Electrical responses to extracellular stimuli	[69; 70]
pH, DO	Changes in metabolic products and/or substrates	Indirect, can be inaccurate as metabolism is not always linear with cell number or growth rate	[72]
Optical			
Reporter genes:			
Luciferase	Cell number, cell mass, specific cellular function unit	Requires substrate addition	[4]
Various GFP mutants		Dynamic, no substrate addition, requires excitation with light	[74; 175; 176]
Galactosidase		Endpoint analysis	[4]
Nanoparticles	Specific cellular function unit	Good for multi-targets, photostable	[88-91]
Chromatophoric cells	Cell mass or specific cellular function unit	Dynamic, no substrate addition, limited sources of cells and functions	[93]
Cells sensitive to infrared radiation			[94]

DO, dissolved oxygen; GFP, green fluorescent protein

Table 2.1. Non-invasive in situ detection methods for cell-based toxicity assays.

Specific lineage	Gene cassette (Promoter and related reporter gene)	References
Heart		
Cardiomyocyte	α-cardiac MHC-G418	[123; 177]
	α-cardiac MHC-LacZ	[178]
	α-cardiac MHC-EGFP	[179]
	MHC-SEAP	[180]
	MLC-2v-EGFP	[181-184]
	MLC-2v-beta-galactosidase	[185]
	Cardiac α-actin-GFP, G418	[129; 186]
	Cardiac α-actin-GFP	[187]
	HSVtk/GCV	[188]
	GFP-IRES-PAC	[188]
Chamber myocardium	Cardiac-specific distant upstream part of the Na^+/Ca^{2+} exchanger-EGFP	[189]
Neural		
Neurons	A necdin promoter-PAC	[190]
Neuroepithelial cell	Sox-1-GFP	[191; 192]
Neural precursor	Thymidine kinase promoter/nestin second intron-EGFP	[193]
Dopaminergic neuron	TH-EGFP	[194]
	TH-GFP	[195]
Others		
Vascular endothelium	Tie-1-EGFP	[196]
Endothelium	Flk-1-GFP	[197]
Epithelial	CYP7A1-GFP	[198]
Melanoblast	Dct-LacZ	[199]
Renal progenitor	LacZ/T/GFP	[200]
ESCs (undifferentiated)	OCT4-EGFP	[117; 192; 201]
	Rex-1-EGFP	[118]

EGFP, enhanced green fluorescent protein; ESC, embryonic stem cel;, GCV, ganciclovir; GFP, green fluorescent protein; HSVtk, herpes simplex virus thymidine kinase; IRES, internal ribosome entry site; MHC, myosin heavy chain; MLC, myosin light chain; PAC, puromycin *N*-acetyltransferase; SEAP, secreted alkaline phosphatase; TH, tyrosine hydroxylase.

Table 2.2. Some cell-lineage-specific promoters and reporter genes used in ES cell studies.

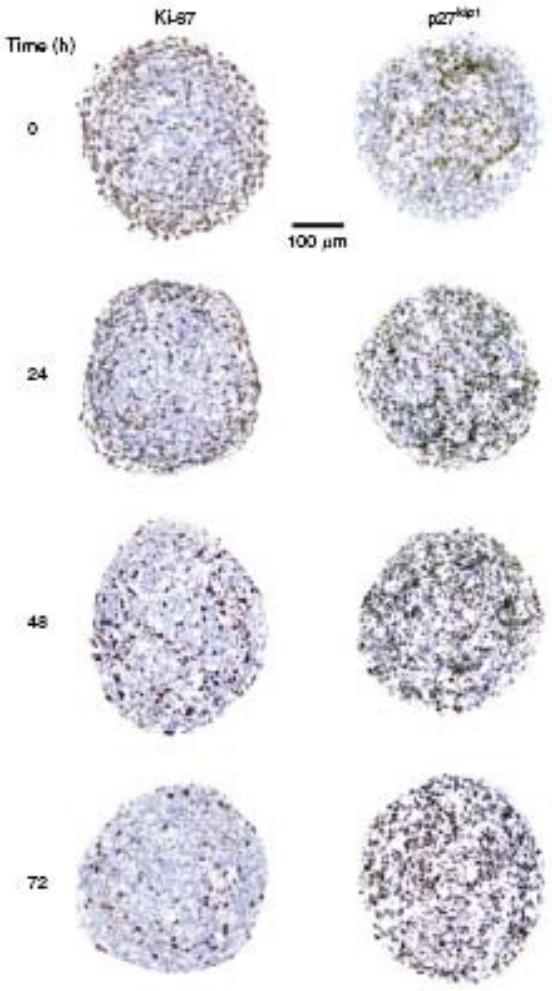

Figure 2.1. The expressions of p27kip1 and Ki-67 in tumor spheroids after serum withdrawal studied with immunohistochemistry. (from British Journal of Cancer 2005; 93(3): 302-309)

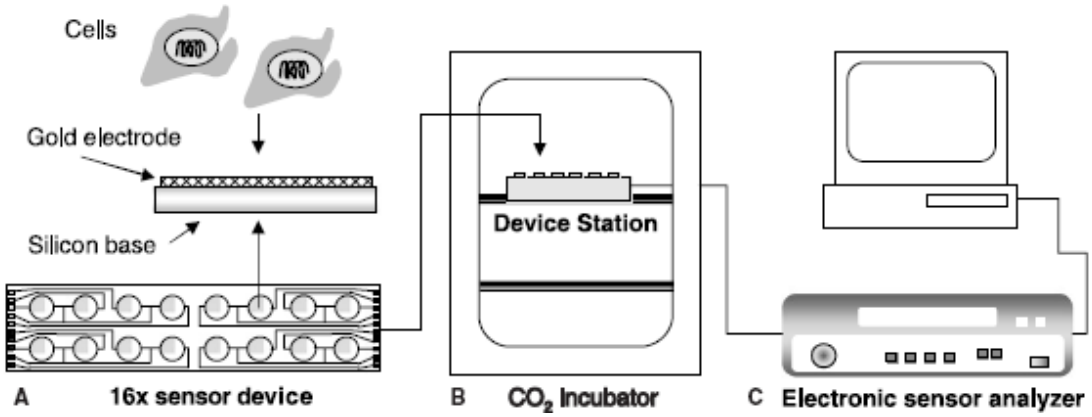

Figure 2.2. Real-time cell electronic sensing system. A. The array had 16 cell culture units and 16 electrodes on a glass slide. B. The array in a cell culture incubator. C. A sensor analyzer to continuously measure the selected wells automatically. (from Toxicology in Vitro 2006; 20(6): 995-1004)

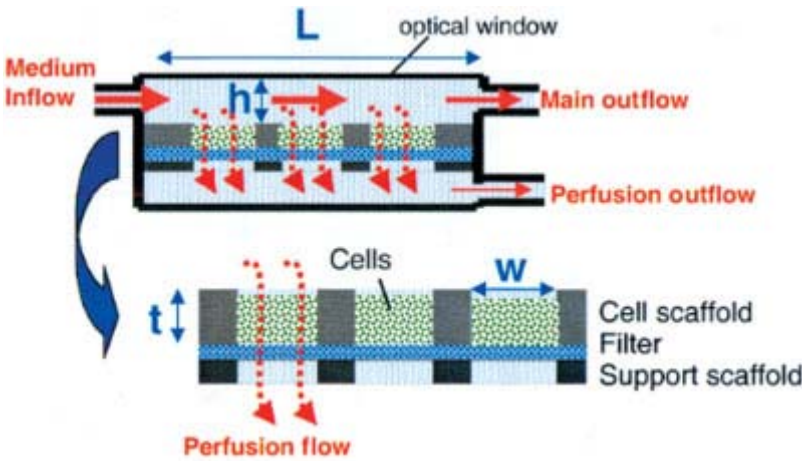

Figure 2.3. The structure and medium flow of the microbioreactor array. (from Biotechnology and Bioengineering 2002; 78(3): 257-269)

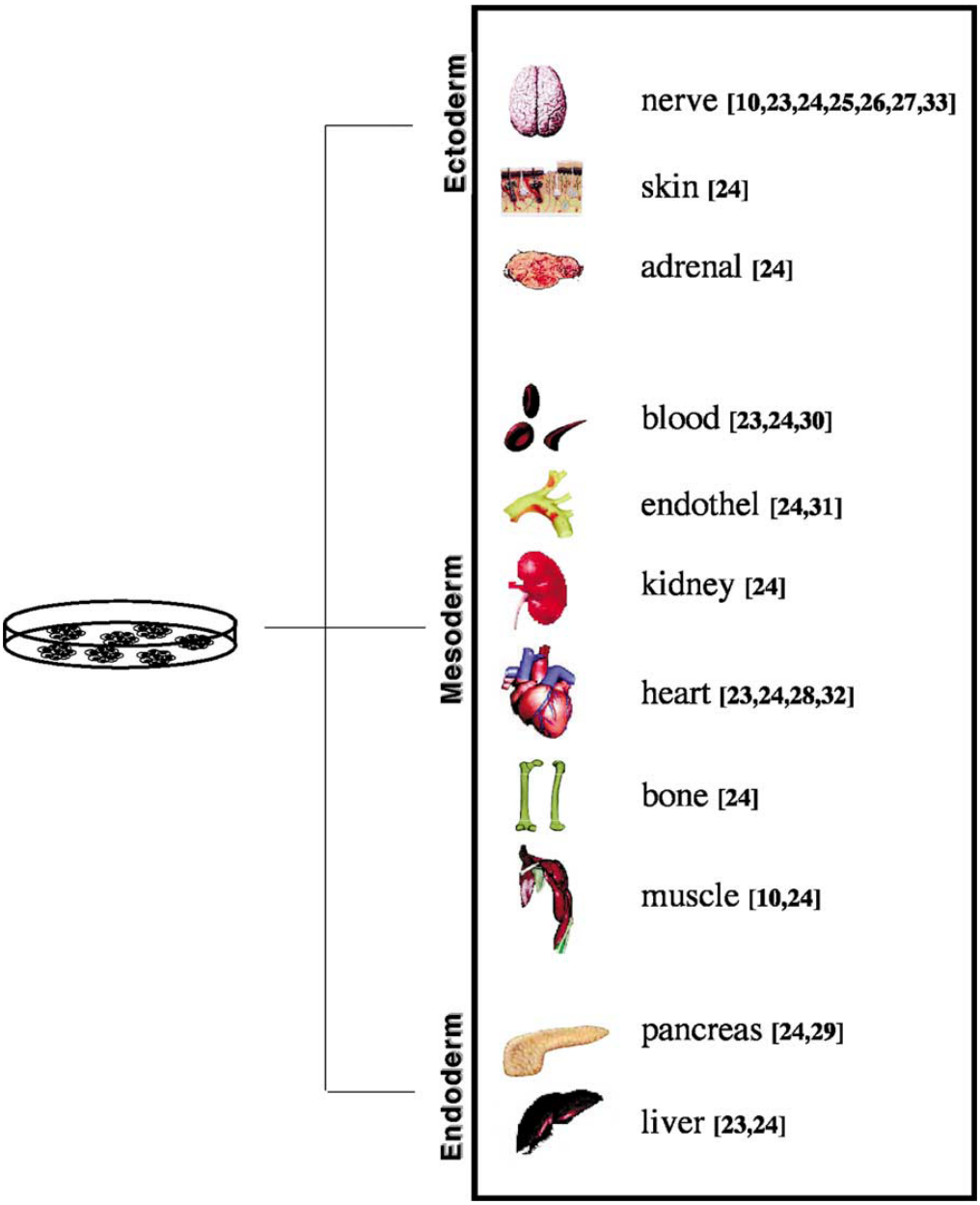

Figure 2.4. The diagram of ES cell differentiation. (from FEBS Letter 2002; 529(1): 135-41)

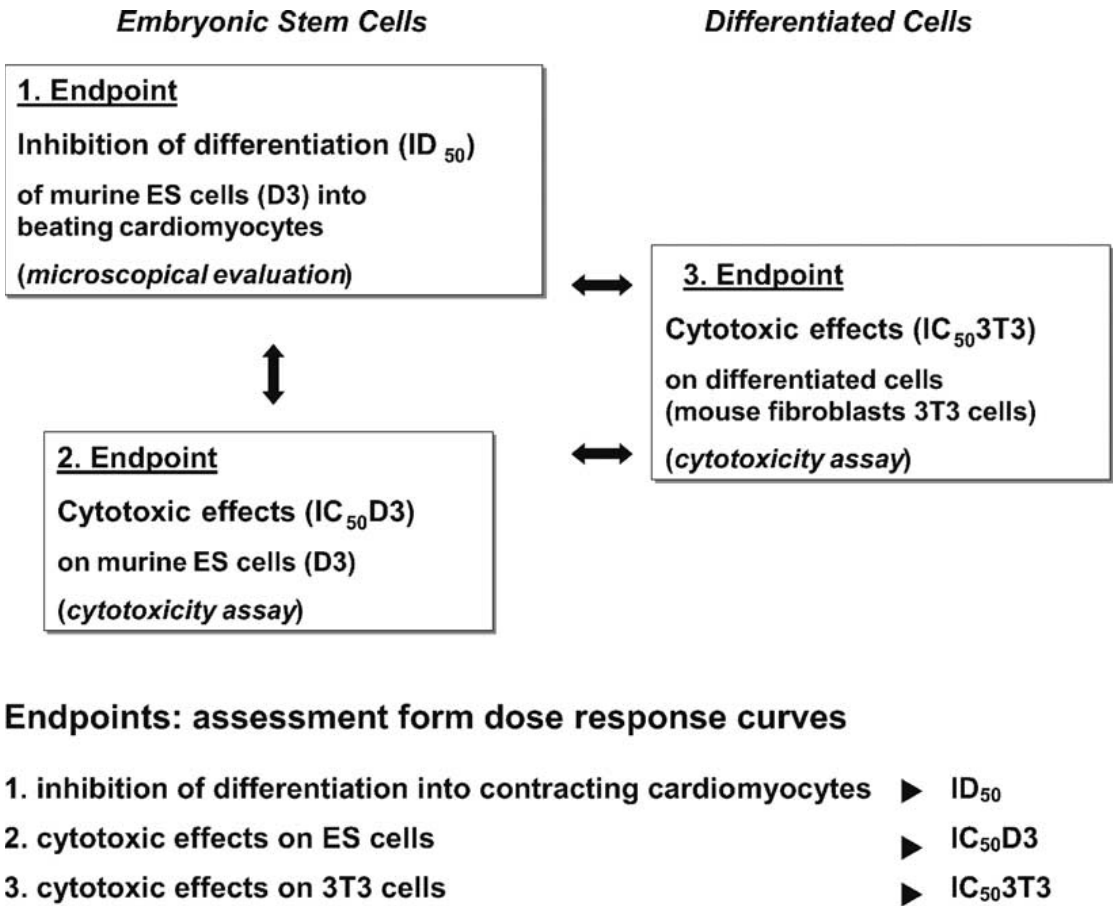

Figure 2.5. The embryonic stem cell test (EST). The diagram showing the three endpoints in EST (from Reproductive Toxicology 2004; 18(2): 231-240)

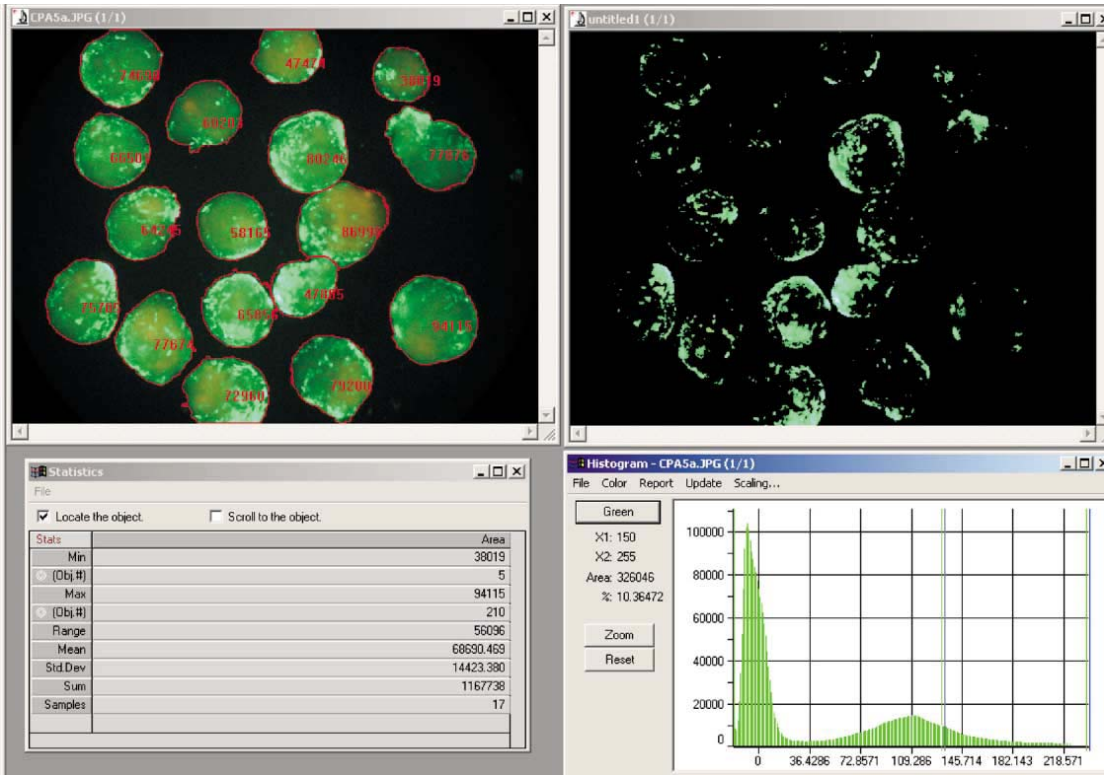

Figure 2.6. Microscopy image analysis to quantify the effects of chemicals on the development of endodermal cells. Images were taken, analyzed and displayed (top left) and the related statistics was calculated (bottom left). Green fluorescent intensity above a certain value was visualized (top right) and analyzed (bottom right). (from Toxicology in Vitro 2002; 16(5): 589-597)

CHAPTER 3

DEVELOPMENT AND VALIDATION OF A HIGH-THROUGHPUT 3-D CELL-BASED ASSAY PLATFORM

3.1. Abstract

Three-dimensional (3-D) cell culture systems are generally better than 2-D cultures for efficacy analysis because 2-D culture models lack proper *in vivo* tissue functions and are inherently prone to error. In addition, the higher cell density in 3-D cell culture gives a potential for higher SNR. We have designed, built and tested a high-throughput, real-time, bioactivity assay based on the three-dimensional (3-D) culture of green fluorescent protein (GFP)-expressing mammlian cells, which can increase SNR by at least one order of magnitude as compared to the conventional 2-D culture system. In this 3-D system, cells with stable enhanced GFP expression were cultured on pre-treated nonwoven polyethylene terephpthalate (PET) fibrous scaffolds. The whole culture system was carried out in modified microplates stacked on a shaker in a cell culture incubator. Fluorometry was used to quantify fluorescence intensity in real time. The effects of fetal bovine serum (FBS) concentration and fibronectin coating on 3-D embryonic stem (ES) cell culture were evaluated in the system. The results from cytotoxicity tests of

dexamethasone (DM) on low density ES cells and 5-fluorouracil (5-FU) and gemcitabine on high density colon cancer cells HT-29 demonstrated that the 3-D culture system could afford parallel, automated, accurate and long-term toxicity test. The system also showed the capability to monitor acute cellular responses. The applications and advantages of this new potential high-throughput cell assay system in drug discovery and bioreactor process optimization are discussed in this chapter.

3.2. Introduction

Drug discovery is a process involving biological target identification and validation, the selection of lead compounds for optimization, and clinical trials [1]. High-throughput screening plays an essential role in the initial identification of leads because the pool of chemical compounds and biological targets is rapidly expanding. After initial screening, compounds selected (hits) are evaluated for many criteria, including efficacy and toxicity to identify leads. In this step, cell viability and proliferation assays in cell culture models are always applied. Currently, pharmaceutical firms spend large amount of money on the compound efficacy and cytotoxicity test [2]. However, there is still a 78% failure rate for all drugs, which may be devastating to developing companies. Effective compounds *in vitro* may be non-effective *in vivo* for many reasons, including differences between *in vitro* and *in vivo* target biology, interrelated biochemical mechanism, metabolism, poor penetration into solid tissues, etc. Almost all cell-based assays or biosensors are developed in 2-D culture systems, although conventional 2-D cultures usually suffer from contact inhibition and a loss of native cell morphology and functionality. In comparison with 2-D cultures, 3-D cell models create a more realistic representation of real human

tissues, which is critical to many important cell functions, including morphogenesis, cell metabolism, gene expression, differentiation and cell-cell interactions [3]. Discrepancies in predicted drug treatment effectiveness in 2-D and 3-D cultures implicate the advantage of using 3-D culture systems [4-6]. For cell-based sensing, particularly in studying cytotoxicity and drug discovery, maintaining cells in their native functional state in a proper 3-D environment would improve predictions and have the potential to reduce clinical trial failures. Smitskamp-Wilms et al. (1998) exposed colon cancer cells to gemcitabine in 2-D and 3-D culture and found that 3-D multilayer cells were over 1000 times more resistant to gemcitabine than cells in 2-D cultures [6]. These results coincided with the lack of *in vivo* efficacy of this drug for colon cancer in clinical trial. However, 3-D culture models have proved difficult to be used in high-throughput screening (HTS), although they have significant advantages over 2-D culture.

3-D culture could also enable a sensor to test the cytotoxicity of those analytes requiring a longer period to take effect. Recently, we discovered that cow luteal cells cultured in a 3-D environment were able to maintain their *in vivo* function for a longer period than cells cultured in 2-D. The ability and the time for cells to maintain their normal functions and responses to environmental stimuli are critical in the development of cell-based biosensors for field applications [7].

Fibrous materials, such as nonwoven polyethylene terephthalate (PET), are shown to be advantageous 3-D scaffolds to support cells because of their high specific surface areas, mechanical properties, and void volume in addition to easy manipulation, and are the proper matrix material to exploit the advantages of 3-D cell models in HTS [8].

In the other hand, mammalian live cell fluorescent assays by fluorometry are always desired by pharmaceutical industries, safety evaluation agencies and even fundamental scientific researchers because they are highly suitable for automatic high-throughput assay design and enable the characterization of acute cellular events. However, their low signal to noise ratio (SNR) is the bottleneck limiting their popularity. Considering that high density cell growth supported by 3-D environment gives the potential to increase fluorescent signals, in this study we dedicated to the combination of fluorometry detection and 3-D culture in order to improve signal quality and at the same time apply 3-D principle.

3.3. Material and methods

3.3.1. Maintenance and transfection of cells

Murine D3 ES cells were maintained on gelatin pre-coated T-flasks. Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% FBS, Gibco), 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µM monothioglycerol and 1000 U/ml leukemia inhibitory factor (LIF, Chemicon) was used as culture medium. DMEM plus 10% FBS was used to maintain colon cancer cells HT-29-GFP.

Both ES and HT-29 cells were transfected with PEGFP-N3 (Clontech, Palo Alto, CA) using Lipofectamine 2000 (Invitrogen). The expression of EGFP reporter gene and G418

resistance gene was under the control of human cytomegalovirus (CMV) promoter, which is a strong constitutive promoter that controls expression relatively independent of environmental effects. After 10 days of selection with G418, green colonies were selected by a fluorescence microscope. After amplification, a genetically engineered cell line was subcultured in the absence of a selective pressure (G418, Gibco) for over 20 passages. The stability of the cell lines (ES-GFP and HT-29-GFP) was verified by FACSCalibur (BD, CA) with the percentage of fluorescent positive cells higher than 97% compared to the negative control. The data showed that the status and the growth rate of the stable transgenic cell line were not affected by EGFP expression. In addition, the SSEA-1 expression maintained the same level as w/t ES cells (w/t: 95%; ES-GFP: 97%).

In this study, ES-GFP cells were used in all experiments except the cytotoxicity test in modified 384-well plates.

3.3.2. Treatment of non woven polyethylene terephthalate(PET) fibrous scaffolds

Needle-punched polyethylene terephthalate PET fabric (fiber diameter, ~20 μm; fiber density, 1.35 g/cm^3) was used as cell culture scaffolds. Different sizes of cake-shaped scaffolds were cut (large size for modified 96-well plate: 0.1 cm height and 0.3 cm^2 bottom surface area; small size for modified 384-well plate: 0.1 cm height and 0.12 cm^2 bottom surface area). PET matrices were treated before use to increase the cell attachment efficiency. Solution with 1% (w/v) Na_2CO_3 and 1% (v/v) Tween-20 was heated to 60 ^{0}C and PET matrices were soaked in the above solution for 30-60 min at 60 ^{0}C. After being rinsed with distilled water several times, PET matrices were transferred to

1% (w/v) NaOH solution and boiled for 30-60 min. After being thoroughly washed with distilled water again, scaffolds were then soaked in PBS, sterilized in an autoclave at 121 ^{0}C for 20 min and stored in room temperature for future uses. Scaffolds were soaked in growth medium for 12h before experiment. Before inoculation with cells, the growth medium was removed from the scaffolds.

3.3.3. Correlation between the fluorescence signal and the cell number suspended in PBS

The correlation between fluorescence signal and ES-GFP or HT-29-GFP cell number was acquired in phosphate buffered saline (PBS) suspension with the method described [9]. Briefly, cells were diluted in PBS and loaded into a 96-well plate (BD Optilux™, Black/clear bottom) with different concentrations. Fluorescence intensity was then measured by a Cytofluor Series 4000 from Applied Biosystems, Foster City, CA (excitation at 485nm with a bandwidth of 20 nm and emission at 530 nm with a bandwidth of 25 nm). In this paper, cell numbers were all determined by the trypan blue exclusion method or with a Coulter counter.

3.3.4. Correlation between the fluorescence signal and the cell number in 3-D PET scaffolds

The correlation between fluorescence signal and cell number was then studied in 3-D scaffolds. Different cell concentrations were determined before inoculation to the large scaffolds. In addition to the treatment described above, the scaffolds were also treated with 10 μg/ml fibronectin to improve cell attachment. The cells were seeded from the tops of scaffolds and the seeding volume was 25 μl per scaffold. The experiment was

conducted in a 96-well plate (one scaffold per well). 6 h after inoculation, each scaffold was soaked by adding 180 µl medium, and fluorescence signals were measured (only scaffolds immersed in liquid environment elicited fluorescence suitable for quantification). The 6 hour interval between seeding and adding enough medium was not only for the adherence of cells to scaffolds but also for the recovery of fluorescence lost due to light quenching during sample treatment. Experience from 2-D cell cultures showed that cells had little growth within 6 h after seeding. The fluorescence signals given by live cells were calculated using Eq. (1):

$$F_{cells}=F_{total}-F_{scaffold+medium} \quad (1)$$

F_{total} represents the fluorescence signal measured directly from one well with cells growing on a scaffold soaked in 180 µl medium. $F_{scaffold+medium}$ represents the fluorescence signal measured in the same way but without cells.

3.3.5. 2-D live cell autofluorescent assay

2-D cell growth kinetics was monitored in a similar way described Hunt et al., (1999). 5000 ES-GFP cells were inoculated into each well of 96-well plates and fluorescence signals were measured twice per day directly from the plates. In addition to measuring total fluorescence as Hunt et al. (1999) did, we also used the fluorescence given by live cells to quantify cell growth. The total fluorescence was calculated as the fluorescence signal measured directly from a well with cells minus a blank well with the same volume

of medium. The fluorescence given by live cells was calculated as the signal acquired after replacing culture medium with the same volume of PBS minus a blank with the same volume of PBS.

3.3.6. High-throughput 3-D cell culture system setup and quantification of autofluorescence in real time

BD 96-well plates were modified with Computer Numerical Controlled (CNC) machine (Sherline 2010, Vista, CA) to allow 6 small-scale bioreactors to run in one plate. ES-GFP cells were initially grown in T flasks. Harvested with trypsin, 25 000 cells in 25 µl medium were seeded on each scaffold in one well of a 96-well plate. Cells were allowed 6 h to attach to PET scaffolds. The scaffolds were then soaked with 180 µl medium again. One day after inoculation, each scaffold was transferred into the center of the chamber in fabricated 96-well plates with a working medium volume of 3 ml (Figure 3.1A). Transferring ensured that only cells attaching to scaffolds were allowed to proliferate. The fabricated plates with scaffolds were then stacked onto a rotational shaker (Stovall Belly Button* Shaker) in a 37 °C cell culture incubator (Figure 3.1B) in order to allow many "3-D bioreactors" to run in parallel. Rotation speeds ranging from 40 to 60 rpm were optimal. Agitation did not only improve mass transfer to support 3-D cell culture, but also assured the separation of non-specific signals, which was critical for the removal of the errors caused by cell activities. The fabricated plates were taken out of the incubator to measure fluorescence twice per day with the Cytofluor Series 4000. The temperature of the measurement was set at 37 °C.

A microbioreactor array with 8*5 units was also fabricated from BD 384-well plate using CNC machine (Figure 3.1C). In this array, smaller scaffolds (0.1 cm height and 0.12 cm^2 bottom surface area) were used and 16,000 HT-29-GFP cells in 11 μl medium were seeded into each scaffold. Rotation speed was set to be 90 rpm. Other procedures followed the above description.

3.3.7. Effects of serum and fibronectin coating

In a subsequent experiment, we evaluated the effects of FBS, an essential ES medium component and fibronectin, a coating material (Sigma Chemical Company, St. Louis, MO) to optimize the 3-D ES cell culture system. To optimize serum concentration, medium with different serum concentrations ranging from 0% to 10% was applied to different samples one day after inoculation. To evaluate the effects of fibronectin coating, PET scaffolds were soaked in 10 μg/ml fibronectin for 12 hours before being soaked in growth medium.

3.3.8. Effects of DM on low density ES-GFP cells

Different doses of DM (Sigma Chemical Company, St. Louis, MO, stock conc. 20 mg/ml in dimethyl sulfoxide (DMSO)) were applied to ES-GFP cells immediately after transferring scaffolds to 6-unit bioreactor arrays in the absence of LIF. DM is one of embryotoxic reference chemicals in the embryonic stem cell test (EST).

3.3.9 Effects of 5-FU and gemcitabine on high density HT-29-GFP cells

HT-29-GFP cells were cultured in a microbioreactor array with 40 units for 108 h until cells reached high density. Different doses of 5-FU (Sigma Chemical Company, St. Louis, MO) and gemcitabine (Eli Lilly, Indianapolis, IN) were then applied. 5-FU is a typical chemotherapeutic drug in colon cancer treatment, while gemcitabine is not. A GENios Pro from Tecan (Durham, NC) was used for fluorescence detection.

3.3.10. Characterization of acute cellular events caused by Triton X-100

The 3-D system was applied to test the response of ES-GFP cells to Triton X-100, (Sigma Chemical Company, St. Louis, MO). Cells in a modified 96-well plate were cultured to reach a high density with fluorescence signals about 2000 RFU. After different doses of Triton X-100 were applied, the plate was immediately put into the plate carrier of a Cytofluor Series 4000. The cycle time was set as 3 min. The mixing time before each measurement was 10 seconds. The temperature of measurement was 37 °C.

3.4. Results

3.4.1. Correlation between fluorescence signals and the cell numbers

A linear correlation of fluorescence signals and ES-GFP or HT-29-GFP cell numbers was found for all cell concentrations tested no matter cells were suspended in PBS or attached on 3-D PET scaffolds (Figure 3.2A and 3.2B). This matches the results of Hunt et al. (1999) that the layering of cells does not significantly impair signal detection. The fact

that the slope corresponding to cells on scaffolds was about 3-4 times more than that from cells in suspension was not expected by the authors. Repeated experiments showed similar results. It might be because of 6 hours' recovery of fluorescence from cells in scaffolds. However, even with 6 hours of recovery the slope for suspended cells was only a little more than that without recovery in suspension and still much less than that on scaffolds. It indicated that there were other factors involved. Another assumption was that the optical combination of fluorescence from the EGFP proteins and PET fibers can enhance fluorescence signals.

3.4.2. 2-D fluorescent kinetics during cell growth of wild type ES cells and ES-GFP cells

For ES-GFP cells, a large discrepancy existed between the fluorescence given by live cells and the total fluorescence, which increased over time (Figure 3.3). For a w/t ES lineage, this discrepancy was only a little less than that for the ES-GFP lineage at 146 h after inoculation. This indicated that in addition to GFP released from dead cells, changes in the medium components caused by cell activities and cell debris contributed most of the fluorescent discrepancy between the live cell fluorescence and the total fluorescence for the ES-GFP lineage. This is inconsistent with the suggestion from [10] that only the stability of GFP released into the medium contributed the discrepancy between the total fluorescence and the viable cell number. In addition, there was a 13 hour interval between the time points for the maximum live cell fluorescence and the maximum total fluorescence for ES-GFP cells. The significant differences with or without medium

replacement suggested that the total fluorescence could not be applied to in situ counting of cell numbers unless most of the errors caused by cell culture medium component changes, cell debris and GFP released from dead cells were eliminated.

It should also be noted that fluorescent compounds in the medium, which were susceptive to natural light, elicited an approximately 500 RFU background signal, more than 2 times of the maximal signal given by live cells. Therefore, the removal of medium before each measurement is also necessary to improve the accuracy of cell number counting in the 2-D culture. Therefore, the fluorescence from live cells instead of the total fluorescence was used for later cytotoxicity tests in 2-D system.

3.4.3. 3-D cell growth study

Fluorescence was measured to reflect 3-D cell growth kinetics (Figure 3.4). The fluorescence of the live cells in our system (Figure 3.1B) was calculated using Eq. (2):

$$F_{cells}=F_{center}-F_{scaffold}-F_{surround} \quad (2)$$

F_{cells} represents the fluorescence signal given only by cells. F_{center} represents the fluorescence signal measured directly from the central well with cells growing on a scaffold. $F_{scaffold}$ represents the fluorescence signal of a scaffold soaked in PBS, a constant during the entire culture process. $F_{surround}$ represents the average fluorescence signal measured from 8 wells surrounding the central well. F_{center} and $F_{surround}$ can be obtained directly by fluorescence measurements twice per day. $F_{scaffold}$ can be measured before the inoculation of cells.

The maximum specific growth rates (μ_m) of these three batches were calculated to be 0.0355, 0.0364, 0.0315 h^{-1}, respectively (all R^2>0.999). The average was 0.0345 h^{-1}($T_{1/2}$ =20.1 h).

3.4.4. Effects of serum and fibronectin coating

The increase of growth rate between 0% and 1% FBS was significant (Figure 3.5), but not much change was found above 1%. Therefore, 1% FBS is probably enough to economically support ES cell growth in large-scale bioreactor production.

Cells were seeded from the top of the scaffolds and cells became trapped into gaps between fibers. The adhesion to PET fibers is important to determine the following cell growth. Fibronectin is involved in many cellular processes, one of which is to serve as a general cell adhesion molecule to help cells anchor to proteoglycan or collagen substrates [11]. As shown in Figure 3.6, fibronectin coating contributed a distinct enhancement to μ_m. With fibronectin coating, μ_m increased from 0.0335 h^{-1} (doubling time 20.7 h) to 0.0458 h^{-1} (doubling time 15.1 h), which was a 37% enhancement. When the cell culture with fibronectin coating reached the maximum fluorescence intensity (128 h after inoculation), the fluorescence intensity with coating was 2.5 times of that without coating. Figure 3.7A, 7B from a fluorescent microscope (Nikon Ecllipse TE2000-U) show that the fibronectin coating benefited cell adherence to PET fibers and cell spreading. In addition, cells with fibronectin coating were distributed more homogeneously along the fibers, while those without fibronectin coating formed larger aggregates (Figure 3.7C, 3.7D). Homogeneous distribution with fibronectin coating benefited mass transfer such as in- or

out gassing so that cells lived in a constant pH environment with a supply of nutrients and oxygen and the removal of metabolites. This might cause the improvement of 3-D culture.

3.4.5. Cytotoxicity studies

To demonstrate the feasibility of our system for toxicological study, ES-GFP cells in the 6-unit platform were exposed to DM 24 h after inoculation. The fluorescent kinetics with different DM doses was shown in Figure 3.8A. The growth rates decreased as the concentration of DM increased.

Toxicity of 5-FU and gemcitabine on high-density HT-29-GFP was compared in the 40-unit microbioreactor array in a high-throughput fashion (Figure 3.8B). In this assay, high density HT-29-GFP cells were exposed to these two chemicals after 108 h of culture. Fluorescence was increased by a low dose of 5-FU (100 μM), but high doses of it decreased fluorescence signal significantly (1000 μM and 10000 μM) ($P<5\%$). However, no fluorescent decreased were found in all gemcitabine doses compared to the control. Mean value derived from triplicate repeats for each dose except 0.05 μM gemcitabine.

3.4.6. Assessment of acute cellular events

Because online continuous measurements can be made with no human attention required in live-cell kinetic assays, acute cellular events can be readily characterized. Figure 3.9A uses relative fluorescence (fluorescence measured / fluorescence at the time of adding Triton X-100 ($t = 0$ min)) kinetics and different Triton X-100 concentrations to show the acute toxicity of Triton X-100. Triton X-100 is a non-ionic detergent commonly used to

solubilize membrane proteins. The slow decrease of the fluorescence signal in the control might be due to an increase of environmental pH from the cell culture incubator to the plate carrier of the fluorometer, which affected the fluorescent components in the whole culture system. The specific death rate k_d for each Triton X-100 concentration is the negative slope of the curve for that concentration in the semi-log plot Figure 3.9B. ($Ln(\text{relative fluorescence}) = -k_d \times t$). Figure 3.9C shows the correlation between Triton X-100 conc. and k_d.

3.5. Discussion

Hunt et al. (1999) used fluorescence signals to assess the density of CHO cells with stable GFP expression in a 96-well microplate with a typical fluorometer without any need for sampling. The method is fast, non-invasive and more robust than trypan blue exclusion or particle counter analysis in the presence of aggregates. However, fluorescence signals generated in such assays are generally too weak to be quantified for *in situ* measurements by a fluorometer. This is mainly due to nonspecific effects on assay responses caused by the changes in biological environment due to cell activity [10]. These nonspecific signals could occur at a wavelength close to GFP and effectively mask GFP signals. Therefore, it is difficult for cells to elicit distinguished resulting signals when this method is applied to cell-based sensing using other sensing element and reporters. This is a bottleneck limiting the popularity of this method. Instead of trying to have higher cell specific fluorescence (average fluorescence of each cell) in order to improve SNR, which is very

difficult for stable established cell lines, we increased the cell number per area unit dramatically with 3-D culture to obtain at least 20-fold higher level of cellular fluorescence with an unchanged background level. Another major strength of our approach is that 3-D scaffolds are able to immobilize live cells, allowing separating noises due to biological environmental changes from desired signals in live cells after mathematical correction. Therefore, these errors would be eliminated mostly if not thoroughly.

The determination of signals correlated to cellular responses is fundamental, no matter what principle of a cell-based assay or a biosensor is used. However, these signals are always too weak to be easily detected with conventional methods [12]. Thus, signal to noise ratio (SNR) is one essential criterion to evaluate an assay or sensor. CMV is a strong constitutive transcription activator which is much stronger than most cis-acting genetic elements corresponding to specific functional units [13; 14]. In addition, EGFP is also a strong autofluorescent protein. As our results show, in standard 2-D cultures resulting signals were weak compared to background noises, even with the combination of these two strong factors, so fluorescence quantification could fail due to low SNR when other genetic elements or other autofluorescent proteins were applied. Here, our 3-D system might provide a solution. Compared with 2-D cultures, our 3-D system has an extra 180-240 RFU background fluorescence signal because of the PET scaffolds. However, the background fluorescence signals from scaffolds remained the same throughout the entire experiment process, unlike culture medium which is susceptible to light. Therefore, the experimental noises of 3-D are close to those of 2-D. On the other

hand, the maximum specific fluorescence signal given by our 3-D culture can be higher than 5000 RFU, while the maximum specific fluorescence signal in 2-D is less than 250 RFU for the same ES-GFP cell line. Therefore, our 3-D system increases SNR at least one order of magnitude compared with 2-D cultures and thus allows the realization of many cell assays which were inapplicable in conventional monolayer culture due to low SNR.

The exact signals representing cell responses are another issue which must be considered for any cellular assay or biosensor. The autofluorescence from released GFP, cell debris and the fluorescent fluctuation of medium due to cell activities or environment changes were other drawbacks preventing the popularity of live-cell GFP fluorescent assays. These raise a more serious problem for cytotoxicity studies in which cell death is critical. In our 3-D system, the fluorescence from live cells is all centralized in the center well (about 0.3 cm^2 area) and the background fluorescence is homogenously distributed in the whole culture area (about 9 cm^2 area) due to agitation. Therefore, theoretically, the errors caused by released GFP, cell debris and fluorescent fluctuation of medium are about 1/30 of that from standard 2-D culture systems. Actually, in all of the data we used Eq. (2) to calculate live cell fluorescence, so these errors could have been further reduced. Because of the convergence of live cell fluorescence signals, both signal magnitude and accuracy are highly improved in our 3-D system. At this point, our system can serve as a very good prototype of cellular assays and biosensors.

Large-scale high-throughput screening and routine lab uses are in favor of assays with simplicity. In our system, the online quantification of autofluorescence signals can be

realized simply with PET scaffolds, fabricated 96-well plates, a standard bench shaker, and a simple fluorometer in addition to regular cell culture materials. For manipulation, our system has additional requirements for scaffold treatment and plate fabrication compared to 2-D systems. However, since PET is a durable material, scaffolds can be prepared and treated in large quantity and stored for a long period of time (several months). Both the automatic fabrication of plates and the large-scale treatment and storage of scaffolds could make the system ready for mass production if it was commercialized. We can imagine that when fabricated plates in which treated scaffolds were prefixed were commercialized, the entire experiment setup would only include cell inoculation and the addition of analyte-containing medium. In addition, installation of the detection part of fluorometry in a cell culture incubator would allow us to conduct assays without any user attention after initiation.

In addition to providing a longer time for field applications and analyte testing, our system allows the characterization of acute cellular events because the realization of live-cell kinetic assays avoids the need for sample preparation, enzyme reactions, and antibody development. In this case, it provides a friendly platform to detect acute cellular events, which is not easily detected by fixed endpoint assay methods but always desired.

Cytotoxicity was usually expressed as the drug concentration causing 50% reduction in cell number at a certain time point (IC_{50}) [15; 16]. Because ratios of cell numbers in drug-treated samples to the control always varied from time to time (Figure 3.8A), the values of cytotoxicity with this definition varied according to different time points chosen. Thus, it cannot provide a constant standard for cytotoxicity tests. For example, if the evaluation

of cytotoxicity of one analyte on 2 cell lines with different growth rates is required, using the same fixed time point to calculate the cytotoxicity will always cause errors. In this context, the concentration of an analyte inhibiting the cell maximal specific growth rate by 50% of the control level ($IC_{\mu 50}$) could be a better choice because $IC_{\mu 50}$ is independent on the time point chosen. However, the application of this expression for large-scale cytotoxicity tests would create an undaunted work load with conventional counting methods. Our real-time system eliminated all cumbersome steps in manual sample preparation or reaction for counting and readily enabled the application of this new expression. At this point, it could be a better cell-based assay for the cytotoxicity test.

Assessment of high-throughput screening (HTS) is taking on a new role with great significance when libraries in initial drug discovery composed thousands of compounds. Our system currently allows up to 40 samples in one plate and it can be amenable to high-throughput experiments by processing many microbioreactor arrays in parallel. In other words, drug discovery and screening could be performed on 3-D tissue cultures in a high-throughput fashion.

Process optimization for 3-D adhesive mammalian cell cultures such as the fibrous bed bioreactor [17] is comparably difficult due to the difficulty of cell sampling. Although static experiment can be conducted in parallel, reducing labor and cost, static microplate cultures are not able to confer enough agitation to support 3-D cell cultures with high cell densities. Our 3-D system overcomes most of these limitations. Because it enables the monitoring of cell growth kinetics on line in real time automatically and parallely, the system can serve as a very powerful tool for scale-down, providing crucial information

for the bioprocess refinement of bench or pilot systems. According to the results above, applying fibronectin coating, large scale designs might achieve a much higher efficiency due to the increase of growth rate and the maximum cell density.

Cell number or cell mass counting is still an issue preventing the further development of 3-D tissue engineering [18]. Using an indirect way to automatically count cell number, our system provided a simple but efficient platform to explore the benefits of 3-D tissue engineering. In this paper, the optimization of 3-D cultures showed this power. In addition, with different fluorescent proteins applied at the same time, the automatic monitoring of co-cultured tissue models will also be realized.

Cells in 2-D cultures and 3-D cultures were proved to have different cellular biology. The systematical understanding of these differences will benefit the studies in biomedicine and pharmacology. To date, only a few studies have been carried in this field due to the difficulties of 3-D culture research. With our method established to reveal cellular biology in 3-D, the systematical discovery of these discrepancies is ready. In addition, different behaviors of anti-cancer drugs in 2–D and 3-D cultures could be analyzed in comparison with the results from animal experiment and clinical data and the superiority of 3-D cultures over 2-D cultures would be proved.

3.6 Conclusion

Our system offers an *in vivo* mimic, convenient, automatic, on line in real-time method with high-throughput potential for the measurement of cell numbers or cell mass. It potentially offers a platform for application to many different disciplines including high-

throughput screening of novel drugs, portable cell based sensors, bioreactor optimization, and basic biomedical research such as functional genetics and quantitative cell biology. Compared to currently used live-cell autofluorescent assays, the 3-D system has several advantages. The reliability is improved because cells in 3-D culture have more desired functional attributes representing the *in vivo* native counterparts. The fact that our 3-D culture system can enhance signals by 20 folds compared with 2-D culture system reflects a higher sensitivity and stands a higher chance to realize on-line automation using weaker biological recognition components and fluorescent proteins. Higher accuracy is achieved because errors due to cell activities are almost eliminated by the convergence of target signals. The elimination of sample preparation during counting makes the constant expression of cytotoxicity ready and gives more chances to monitor cellular acute events. Finally, the simple design offers high adaptability for both large-scale drug discovery and routine lab cell assays. Currently, an optimal combination of these attributes is unmatched by any other cell assay or biosensor found by the authors.

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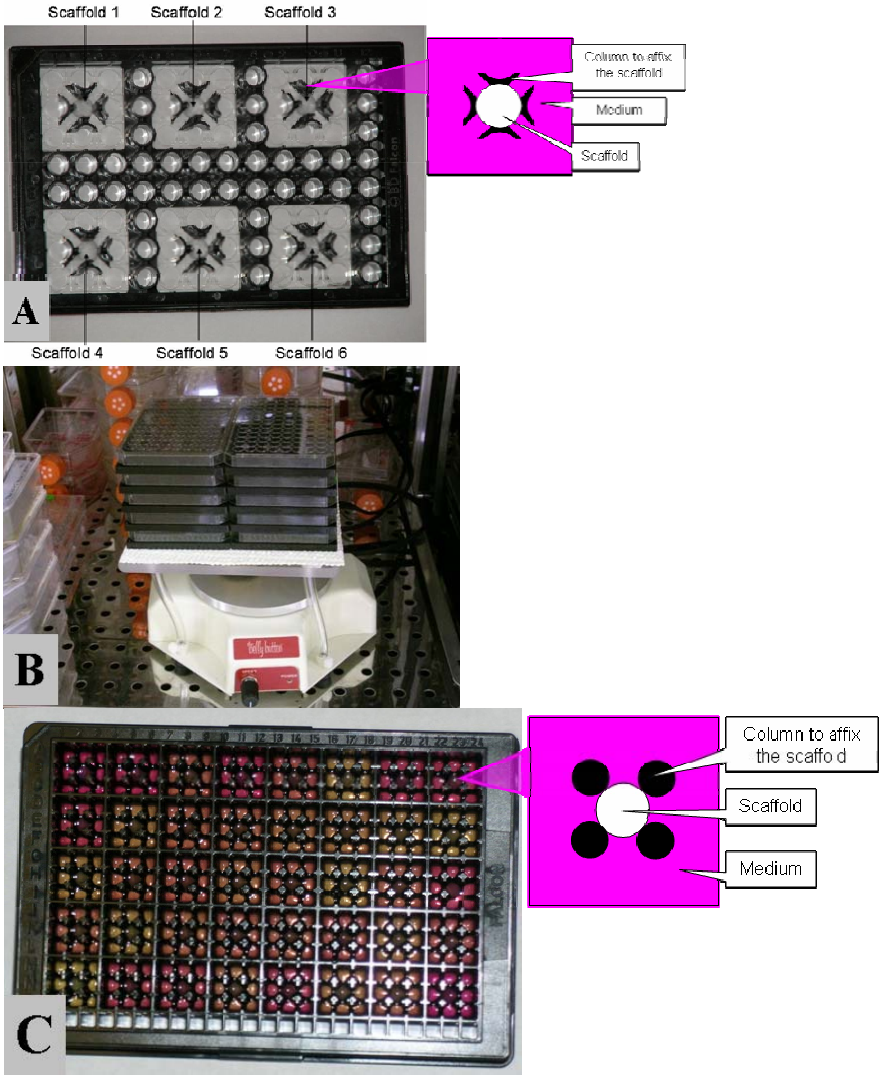
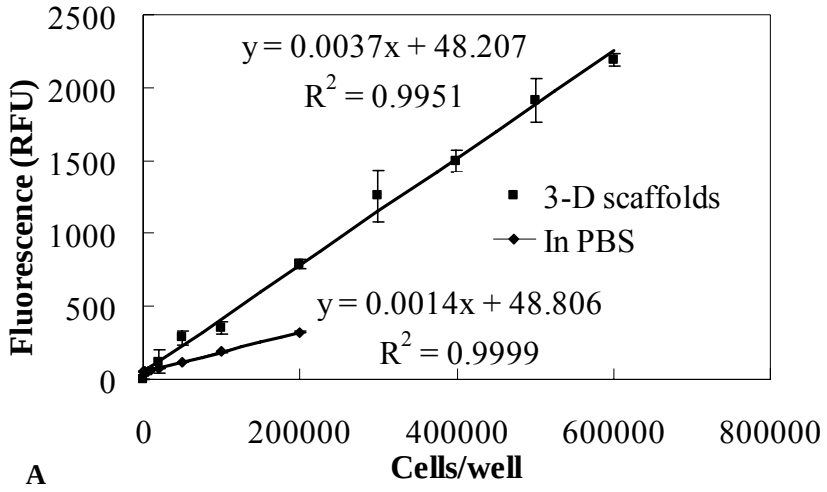


Figure 3.1. The establishment of the 3-D cell culture system. A. A 6-unit bioreactor array (modified 96-well plate) with large cake-shape scaffolds (0.1cm height, 0.3 cm^2 bottom; working volume per unit 3 ml). Most of walls inside of chambers were removed so that medium was able to flow everywhere in these 9-well integrities to provide enough mass transfer after the plate was put on a shaker. B. Cells were cultured on the scaffolds in fabricated plates stacked on a shaker in a 37 ^oC incubator to allow many 3-D bioreactors to run in parallel. C. A 40-unit microbioreactor array (modified 384-well plate) with smaller cake-shape scaffolds (0.1cm height; 0.12 cm^2 bottom; working volume per unit 1 ml).



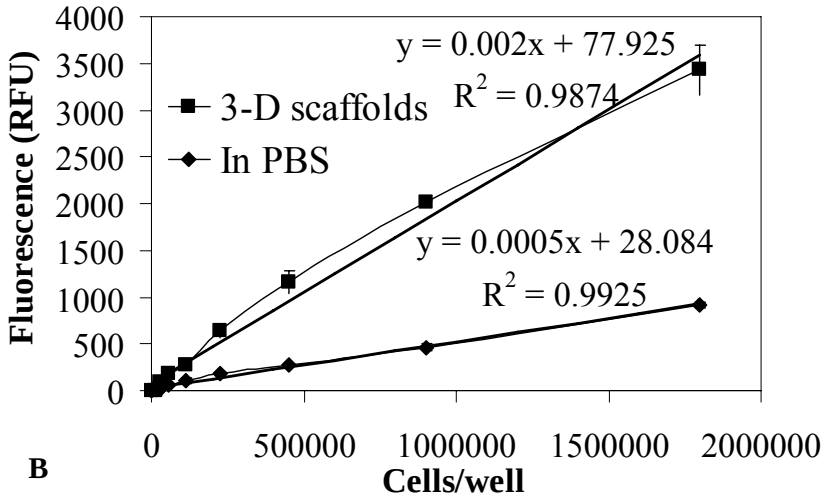


Figure 3.2. Fluorescence intensity is proportional to GFP expressing protein. ES-GFP (A) and HT-29-GFP (B) cell number either in PBS suspension or on 3-D scaffolds. Each point represents the average fluorescence intensity from triplicate samples minus the average fluorescence intensity of blanks. The blanks for suspension are the fluorescence from the same volume of PBS without cells. The blanks for 3-D consist of the fluorescence signals from the same volume medium and scaffolds without cells

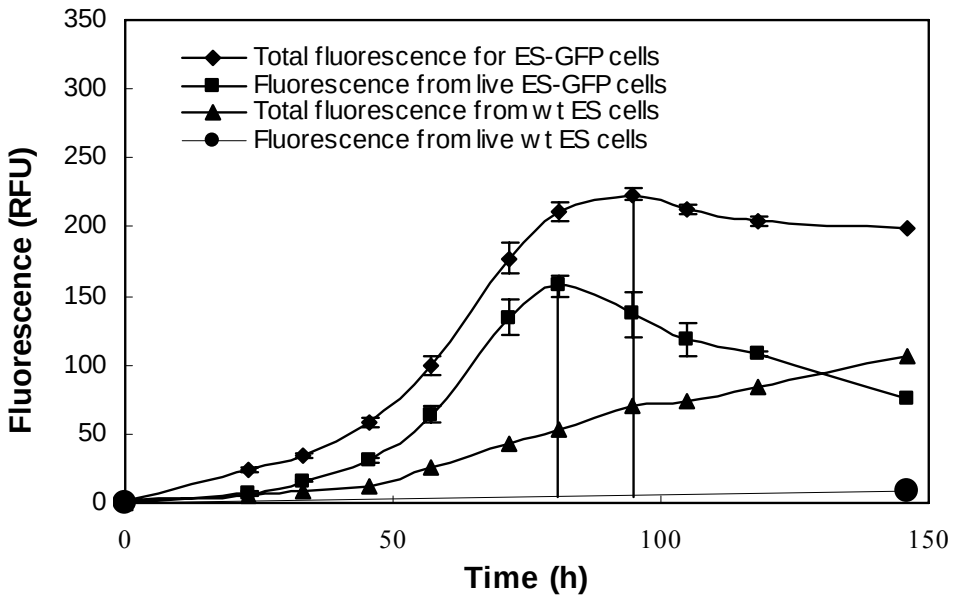


Figure 3.3. Difference between the total fluorescence and the fluorescence given by live cells for the ES-GFP lineage and the w/t ES lineage. In the first two curves, each point represents the average fluorescence intensity from triplicate samples minus the blank of medium or PBS. In the last two curves, each point represents the fluorescence intensity from one sample minus the blank of medium or PBS.

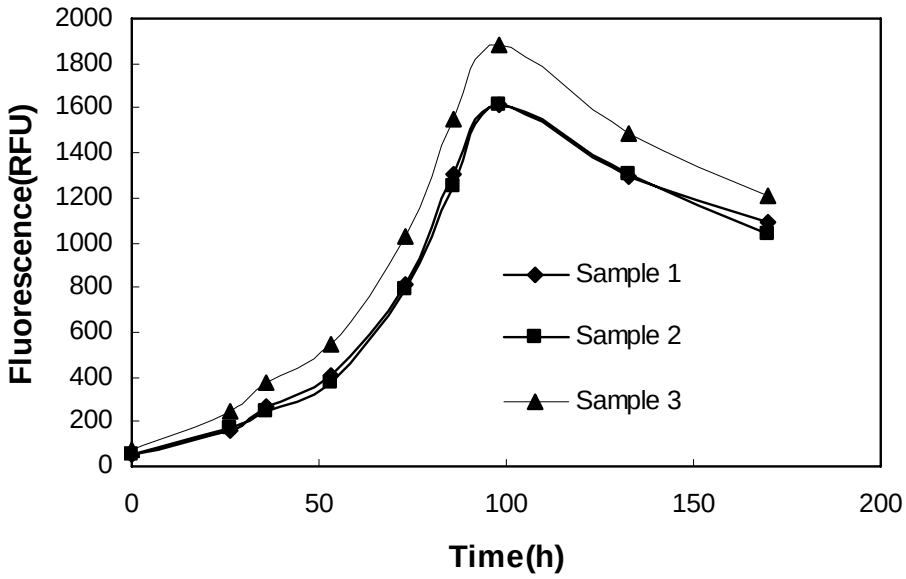


Figure 3.4. Estimation of cell growth kinetics by fluorescence signals. Batch kinetics of cell growth was monitored by fluorescence intensity for ES-GFP cultured in fabricated 96-well plate with PET matrices. The growth kinetics as monitored with the GFP fluorescence intensity was consistent in triplicate runs and could be used to determine the specific growth rate of the culture under the culturing conditions.

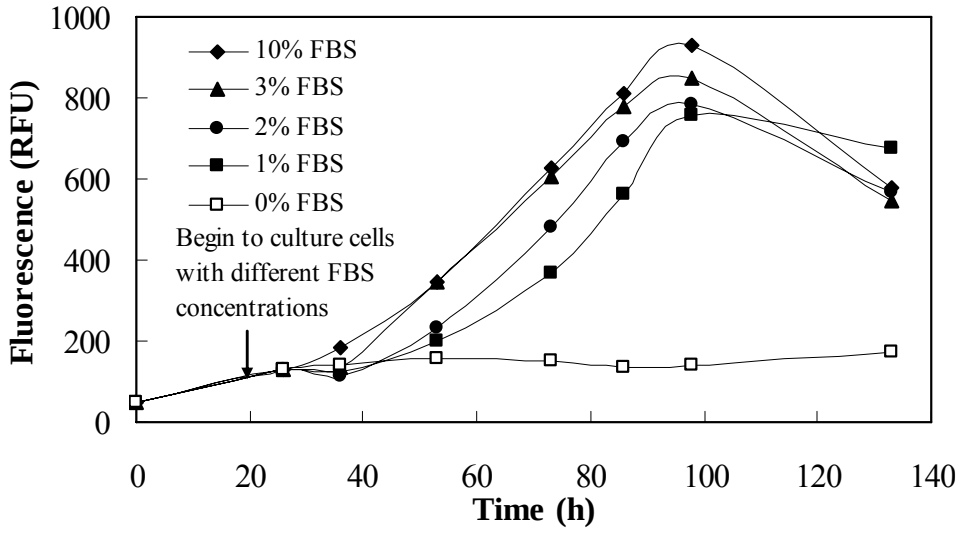


Figure 3.5. ES-GFP cultures to investigate the effects of FBS on cell growth.

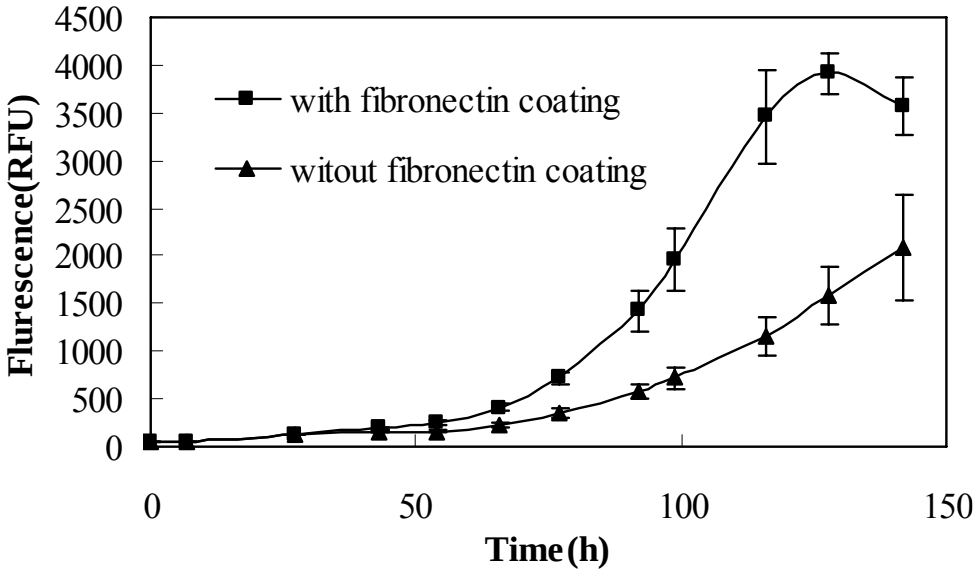


Figure 3.6. ES-GFP cultures to investigate the effect of fibronectin coating on cell growth. Three samples were with fibronectin coating, while the other three samples were without fibronectin coating. Each point represented the average fluorescence from triplicate samples.

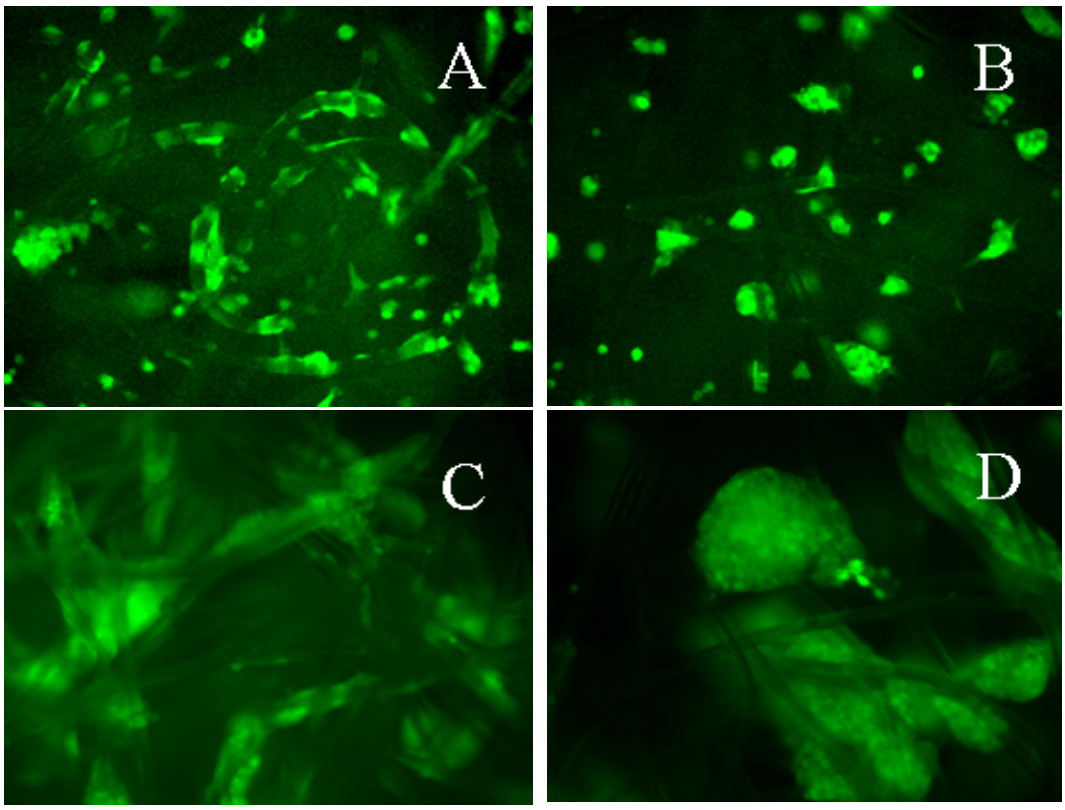


Figure 3.7. Fluorescent microscopic images of ES-GFP cells on 3-D scaffolds. A. one day after inoculation with fibronectin coating; B. one day after inoculation without fibronectin coating; C. 4 days after inoculation with fibronectin coating; D. 4 days after inoculation without fibronectin coating. An inverted fluorescent microscope (Nikon Ecllipse TE2000-U) was used for detection.

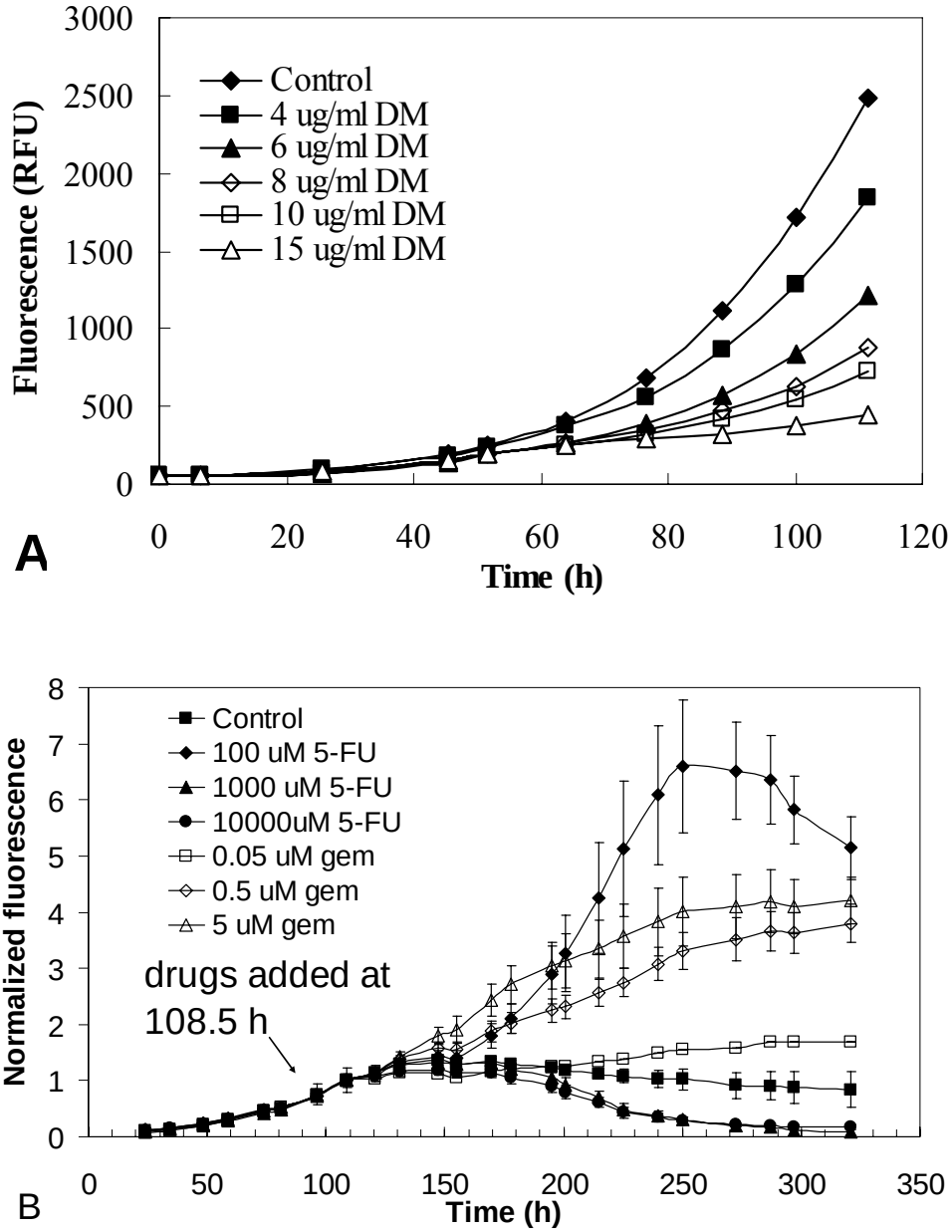


Figure 3.8. Study of cytotoxicity on 3-D cultures. A. Fluorescence kinetics of ES-GFP cell growth with different doses of DM in a 6-unit small-bioreactor array. Chemicals were applied at low cell density. B. Fluorescence kinetics of HT-29-GFP cell culture with different doses of 5-fluorouracil or gemcitabine in a 40-unit microbioreactor array. Chemicals were applied at high cell density.

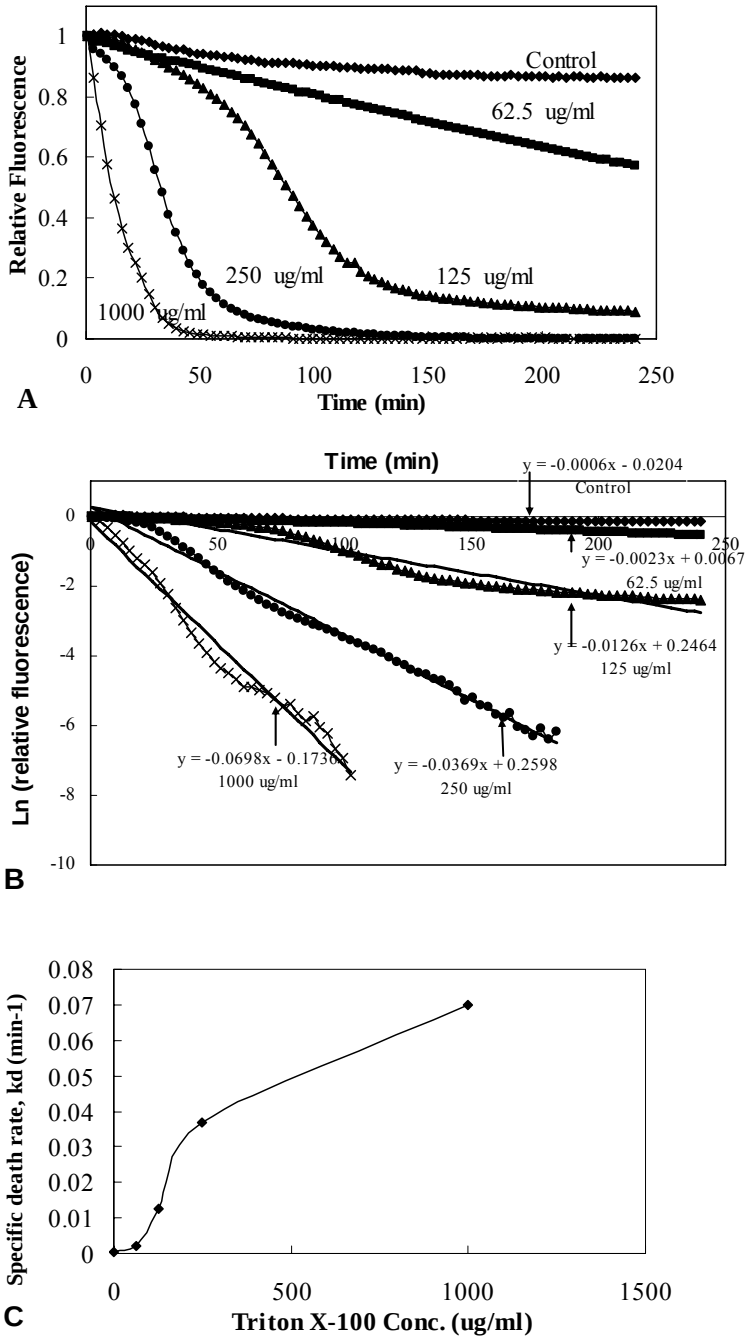


Figure 3.9. Acute cellular responses to Triton X-100. A. Relative fluorescence kinetics with different Triton X-100 concentrations. B. Semi-log plot of fluorescence kinetics to calculate k_d. C. Correlation between Triton X-100 conc. and k_d.

CHAPTER 4

APPLICATION OF HIGH-THROUGHPUT REAL-TIME GFP-BASED ASSAYS TO REVEAL THE DISCREPANCIES OF EMBRYOTOXICITY ON 2-D AND 3-D CULTURES

4.1. Abstract

Recently, research on chemical activities to three-dimensional (3-D) cell cultures is more productive than ever. However, people has focused on the efficacy of therapeutic agents on 3-D heterogeneous cancer spheroid models, while few of them concerned with chemical toxicity on microregions with well-developed vasculature, a physiologically relevant morphology of normal tissues *in vivo*. We have recently developed a 3-D ES cell culture system, which can afford parallel, automated, and long-term cell bioactivity assays accurately. In the present work, we further studied the microenvironment characteristics of this system and used it to explore chemical toxicity in 3-D cultures. First, by histological study and simple theoretical modeling it was confirmed that cells cultured in a 3-D environment grew without diffusion limitation, even in long term cultures, and resembled the microenvironment *in vivo*. The responses of cell-specific fluorescence to different chemicals were then investigated and it was found that only

cells treated with S-phase specific chemicals 5-fluorouracil (5-FU) and gemcitabine could give higher cell-specific fluorescence. This was consistent with the fact that the promoter CMV controlling GFP expression is S-phase specific. Next, the embryocytotoxicy of dexamethasone (DM), diphenylhydantoin (DPH), Penicillin G and 5-FU in different culture models was studied. The effects of these chemicals on low density cells (proliferation assays) and high density cells (tissue-like response assays) in 3-D cultures were investigated in an online high-throughput manner and compared to the data from 2-D cultures (2-D proliferation assays). Cells were a little more sensitive to DM, 5-FU, and penicillin G in 3-D proliferation assays than in 2-D proliferation assays, but cells showed different levels of resistance in the tissue-like response assays. Compared to proliferation assays, 3-D multi-cellular organizations were 5.7, 15.4 and 30.7 times more resistant to DM, DPH and Penicillin G, respectively, while 5-FU was almost non-effective in multi-cellular models. These results suggested that the 3-D culture system was able to reveal physiological responses of chemicals and enable toxicological studies in a high-throughput manner. In general, we applied tissue engineering approaches to construct cell microenvironment, which enabled a similar *in vivo* cell cycle progression resulting to multi-cellular resistance to external stimuli, and transformed this culture system to online high-throughput cell-based assays for future chemical toxicity evaluation and drug development.

4.2. Introduction

Both USA and EU have incepted programs to evaluate thousands of chemicals toxicologically, such as the U.S. High Production Volume (HPV) Challenge Program and

a future European Chemicals Policy (Commission of the European Communities, 2001). More than 10 million animals were estimated to be used in the near future [1]. In addition to their high cost, intensive labor, and increasing protection by animal rights advocates and governments, animal models are just proxies for human. In this case, *in vitro* cell-based assays provide alternative predictive approaches to reduce the number of animal experiments and increase testing efficiency in a high-throughput manner.

For a chemical with direct toxicity *in vivo*, it must not only be able to kill the targeted cells, but also reach the cells with optimal quantifies [2]. First, a possible limitation of toxicological evaluation using 2-D cell cultures is the overestimation of drug toxicity because these culture models expose all cells to the same dose as in the medium while in 3-D multi-cellular organization the access to drugs is limited due to mass transfer resistance. Second, the responses of individual cells to chemicals might alter due to many mechanisms such as gene expression perturbation in 3-D microenvironment [3-5]. In addition, long-term tissue responses to external agents can rarely be reached due to metabolic-waste accumulation and contact inhibition in static cultures. Therefore, although conventional 2-D cell cultures could be used in toxicological tests with low cost and high speed, they cannot reproduce complex 3-D microenvironment *in vivo* in a long term and thus often give unsatisfactory and misleading results for the prediction of *in vivo* responses [3; 6]. In this aspect, 3-D *in vitro* cell models serving as a link between single cells and organs have a more physiologically relevant morphology, resemble cellular environment, and reproduce *in vivo* cell metabolism, gene expression and differentiation considerably [7]. These resemblances can determine similar biological behaviors of cells

to chemicals, which 2-D cell cultures are not able to offer [8-12]. However, although the advantages of 3-D cell models are significant over conventional 2-D cell models, they are very difficult to apply in high-throughput toxicological tests. To date, high-throughput toxicological tests of chemicals are limited mostly to biochemistry assays and 2-D cell culture assays, and there are no mature methods available currently using 3-D cultures in HTS to test chemical toxicity.

In the pharmaceutical industry, intercellular contact and cell-matrix interaction regulate cell responses and should also be considered to improve the predictability of drug potency. However, in drug discovery processes, the development of *in vitro* models with cells in their inactivated or quiescent state was always glossed over, while such states would be critical to reconstruct natural tissue properties, such as permeability, multi-cellular resistance and compound metabolism [6].

Recently, one of the most exciting areas of 3-D culture research is the study of embryoid bodies derived from stable ES cell lines. ES cells normally require the formation of aggregation structures, termed embryoid bodies (EBs), for *in vitro* differentiation to three primary germ layers and further specialization into somatic cells. During differentiation, specific genes, proteins, and ion channels are expressed in a similar continuum as the developmental processes of embryogenesis. These specific genes, proteins and ion channels provide very useful markers for the study of embryonic development. Currently, ES cells are the only truly immortal stem cells with a normal diploid karyotype, which makes them more suitable for developmental studies than other immortalized cell lines or primary cells [13]. Furthermore, since different somatic cell lineages such as

cardiomyocytes and neurons can be obtained by currently established methods, differentiation of ES cells provides unlimited homogeneous somatic cells, while primary cells are various according to different donors. Because of the unique characteristics and many potential advantages provided by ES cells, cytotoxicity based on the embryonic stem cell test (EST) has become increasingly used in drug screening [14]. Three endpoints were used to determine the embryotoxicity of chemicals: morphological analysis of beating cardiomyocytes after differentiation in the medium containing tested chemicals, and cytotoxicity tests on pluripotent ES cells and differentiated 3T3 fibroblast by 2-D proliferation assays. With a stable ES cell line expressing GFP under the control of a cardiac alpha-actin gene promoter, Bremer et al. (2001) used FACS flowcytometry analysis to determine the effects of 15 chemicals on cardiac differentiation [15]. Their results showed that fluorescence given by GFP inside of the cardiomyocytes could provide an appropriate quantification to determine the toxicity of chemical compounds on developing cardiac cells. Furthermore, this genetic engineering technique offers an objective measurement, instead of original evaluation by an experienced person. However, FACS is suboptimal for the design of HTS cell assays. An easier way to measure the fluorescence of GFP in EB bodies is the quantitative image analysis [16]. Although the robustness was enhanced and the measurement could be repeated at different time points in the same sample, quantification of cells in 3-D aggregates with a microscope would cause errors due to various foci. One of the limitations of EST is that only the first endpoint was established applying 3-D principle, while the other two used regular 2-D proliferation assays. This disharmony might cause errors in toxic evaluation of chemicals. Finally, it needs to be noticed that a huge number of new bioactive

compounds produced everyday by combinatorial synthesis fuel an increasing need of screening with high-throughput capability, which the current EST doesn't have. In this case, 3-D high-throughput real-time cell assays offer great potentials for further optimization of EST.

A microplate-based 3-D culture system was developed to bridge the gap between monolayer cell cultures and animal models. The system demonstrates the appropriation of HTS technology: enhancement of signal to noise ratio, elimination of labor intensive manual sample preparations, improvement of accuracy and minimization of errors. This study dedicated to the discovery of the discrepancies of cellular responses to external stimuli existing between different cultures. The cytotoxicity of several embryotoxic relevant chemicals was tested in this study.

4.3. Material and methods

4.3.1. Maintenance and transfection of ES cells

A stable transgenic ES cell line was established from murine D3 cells (ATCC). D3 cells were transfected with PEGFP-N3 (Clontech, Palo Alto, CA) using Lipofectamine 2000 (Invitrogen) followed by isolation and amplification. Therefore, EGFP expression was under the control of a human cytomegalovirus (CMV) promoter. The stability of the cell line was verified by FACS and SSEA-1 expression after 20 passages of subculturing without G418 (Gibco, USA).

4.3.2. Measurements of cell number, cell size and cell specific fluorescence

50,000 cells were seeded into each well of 12-well plates. One day after inoculation, cells were exposed to the test chemicals in non-LIF ES media. On day 3, the cell number was counted using a hemocytometer and cell-specific fluorescence was calculated as the mean fluorescence value detected by a flow cytometer (BD FACS Calibur, Franklin Lakes, NJ). Cell size was measured by Beckman Coulter Multisizer II (Fullerton, CA).

4.3.3. Treatment of non-woven PET fibrous scaffolds

Disk-shaped PET scaffolds (0.1 cm height, 0.6 cm diameter) (Figure 4.1A) were immerged into a solution containing 1% (w/v) Na_2CO_3 and 1% (v/v) Tween-20 at 60 ^{0}C for 30-60 min. After rinsed with distilled water, PET matrices were boiled in 1% (w/v) NaOH solution for 30-60 min. After rinsing again and soaking in PBS, scaffolds giving similar fluorescent backgrounds were selected by using a Cytofluor Series 4000. Scaffolds were then sterilized and stored in room temperature. Before use, scaffolds were soaked in 10 μM fibronectin and growth medium in series and the growth medium was removed from the scaffolds 1-2 hours before seeding.

4.3.4. Histological examination with H&E stain

To characterize multi-cellular solid structures in scaffolds, PET matrices with 6 days and 10 days of cell cultures were fixed in 10% formalin for one day, embedded in paraffin and then cut into 4 μm thick layers. They were stained with Hematoxylin and Eosin (H&E) and viewed with a microscope.

4.3.5. Exposure to chemicals in the 2-D system

2-D autofluorescent cytotoxicity assays of the chemicals were performed in 96-well plates. 5,000 cells were inoculated into each well with 150 µl non-LIF ES medium. To obtain a similar drug exposure time to the 3-D assays, ES-GFP cells were exposed to the chemicals one day after inoculation. Fluorescent signals were measured twice per day in the culture plates. The fluorescence given by live cells was calculated as the signals obtained after replacing culture medium with the same volume of PBS minus the blanks.

4.3.6. Exposure to chemicals in the 3-D system

25,000 cells in 25 µl medium were seeded into each pretreated PET scaffold. After 6 h attachment, 180 µl of media were added. The scaffolds with seeded cells were then transferred to the centers of the chambers in modified 96-well plates (Figure 4.1B) with a working medium volume of 3 ml one day after inoculation. The plates were then stacked onto a rotational shaker and put into a 37 °C cell culture incubator (Figure 4.1C). At each time point, the plates were taken out and the fluorescence was measured twice per day with a Cytofluor Series 4000.

Two weak embryotoxic chemicals, DM, and DPH, one non-embryotoxic chemical, Penicillin G, and one strong embryotoxic chemical, 5-FU were applied with different concentrations to low density ES cells (exposure one day after seeding) or high density ES cells (exposure at late exponential growth phase). All of these four chemicals were from Sigma Chemical Company, St. Louis, MO. Fluorescence signals from live cells were calculated as the values from the central wells of the small bioreactors minus the

background from medium and scaffolds. Measurements were usually performed twice per day.

4.4. Results

4.4.1. Cell culture microenvironment

3-D cell cultures have been used in biomedical research since early last century. Research mainly focuses in the areas of multi-cellular tumor spheroids as a model to study tumor behaviors and clinical cell-based tissue engineering for transplantation. Variations in oxygen pressure, nutrition concentration and pH etc. due to poor mass transfer in 3-D architectures have physiological implications and thus result in different cellular responses. These variations are critical to both of these two areas. Proper access of oxygen and nutritions and clearance of metabolic wastes are critical to the survival of transplanted cells *in vivo* [17]. Heterogeneous microenvironmental conditions such as oxygen pressure, glucose concentration, and pH influence the responses of tumor cells to therapy and remain one of the biggest obstacles for cancer therapy [7; 12]. Because of well-developed vasculature, normal tissues *in vivo* rarely have any cellular heterogeneity caused by poor diffusion. Therefore, *in vitro* models consisting of well-vascularized microregions would be better substitutes to normal tissues than those with heterogeneous microregions for chemical toxicity testing. H&E staining was used to study microenvironment inside of our cell-matrix samples 6 and 10 days after inoculation. Because the homogeneous distribution of fibers with high porosity in our scaffolds provides stable microcirculation regions, no necrotic cores or various zones were found.

All cells were loosely attached to each other. Figure 4.2 shows the two sections of the largest aggregates found from all H&E slices. The radiuses of both were less than 100 μm.

Among many variables, oxygen plays a key role in cell cultivation, because of its low diffusion rate and high consumption rate compared to other factors such as glucose and amino acids [17]. In addition, it has been widely reported that hypoxia caused significant resistance to chemicals or irradiation [18-20]. Oxygen diffusion and consumption kinetics can be calculated as:

$$D\nabla^2 C = Q_{O2}$$

where C is the local oxygen concentration, D is the oxygen diffusion coefficient in cell aggregates and Q_{O2} is the volumetric oxygen consumption rate and equal to the product of the specific oxygen uptake rate q_{O2} and the concentration of cells in aggregates C_{cell}.

Approximating cell aggregates in our 3-D cultures as cylinders and assuming that C only depends on the radial position (r) gives the equation:

$$\frac{1}{r}\left[\frac{d}{dr}\left(r\frac{dC}{dr}\right)\right] = \frac{Q_{O2}}{D}$$

Which is subject to the following boundary conditions:

$r = R$ (at the outer boundary of the cylinder), $C = C_0$ (saturation oxygen concentration)

$$r = 0 \ , \frac{dC}{dr} = 0$$

The oxygen diffusion coefficient D in normal tissues is 2×10^{-5} cm^2/s [17]. The medium oxygen concentration in cell culture incubators with 20% oxygen pressure is 2.14×10^{-7} mol/ml. Because of good agitation, C_0 was assumed to be equal to this value. With ES cell specific oxygen uptake rate q_{O2} about 2.9×10^{-8} nmol/cell·s [21] and 10^9 cell/cm^3 cell density in real tissues, the volumetric oxygen consumption rate of ES cells Q_{O2} was estimated to be 2.9×10^{-8} mol/s·ml.

The solution to the differential equation with above boundary conditions gives oxygen distribution in the cylinder as follows.

$$\frac{C}{C_0} = 1 + \frac{Q_{O2}R^2}{4DC_0}\left(\left(\frac{r}{R}\right)^2 - 1\right) = 1 + \phi^2\left(\left(\frac{r}{R}\right)^2 - 1\right)$$

where ϕ^2 is the parameter to describe the ratio of oxygen consumption and diffusion. When ϕ^2 is equal to 1, C is zero at the center of the cylinder; when $\phi^2 = 0.5$, $C = 0.5C_0$ at $r = 0$. Oxygen tension in most of current cell culture incubators (20%) is much higher than that in the physiological environment (2-6%) [22; 23]. Especially, mouse embryo fibroblasts *in vivo* were under 3% oxygen tension [24]. Furthermore, 5% and 20% oxygen tension made no significant difference for the culture of undifferentiated stem cells [21]. In this case, we can assume that when $\phi^2 < 0.5$, there is no oxygen limitation. R is calculated to be less than 172 μm to fulfill $\phi^2 < 0.5$. As shown in the H&E staining sections (Figure 4.2), all cell aggregates had a radius less than 100 μm, suggesting that

microregions inside of scaffolds were well oxygenated and could mimick the well vascularized tissues.

4.4.2. Effects on cell specific fluorescence

With fluorescence to represent cell number in cytotoxicity tests, it is important to ensure that the cell specific fluorescence can maintain the same level upon the treatment of different chemicals. Gu et al. (1993) reported that the expression of β-galactosidase controlled by CMV was higher in S phase than in other phases [25]. Basu (2004) also observed that CHO-GFP cells with GFP expression under the control of CMV expressed more GFP in S-phase [26]. In this regards, it is worthy to study the effects of cell cycle specific chemicals on cell specific fluorescence. Among the chemicals we studied, exposure to 5-FU [27; 28] could cause S-phase arrest, while DM [29-31] and DPH [32] resulted in cell cycle arrest in G1 and G0/G1, respectively. No reports on the cell cycle specificity of penicillin G were found. In the study, flow cytometry was used to study if chemicals influenced cell cycle progression and thus resulted in different cell specific fluorescence. Table 4.1 shows the toxicity effects of DM, DPH, penicillin G, and 5-FU on cell number and cell specific fluorescence. As the doses of S-phase specific chemical 5-FU increased, cell specific fluorescence could be up to 3.3 times higher than that of the control. However, the cells treated with the other three chemicals had similar levels of specific fluorescence as that of the control. Gemcitabine (Eli Lily), as another S-phase chemical, had a similar effect as 5-FU on cell specific fluorescence (data not shown). The results were consistent with S-phase specificity of CMV, which suggested a possibility of

drug screening approaches using engineered cells with expression of reporter genes in a specific cell cycle.

4.4.3. Cytotoxicity on both 2-D and 3-D low density cells

In the subsequent experiments, we exposed four embryotoxic reference chemicals DM, DPH, Penicillin G and 5-FU one day after inoculation in both 2-D (Figure 4.3) and 3-D cell cultures (Figure 4.4). As shown in Table 4.2, all chemicals demonstrated considerable toxicity in both cultures. The extents of cell kill at the maximal doses were all higher than 80%, which indicated that most of the cells were sensitive to the chemicals in these two cultures.

Although as the 5-FU dose increased in 2-D cultures, there was a clear decrease of fluorescence due to the toxicity of 5-FU, at 70 h the high doses of 5-FU caused the total fluorescent signals of 2-D cells higher than that of the control (Figure 4.3D). It could be explained by the increased cell specific fluorescence with 5-FU treatment as discussed in the previous section. However, this disharmony didn't exist in 3-D proliferation assays (Figure 4.4D), suggesting that fluorescence could better represent cell number upon the treatment of chemicals in 3-D cultures. In addition, only 5-FU increased cell size in 2-D cultures (Figure 4.5). This might be because cell treated with 5-FU stopped DNA synthesis, while cells continued growth. Based on these experiments, we postulated that multilayer 3-D architectures could limit cell size, cause gene expression perturbation, and thus prevent an increase in cell specific fluorescence upon exposure to chemicals.

Figure 4.6 shows the relationship between the specific growth rate and chemical dose in both 2-D and 3-D cultures. Here, the cytotoxicity was defined as the concentration of a chemical causing 50% reduction of the specific growth rates ($IC_{\mu 50}$). Table 4.2 compares the cytotoxicity test results in the 2-D and 3-D systems and the reported results in the literature [33]. In general, $IC_{\mu 50}$ values were lower in 3-D than in 2-D and those previously reported [33]. For DM and Penicillin G, $IC_{\mu 50}$ in 2-D were 3 and 6 times of those in 3-D, respectively. The difference of 5-FU cytotoxicity in 3-D and 2-D was not significant. $IC_{\mu 50}$ of DHP in 2-D was similar to 3-D, which was lower than the results from [33]. It should be noted that in 2-D cultures, DPH formed crystals on the bottom of wells. The higher the concentration, the more the crystals formed. This might lead to a strong effect on cells attaching to the bottom in 2-D cultures. On the other hand, in our 3-D culture system, there were comparatively fewer crystals formed due to continuous agitation and for both EST tests, medium was replaced on day 3 and day 5 during the culturing, which might also prevent the accumulation of crystals. The fact that $IC_{\mu 50}$ of DPH in 2-D was even lower than in 3-D, which was not like the other three drugs, partially confirmed the assumption that chemicals with low solubility have a stronger effect in static 2-D cultures due to the formation of crystals in the bottom, while agitation in 3-D cultures can help to dissolve chemicals, mimicking the *in vivo* situation. In conclusion, our fluorescence 3-D proliferation assay was more sensitive than in 2-D and the standard EST test.

4.4.4. Cytotoxicity on high density 3-D cell culture with tissue-like morphology

Culture organization can modulate cellular molecular mechanisms and result in changes in cell cycle progression, which further alters the responses of individual cells to chemicals. In both tumors and normal tissues *in vivo*, most of cells are in a quiescence state with a very small faction of the total population being the actively dividing cells [34]. Multi-cellular tumor spheroids are well-established 3-D *in vitro* model systems which reflect tumor pathophysiological situations and show similar responses to external stimuli as their *in vivo* counterparts. They are better than 2-D monolayer cultures to evaluate drug efficacy. Experiments to study the differences between monolayer cells and multilayer cells showed a reduction of cells in S-phase in multilayer cultures accompanied by a change of cell-cycle specific protein expression and nucleotide pools [35]. Using BrdU-PI dual-staining confocal laser scanning microscopy, Ma et al. (2000) discovered that long-term cultures in PET scaffolds had much fewer cells in S-phase as compared to a 3-day culture, and were accompanied with decreased expression of a proliferating marker cyclin B1 and increased expression of quiescence marker $p27^{kip1}$ [36]. Due to different cell cycle specificity of chemicals, high-density cells such as tumor spheroids or tissue constructs in PET scaffolds were expected to show different levels of resistance as compared to conventional 2-D cultures with highly proliferating cells.

Figure 4.7 shows the effects of various chemicals on cell growth in high-density 3-D cultures. Drug cytotoxicity has been evaluated based on toxicity index such as IC_{50} (50% growth inhibition), TGI (total growth inhibition) or LC_{50} (50% cell killing). However, none of these toxicity indices can be used for high density cultures because they are

either not available or irrelavant to the situation. Therefore, EC_{50} (the concentration required to obtain 50% of the maximum toxicity effect) is used to define cytotoxicity in high-density cultures. If the elimination of cells is considered as the maximum effect of a chemical, an EC_{50} is not always available [35]. As can be seen in Fig. 4.7, a chemical such as 5-FU even at its highest possible concnetration might still not be able kill all cells or reduce all the fluorescence in high-density cultures. In this case, we define the maximum effect as the fluorescence reduction caused by the saturation (DM and DPH) or maximum (Penicillin G and 5-FU) concentrations used in the media, which were much higher than those used in 2-D assays. In addition, we also noticed that maximal effects changed along with time, so the time point with the highest value of the maximal effects was used for the cytotoxicity definition. A resistance factor or Rf is herewith defined as the ratio between EC_{50} and $IC_{\mu 50}$. Rf = 1 indicates that the cytotoxicity of a chemical to 3-D high density cultures is similar to that of a low-density culture, while a high Rf means a large difference of cytotoxicity between these two cultures.

Despite a very high dose used (up to 2857 times of $IC_{\mu 50}$), 5-FU was almost non-effective to cells in high-density cultues mimicking tissue constructs. The extent of cell kill at the maximum dose of 5-FU was only 20%, much lower compared with the other chemicals. Because of the lack of effectiveness, EC_{50} of 5-FU was not meaningful. In contrast, DM, DPH and penicillin G all showed considerable toxicity in tissue constructs, with an extent of cell kill 80.9%, 52.2% and 62.3%, respectively.

The toxicity of all these four chemicals decreased in 3-D high density cultures when compared with 2-D monolayer or 3-D low density cultures (Table 4.3). Although Rf

values were different for the first three chemicals, in general these chemicals could cause sufficient cytotoxicity at a decent dose (less than 50 times of those in 3-D low density cultures). However, 5-FU had no significant effect even at a high concentration of 100 μg/ml, over 2,000 times of the $IC_{\mu 50}$ found in the proliferation assay. In other words, 5-FU was almost non-effective to the tissue constructs. 5-FU is small water-soluble molecule and easy to penetrate into tissues. Several hours were enough for it to diffuse cross more than 100 μm thick solid tissue with a certain amount [37-39]. Therefore, 5-FU seems to be able to reach each cell in tissue constructs with a certain dose and the penetration of this chemical should not be the main obstacle for its efficacy. Considering the S-phase specificity of 5-FU and the reduction of S-phase cells in PET 3-D long-term cultures, we can hypothesize that in comparison with 2-D or 3-D low density cells, the 3-D tissue constructs have a different cell cycle progression, which can cause various cellular responses to S-phase specific and non S-phase specific chemicals.

4.5. Discussion and conclusion:

In our previous study, 3-D cultures in PET scaffolds have been provn to be a good alternative *in vitro* system resembling cell growth microenvironment and cells cultured in these scaffolds exhibited morphology and interaction similar to an *in vivo* 3-D milieu up to several months [40-42]. In this study, a high-throughput 3-D GFP-based cell assay was used as an *in vitro* model to predict *in vivo* performance of several chemicals. Cell aggregates in PET scaffolds can be considered as substitutes to well-vascularized normal tissues as found in the investigation of biological characteristics and model calculation. CMV-controlled cell specific fluorescence was useful to screen chemicals with cell phase

specificity. 3-D assays with exponentially growing cells seemed to be a little more sensitive than 2-D proliferation assays and the interference of cell specific fluorescence by cell cycle specific chemicals was unconspicuous in this study. 3-D tissue constructs raised significant difference in cytotoxicity and the difference could be explained by cell cycle progression altered in different culture models.

Proliferating cells are targets for many chemotherapeutic drugs. However, the growth fraction in real tumors is much lower than in established cancer cell lines cultured *in vitro* [34]. 3-D *in vitro* tissue constructs could restore cell cycle progression and rescue multi-cellular drug resistance *in vivo*. The metabolites of 5-FU could be misincorporated into RNA and DNA and 5-FU could deplete thymidine by binding with thymidylate synthase. 5-FU is an S-phase specific chemical causing S-phase arrest. It was developed in the 1950s by observing that pyrimidine uracil was used by rat hepatomas more rapidly than other tissues. However, compared to *in vitro* cytotoxicity tests, the performance of 5-FU was not as exciting as expected (10% response rate for 5-FU as a single agent in advanced colorectal cancer [43]). This is explained by several established 3-D models that a solid tissue environment has a much higher level of drug resistance [35; 43]. Using live cell fluorescence, we discovered that tissue constructs with less proliferating cells were almost unaffected by 5-FU, which was consistent with the increased cell specific fluorescence controlled by an S-phase specific promoter CMV upon the 5-FU treatment in monolayer. Finally, it needs to note that within 10 days after fertilization, mouse embryos begin to develop all organs and early embryos are highly proliferating in vivo, which is not like somatic cells in mature mice. The exception might cause a high toxicity

of 5-FU *in vivo*, which is consistent with the results from the 3-D low density model instead of the high density model. This can explain that 5-FU was shown as a chemical with strong embryotoxicity in animal experiments [44].

Because most of differentiation processes require ES cells to form aggregates such as EBs and then simultaneously develop into various cell phenotypes, an *in vitro* quantitative assay with wild-type ES cells during development processes is complicated. Normally, immunological or molecular methods are required to quantify specific cell types. These are time-consuming and therefore not suitable for the design of automated, high-throughput screening cell assays and the kinetic studies of the entire differentiation process. Because cell layering did not impair significantly signal detection using a fluorometer [45] and the signal to noise ratio was largely increased by cell layering, our 3-D system would stand much chance of monitoring full-scale differentiation to specific lineages in EBs if CMV was replaced by a tissue-restricted promoter as Bremer et al. (2004) and Paparella et al. (2002) did [16; 46]. In addition to 3-D ES cell differentiation, other engineered cell lineages containing reporter genes coupled to particular biological recognition components can be applied in order to study other 3-D tissue models. The measurement of fluorescence with fluorometry may provide a more accurate and convenient way for full-scale online quantification of specific function units in 3-D structures. Our work addresses a prototype for advanced 3-D tissue culture arrays.

In general, non-proliferating cells tend to be less sensitive to many conventional chemicals. Modeling study suggested that the presence of a large number of G0 cells had the largest implication for the resistance of chemotherapy [47]. However, most of current

in vitro chemical cytotoxicity tests are based on monolayer cell cultures lacking in a similar cell cycle progression as normal cells *in vivo*. It is therefore important to develop a system better resembling a multi-cellular environment in the body to evaluate chemical toxicity. In "V-bottomed" microtitre plates, postconfluent multilayer cells presented a low proportion of S-phase cells and caused more resistance to S-phase specific chemicals [35]. Nevertheless, there are some major problems limiting the extensive usage of their methods. First, the medium had to be refreshed every one or two days in order to prevent exhaustion and this limited the system automation. Second, due to using a fixed point assay, they lacked dynamic data to evaluate the performance of chemicals accurately. In our study, similar results were obtained with these drawbacks overcome. For the toxicological evaluation of chemicals, the future system will need to have online detection in an automatic high-throughput fashion, combining with current well-developed computational techniques to predict the performance of thousands of chemicals *in vivo* efficiently and detailedly.

Drug discovery also requires screening large libraries composed of hundreds of thousands of chemical compounds [48] due to the rapid advances of combinatorial chemistry for chemical synthesis and advanced biological tools to identify targets. Current status of cell-based screening is not reliable partially because *in vitro* cells are highly proliferating while normal cells *in vivo* are in their quiescent state. This brings an overestimation of compound potency, which results in a large number of candidates waiting for further *in vivo* tests. In the other hand, due to the internality of animal experiments, high-throughput screening might only be able to be realized before this step.

In this case, the removal of drug candidates with low probability of market success should be as early as possible [6]. It is believed that solid tissues normally display a higher resistance to chemicals *in vivo*, which is called multi-cellular resistance [7]. Multi-cellular pharmacodynamics, a newly developed concept, intends to uses multi-cellular tissue constructs to dynamically model and simulate chemicals distribution and other ADMET properties, combining molecular, cellular and tissue level information [49]. It will study the dynamics and diversity of drug effects on a 3-D organization in order to have a better and detailed potency prediction of candidates. However, models up to date are short of the comprehensiveness. On one hand, although many *in vitro* models have been established to study pharmacodynamics in a high-throughput manner (high content cell assays) or multi-celllular behaviors (multi-cell tumor spheroid), none of them can afford both. In the other hand, animal models are less applicable to study multi-cellular resistance since *in vivo* metabolism, distribution, absorption, and biochemistry of the drugs often conceal real tissue responses and complicate the analysis. In this case, a high-throughput 3-D tissue assay with low cost and high efficiency serving as a bridge between 2-D cells cultures and animal models is paramount. One aim of our system is to replicate these specific 3-D engineered cell-based systems faithful to their *in vivo* counterparts in minimized arrays in order to improve predictability in savings of time, labor and expenses in drug discovery processes.

To pursue therapeutic efficacy and avoid side-toxicity, the selectivity of an ideal chemotherapeutic approach is to kill tumor quiescent cells over normal quiescent cells [34]. Base on this idea, the selectivity of toxic potency to solid tumor constructs instead

of normal tissue constructs *in vitro* has much more predictive value than the comparison of toxicity in simple conventional *in vitro* cell cultures. Engineered needle-punched isotropic non-woven PET fibrous scaffolds with 20 μm in diameter and 30-80 μm pore size have been reported to have high surface area to volume ratio and minimal transport limitation with less diversity in pore structures. Currently, most commercially available biodegradable non-woven scaffolds have pore size larger than 200 μm, while 50-100 μm was suggested to be a favorable distance for *in vitro* tissue cultures [50]. In addition, non-biodegradable scaffold is easier to maintain the construct structures to perform long-term *in vitro* toxicity tests. High porosity (94%) and pore interconnectivity improved the conductivity to nutrient delivery and waste removal. ES cells cultured on these scaffolds with chemical and biological pretreatment to improve the cell attachment formed homogeneous aggregates and resembled vascularizd tissues in the body. The established system has been shown to support the formation of both multi-cellular constructs with normal diploid karyotype cells (ES cells) and cancer cells (colon cells). Toxicity test on these 3-D constructs has proved to be in a high-throughput online manner.

4.6. Reference

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	Dose (μg/ml)	Cell number (10^4)	Cell specific fluorescence (RFU)
Control	0	112 ± 3	111 ± 2
DM (G1 arrest)	8	109 ± 5	117 ± 2
	32	72 ± 9	120 ± 4
	128	51 ± 4	113 ± 5
DPH (G0/G1 arrest)	8	92 ± 10	126 ± 1
	32	88 ± 2	115 ± 0
	128	71 ± 6	109 ± 3
Penicillin G (non)	1125	100 ± 11	137 ± 2
	4500	69 ± 2	124 ± 1
	18000	14 ± 1	111 ± 1
5-FU (S-phase arrest)	0.0125	94 ± 4	141 ± 4
	0.045	56 ± 4	199 ± 27
	0.18	25 ± 3	371 ± 4

Table 4.1. Effects of five chemicals on cell number and cell specific fluorescence in 2-D cell culture

Drug	3-D low density cultures		2-D cultures		ZEBET[a]	ECVAM[b]
	$IC_{\mu 50}$ (µg/ml)	Extent of cell kill	$IC_{\mu 50}$ (µg/ml)	Extent of cell kill	IC_{50} (µg/ml)	IC_{50} (µg/ml)
DM	11.5	88.9%	32	82.2%	37	51
DPH	39	91.1%	30	83.7%	102	195
Penicillin G	750	98.4%	4500	93.4%	2100	2000
5-FU	0.035	89.0%	0.045	85.6%	0.09	0.065

[a] ZEBET - Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (Scholz et al., 1999)
[b] ECVAM - European Centre for the Validation of Alternative Methods (Scholz et al., 1999)

Table 4.2. Comparison of cytotoxicity and the extent of cell kill upon exposure to various embryotoxic reference chemicals in low-density 2-D and 3-D cultures .

Drug	EC_{50} (µg/ml)	Extent of cell kill	Rf (= EC_{50}/ $IC_{\mu 50}$)
DM	65	80.9%	5.7
DPH	600	52.2%	15.4
Penicillin G	23000	62.3%	30.7
5-FU	N/A	20.0%	>1000

Table 4.3. Cytotoxicity and the extent of cell kill upon the treatment of different doses of four embryotoxic reference chemicals in 3-D tissue constructs

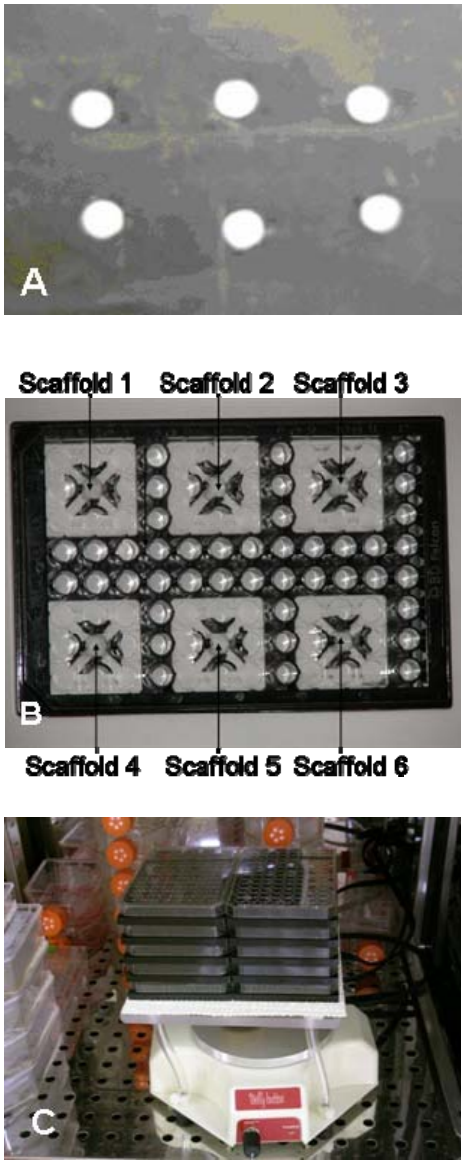

Figure 4.1. The establishment of the 3-D cell culture system. A. Cake-shape scaffolds with 0.1cm height and 0.3 cm2 bottom surface area. B. Each scaffold was fixed into the center of each square chamber, which formed a small bioreactor. Most of walls in chambers were removed so that medium was able to flow everywhere in these 9-well integrities to provide enough mass transfer for 3-D cultures after the plate was put on a shaker. C. Cells were cultured on the scaffolds in fabricated plates stacked on a shaker in a 37 ^{o}C incubator to allow many 3-D bioreactors to run in parallel.

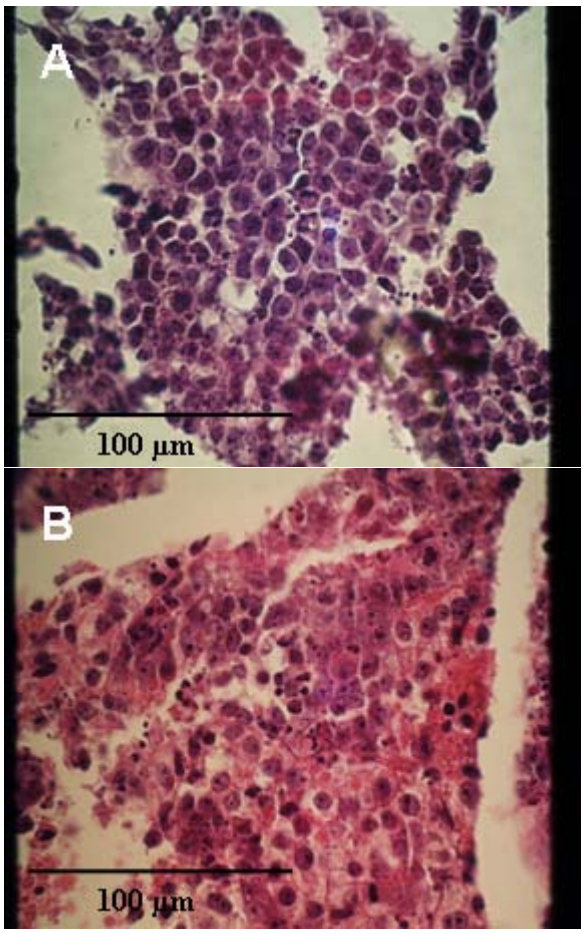

Figure 4.2. H&E stained cross sections of the largest aggregates found in 3-D cultures of ESCs. A. 6 days after inoculation B. 10 days after inoculation.

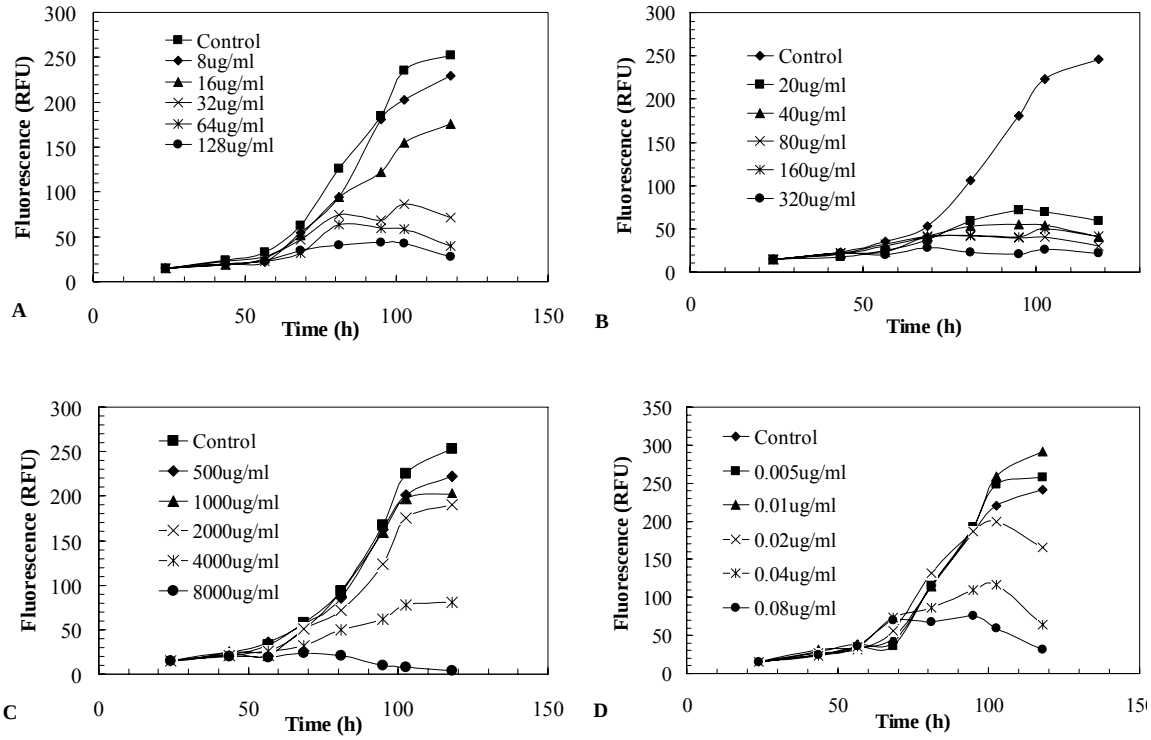

Figure 4.3. Growth kinetics of 2-D cultures of ESCs exposed to various embryotoxic reference chemicals in a dose-dependent manner one day after inoculation in. A. DM; B. DPH; C. Penicillin G; D. 5-FU.

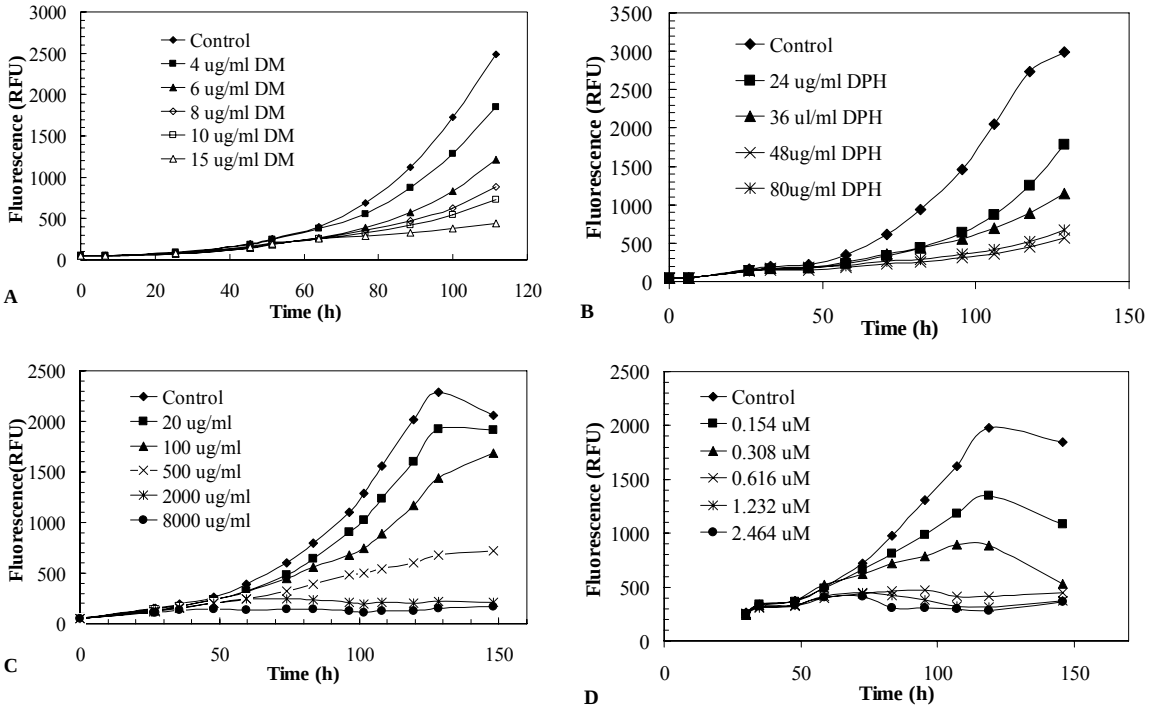

Figure 4.4. Growth kinetics of low-density 3-D cultures of ESCs exposed to various embryotoxic reference chemicals in a dose-dependent manner one day after inoculation. A. DM; B. DPH; C. Penicillin G; D. 5-FU.

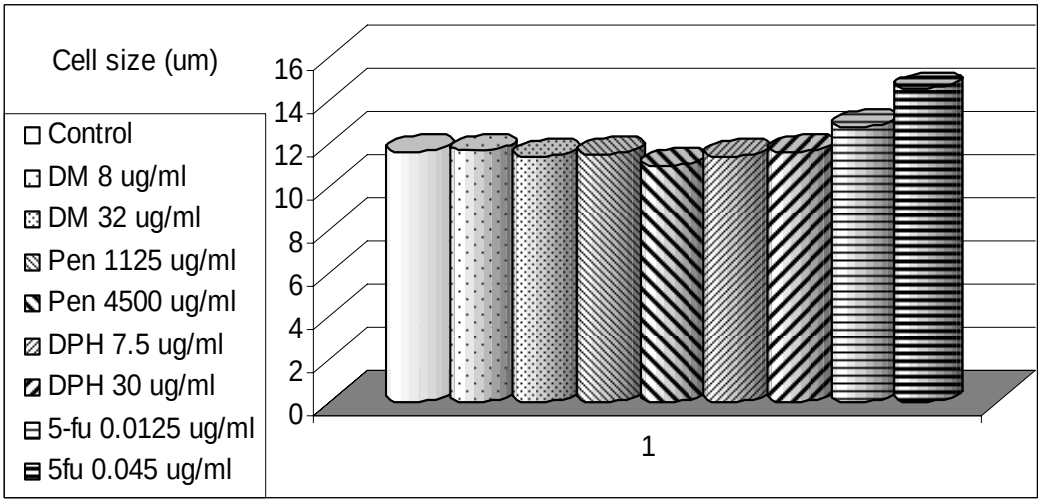

Figure 4.5. Effects of DM, DPH, Penicillin G and 5-FU on cell size.

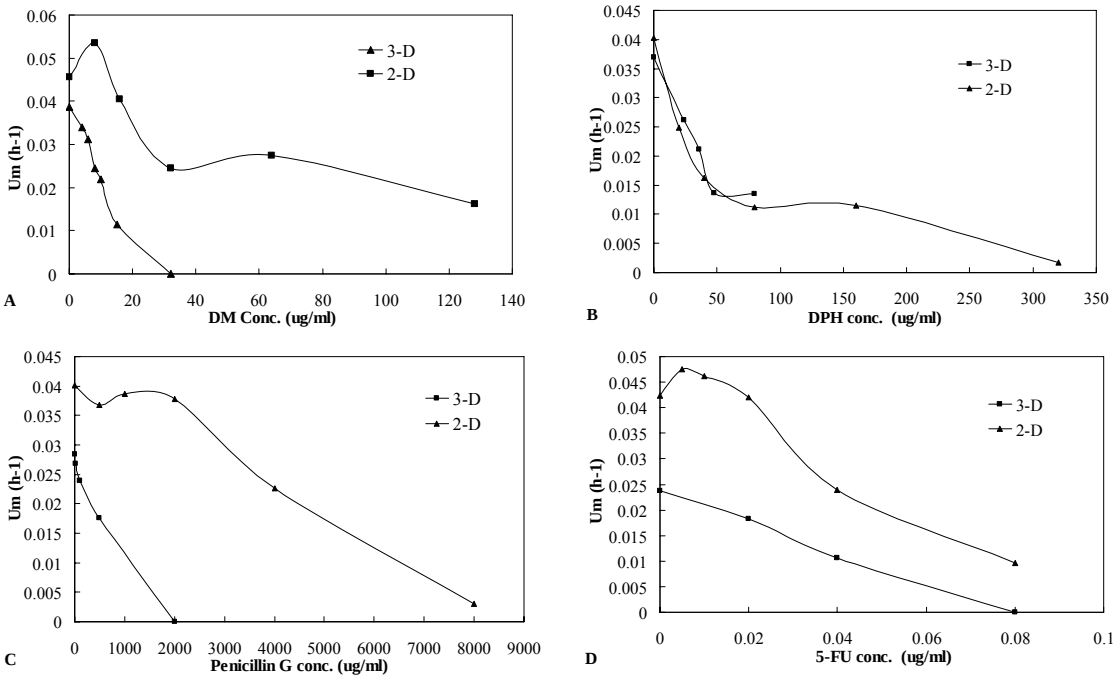

Figure 4.6. Effects of drug concentration on the specific growth rate (μ_m) of ESCs in low-density 3-D and 2-D cultures. A. DM; B. DPH; C. Penicillin G; D. 5-FU.

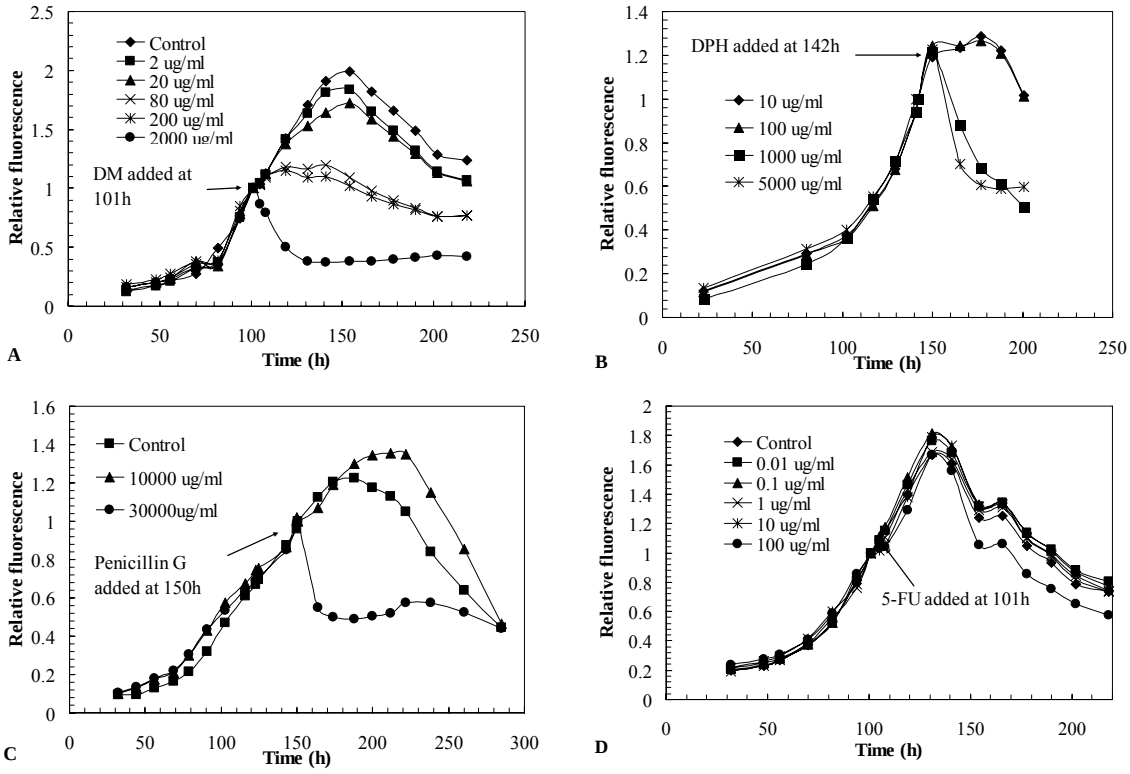

Figure 4.7. Kinetics of high-density 3-D cultures of ESCs in response to various embryotoxic reference chemicals in a dose-dependent manner. Fluorescence intensity was normalized against the signal at the time of chemical addition (= 1) as indicated by the arrow. A. DM; B. DPH; C. Penicillin G; D. 5-FU.

CHAPTER 5

APPLICATION OF 3-D WHOLE CELL GFP-BASED ASSAYS IN HIGH-THROUGHPUT DRUG SCREENING

5.1. Abstract

Most of chemotherapeutic drugs target actively proliferating cells. However, the fraction of proliferating cells in both normal solid tissues and tumors is low *in vivo*. Combining with an autofluorescent technique, 3-dimensional (3-D) multicellular structures mimicking *in vivo* microenvironment was introduced into high-throughput drug screening. Compared to conventional 2-D monolayer cultures, both 3-D cultures with multicellular structures and fresh tissues have lower expression in Ki-67, a proliferation maker, and higher expression in $p27^{kip1}$, a quiescence marker. Three commonly used drugs 5-fluorouracil (5-FU), gemcitabine and sodium butyrate were tested for their toxicity effects on mouse embryonic stem cells (ESCs) and colon cancer cells at low cell density and high cell density (multicellular structures). Finally, an array applying the same principle which could afford 40 micro-bioreactors to run within one plate of standard screening size was demonstrated. The system allows high-throughput study of drug cytoxicity on 3-D multicellular structures representing *in vivo* biology and has a much better predictive value than 2-D monolayer cultures.

5.2. Introduction

Colon cancer is the third most common cancer and the second leading cancer causing death only next to lung cancer in Western countries. Surgical extraction combining chemotherapy, immunotherapy and radiotherapy is the typical treatment for colon cancer [1; 2]. 5-Fluorouracil (5-FU) has been the most commonly used chemotherapeutic agent against colon cancer during the past few decades and new treatments combining 5-FU with other drugs are continually emerging. Currently, advanced colon cancer is treated with 5-FU and folinic acid, but the remission rate is only 23% [3] and response rate is 40-50%.

Current cell-based assays usually suffer from inconsistent culture environments different from their *in vivo* counterparts [4; 5]. Most normal cells and tumor cells (as abnormal cells) are highly proliferating in monolayer cultures. Some normal cells such as mouse ES cells even divide more rapidly than most of tumor cells. Because the targets of many antimetabolites agents are actively proliferating cells, cytotoxicity assays in monolayer cultures usually result in high efficacy to both normal and tumor cells. However, except for developing organisms, most normal somatic cells *in vivo* stop proliferating and stay in a quiescent state, which could cause a high-level resistance to antineoplastic drugs [6; 7]. The percentage of rapid dividing cells in tumors is also very low, although it could be higher than that of normal cells. This, in addition to drug adsorption, penetration and metabolism, could result in resistance of chemotherapeutic agents and could not be

revealed by simple traditional 2-D monolayer cultures. Furthermore, as shown in this study, tests based on monolayer cultures provided confusing results that those antimetabolites showed higher activity against normal cells instead of tumor cells.

There are about 3500 cases of cancer-complicated pregnancies every year in USA [8]. Dilemmas are always raised for physicians because life-saving antineoplastic therapy for mother poses life-threatening concerns for fetal well-being. All chemotherapeutic drugs are potentially very dangerous because they might act on actively proliferating cells, which are more similar to cells in rapidly growing tumor than most of non-proliferating somatic cells. It has been reported that the risk of abortion and malformation will increase if chemotherapy comes up at the beginning of a pregnancy. The potential adversities of these drugs are related to the drug regimen and the timing. However, physicians must rely on retrospective studies from case reports because no clinical trial is likely to be allowed. In this case, the evaluation of chemotherapeutic risk on fetus well-being is difficult to establish. Recently, as the quick advance of embryonic stem cell biology, systematic study of reproductive outcome of cancer treatment regime has been performed with different assays, and embryonic stem cell test (EST) is among the most promising assays for developmental toxicology test [9].

Both the development of fetus and the existence of solid tumors are organized within a 3-D environment. In this study, a label-free high-throughput fluorescence assay with cells expressing an enhanced green fluorescent protein (GFP) grew in a 3-D fibrous scaffold was used as an alternative *in vitro* method to study embryotoxicity and antineoplastic activity of drugs. Three commonly used cancer drugs, 5-fluorouracil (5-FU), gemcitabine

and sodium butyrate, were tested for their cytotoxicity effects on mouse embryonic stem cells (ESCs) and human colon cancer cells. These chemicals were tested in 3 different models: 2-D monolayer cultures, 3-D low-density cultures, and 3-D high density cultures with multicellular structures (tissue-like morphology). In the last model, tumor cells were arranged in a 3-D environment to resemble *in vivo* small avascular tumors.

5.3. Material and methods

5.3.1. Chemicals

5-Fluorouracil and sodium butyrate were from Sigma Chemical Company, St. Louis, MO. Gemcitabine was provided by Eli Lilly, Indianapolis, IN. Both 5-FU and gemcitabine are antimetabolites. They can cause cell cycle arrest in early S phase and their activity is S-phase specific. However, 5-FU has the greatest impact of it in colon cancer treatment, while gemcitabine has limited effects on colon cancer [10-17]. Butyric acid can induce hyperacetylation of histones, which results in the increase of DNases accessibility to chromosome DNA [18]. Cell cycle arrest at G0/G1 phase is commonly observed in the presence of butyrate and accompanied with the induction of a cell differentiated status and finally apoptosis.

5.3.2. Media and cell lines

Both murine ES-D3 cell line (doubling time = 12 h) and colon cancer cell line HT-29 (doubling time = 24 h) from ATCC were transfected with PEGFP-N3 (Clontech, Palo Alto, CA) using Lipofectamine 2000 (Invitrogen) and positive colonies were isolated and

amplified to establish stable cell lines ES-GFP and HT-29-GFP. EGFP was expressed under the control of human cytomegalovirus (CMV) promoter, which is S-phase specific [19; 20]. The growth rates of the transformed cell lines were the same as those of their respective parent cell lines. Also, SSEA-1 expression of ES-GFP after 20 passages maintained at a high level (97%) similar to that of the untransformed ES cells. Both transgenic cell lines were cultured in media without G418.

DMEM plus 10% FBS was used for maintenance and cytotoxicity assay of HT-29-GFP. ES-GFP cells were maintained with DMEM (Gibco) supplemented with 10% FBS, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µM monothioglycerol and 1000 U/ml leukemia inhibitory factor (LIF, Chemicon). During cytotoxicity test, media without LIF was used for ES-GFP cells.

5.3.3. Scanning Electron Microscopy (SEM)

3-D tissue samples in PET scaffolds were fixed with 2.5% glutaraldehyde (Sigma) for 10 hours. After washed with PBS, they were dehydrated with graded alcohols from 20% to 100%. The samples were then soaked into a mixture of increasing hexamethyldisilazane (HMDS) (Sigma) concentration and decreasing ethanol concentration until HMDS concentration reached 100%. Gold-palladium was then used to sputter-coat the samples in a sputter coater (Pelco Model 3). The current was maintained at 17 mA for 120 seconds at a 14 Pa argon pressure. Pictures were taken with a scanning electron microscope (Nova 400 NanoSEM; FEI, Hillsboro, OR).

5.3.4. Measurements of cell number, cell size, and cell specific fluorescence

Cells were cultured in 12-well plates. 50,000 cells of ES-GFP or HT-29-GFP were inoculated into each well with 1 ml medium. One day after inoculation, cells were exposed to the test chemicals at various concentrations. Cell number, cell-specific fluorescence and cell size were then measured with a hemocytometer, a flow cytometer (BD FACS Calibur, Franklin Lakes, NJ) and a Coulter counter (Beckman Coulter Multisizer II, Fullerton, CA), respectively.

5.3.5. Histological examination of biomarkers

To evaluate the cell cycle progression in 3-D scaffolds, a cell proliferation marker Ki-67 and a quiescence marker $p27^{kip1}$ were studied by immunohistochemistry. Fresh human tonsil and HT-29 cells from 1-week monolayer cultures were used as controls.

Agarose-embedded cell pellets were prepared for histological processing of cells from monolayer cultures. Fresh trypsinized 10^7 cells were spun down into a pellet. Supernatant was removed and cells were resuspended by vortex. A solution with 3% agarose in PBS was prepared, cooled down to about 50 °C and added into cells at the same volume. The mixture was vortexed immediately. The agarose-embedded cell pellet was cooled down in -20°C refrigerator for 10 min before fixing.

Fresh human tonsil, 3-D tissue cultures (2 weeks) and agarose-embedded cell pellets were incubated in 10% formalin for 3 hours and then embedded in paraffin wax. Sections

of 4 µm thick each were cut, and then stained with Hematoxylin and Eosin (H&E) or used for immunohistochemical staining.

For immunohistochemical staining, the sections were placed on positively charged slides, and then dewaxed and rehydrated with xylenes and graded alcohols. Slides were peroxidized with 3% H_2O_2 for 5 minutes in order to inhibit endogenous peroxidase before antigen retrieval. Slides were then placed on an immunostaining system Dako Autostainer. After washed with PBS, primary antibody against Ki-67 (1:150 in PBS; Dako, M7203) or $p27^{kip1}$ (1:50 in PBS; Dako M7240) was added at room temperature. This was followed by consecutive additions of biotinylated linking antibody, enzyme conjugated streptavidin, and substrate chromogen DAB. Slides were then counterstained with hematoxylin, dehyrated with graded alcohols, and coverslipped.

5.3.6. Cytotoxicity assays in 2-D cultures

50,000 cells were seeded into each well of 12-well plates. Cells were exposed to the chemicals one day after inoculation. The cell number in each well was counted every day using a hemacytometer under a microscope. IC_{50} was calculated as the concentration of chemicals which resulted in 50% reduction in cell number as compared to the control on day 3 for ES-GFP cells and day 6 for HT-29 cells.

2-D autofluorescent cytotoxicity assays of the chemicals were also performed in 96-well plates. 5000 cells of ES-GFP were inoculated into each well with 150 µl medium. Cells were exposed to the chemicals one day after inoculation. The culture fluorescence was monitored with a flurescence plate reader (Cytofluor Series 4000, Applied Biosystems,

Foster City, CA). The fluorescence intensity was determined as the signals obtained after replacing culture medium with the same volume of PBS. IC_{50} was calculated as the concentration of chemicals resulting in 50% reduction as compared to the control when the control reached the maximum fluorescence.

5.3.7. Cytotoxicity assays in 3-D cultures

Unless otherwise noted, the assays for 3-D cultures were performed on modified 96-well plates, each had 6 microbioreactor units (Figure 5.1A). 25,000 ES-GFP cells or 40,000 HT-29-GFP cells in 25 µl medium were seeded into each PET scaffold pretreated with 1 M sodium carbonate, 1 M sodium hydroxide, 10 µg/ml fibronectin and medium in a 96-well plate. After 6 h for cell attachment, 180 µl of media were added. One day after seeding, each scaffold was transferred into the center of a microbioreactor containing 3 ml of media on the modified 96-well plate (see Figure 5.1A). The plates were then stacked onto a rotational shaker with a speed of 60 rpm in a 37 °C cell culture incubator. IC_{50} was calculated as the concentration of chemicals which resulted in 50% signal of the control when the control reached the maximum fluorescence. Drugs were added to the cell cultures 1 day (3-D low density models), 4 days (3-D high density ES-GFP model), or 8-10 days (3-D high density HT-29-GFP model) after inoculation. All assays with high density models were all triplicate or duplicate. IC_{50} was calculated as the concentration of chemicals resulting in 50% reduction in the fluorescence signal when the control reached the maximum fluorescence.

The cytotoxicity assay was also performed in a modified 384-well plate with 40 microbioreactors, each with a 1-ml working volume (Figure 5.1B). Unless otherwise noted, 10,000 or 16,000 cells in 11 µl of media were seeded into each pretreated scaffold, which was then placed in the microbioreactor containing 1 ml medium. The plate was incubated in a CO_2 incubator, agitated at a rotational speed of 90 rpm. The culture fluorescence was monitored with a flurescence plate reader (GENios Pro, Tecan, Durham, NC).

5.3.8. Statistical Analysis

Analysis of variance (ANOVA) was used to analyze data and significance was considered at $p < 0.05$.

5.4. Results

5.4.1. HT-29-GFP growth in 3-D PET scaffolds

During the culture of HT-29-GFP in 3-D, cell density increased and large aggregates were formed (Figure 5.2).

5.4.2. Drug-induced enhancement in cell specific fluorescence

Tables 5.1 and 5.2 show the effects of 5-FU, sodium butyrate, and gemcitabine on cell growth, cell size, and cell specific fluorescence for ES-GFP and HT-29-GFP cells, respectively. It is clear that all three chemicals were toxic to cells in a dose-dependent manner and they could increase cell specific florescence and enlarge cell size.

Due to the S-phase specificity of a CMV promoter, chemicals inducing S-phase arrest such as 5-FU and gemcitabine would increase cell size and thus cell specific fluorescence. However, studies here showed that the increased cell specific production of a protein under the control of a CMV is not usually, or at least not only a result of cell cycle distribution. It could also be caused by a specific induction that increased the whole cell productivity. Although sodium butyrate caused G0/G1 arrest, it had a significant effect on cell specific fluorescence. Bras-Goncalves et al. (2001) showed that after treating with 38.5 μM 5-FU for 24 h, the percentage of colon cancer cells in S-phase increased from 57% to 87% [21]. Compared to the control, the cell specific fluorescence increased 7.3 and 11.43 times for HT-29 cells exposed to 10 μM and 50 μM 5-FU, respectively, which could not be entirely attributed to the increased level of S-phase cells. On the other hand, there appeared to be a correlation between the increase of cell specific fluorescence and the enlargement of cell size (Figure 5.3). The fact that the increase in the cell specific flurorecence is more than proportional to the cell size (volume) suggests that the expression of GFP was greatly enhanced by these chemicals and the effect cannot be explained solely by changes in cell cycle progression. It is noted that DM (G1 arrest) and DPH (G0/G1 arrest) did not decrease the cell specific fluorescence in ES-GFP (data not shown), which also suggested that drug-induced changes of cell-cycle distribution could not explain the observed drug effects on the cell specific fluorescence.

5.4.3. Proliferation assays with 2-D and 3-D low-density cultures

The culture fluorescent signals can be affected by the total cell number and the cell specific fluorescence. For both 2-D and 3-D proliferation assays based on the GFP

fluorescence measurement, the increased cell specific fluorescence with drug dosage confounded the estimation of cell number or proliferation. In general, this confounding effect was more significant in 2-D cultures (data not shown) than in high-density 3-D cultures. For high-density 3-D cultures, the cells growing in the fibrous matrix might be confined by the available void space within the matrix and thus their cell size was more likely to be limited, thus reducing the effect on increasing the cell specific fluorescence.

IC_{50} for each case studied was estimated from the growth curves (see Figures in Appendix) and is listed in Table 5.3. As indicated by the IC_{50} value, ES-GFP cells were found to be more sensitive than HT-29-GFP cells in 2-D cultures for all three chemicals studied. However, in 3-D (low-density) cultures, ES-GFP cells showed a lower sensitivity to gemcitabine than HT-29-GFP cells.

It was also found that the doubling time for HT-29-GFP cells was much shorter in 3-D cultures (17 h) than in 2-D monolayer cultures (24 h). This might be because cell growth in the 3-D matrix was not limited by the available surface area as in the 2-D cultures on planar surface. The increased growth rate could also be attributed to increased cell contact in 3-D milieu as increasing cell contact can enhance proliferation [22]. However, this phenomenon was not observed in 3-D ES-GFP cultures.

5.4.4. Proliferation and quiescence

Cell proliferation and quiescence were studied through immuno-histochemical staining of Ki-67 and $p27^{kip1}$. Ki-67 antigen is present in all proliferating cells, which are at the active phases of cell cycle (G1, S, G2, and M phases), but it is absent in resting cells (G0).

Therefore, it is an excellent biomarker for proliferating cells [23] and a good indicator of cell responses to chemotherapy in aggressive tumors. [24; 25]. In contrast, $p27^{Kip1}$ can inhibit cyclin-dependent kinases (CDKs) by binding to cyclin/CDK complexes and modulate the transition from G1 to S. It can induce growth arrest, differentiation and apoptosis [26]

As shown in Figure 5.4, the fraction of Ki-67 positive cells was low (10%-20%) in both fresh human tonsil tissue and cell aggregates from 3-D tissue cultures, while a much higher proportion (30%-40%) of cells from 2-D monolayer cultures was Ki-67 positive. In contrary, a significant increase in $p27^{kip1}$ positive cells (30%-40%) was observed in fresh human tonsil tissue and 3-D cultured tissues as compared to a very low percentage (<5%) of quiescent cells in the monolayer cultures. This study confirmed that 3-D cultures with multicellular structures shared the similar cell cycle progression as human solid tissues, while cells from monolayer cultures did not.

5.4.5. 3-D high-density cultures with tissue-like morphology

For 3-D high-density cultures, cells were cultured to reach a high density before exposure to chemicals. The IC_{50} values of the high-density cultures are also listed in Table 5.3. In addition, a resistance factor (Rf), calcualted from $IC_{50,3\text{-D high-density}}$ divided by $IC_{50,2\text{-D}}$, can be used to evalute the effect of multicellular strutures on drug resistance. As expected, the Rf value was larger than 1 for all three chemicals studied.

It is noted, however, that in 3-D high-density cultures, ES-GFP cells showed an extremely high resistance to 5-FU (IC_{50} > 770 μM, Rf > 3875), while 5-FU still had

significant cytotoxicity to HT-29-GFP cells (IC_{50} = 300 μM, Rf = 187) although it was not as effective as in the 2-D monolayer cultures. Clearly, the 3-D high-density cultures showed that 5-FU was effective in killing HT-29 cells present in high density or tumor-like structure, but not toxic to high-density ES cells. This selective cytotoxicity toward HT-29 cells is consistent with the fact that 5-FU has the greatest impact on colon cancer and remains the most popular therapeutic agent for about half a century. However, this selective cytotoxicity effect could not be predicted in 2-D monolayer cultures, which showed that ES-GFP cells were more sensitive to 5-FU with an IC_{50} lower than that for HT-29-GFP.

It should be noted that both the reported peak plasma concentrations of 5-FU (385 μM) [27] and the achieved serum concentration after *in vivo* administration (77 μM) [28] are much higher than IC_{50} from 2-D HT-GFP cultures but are close to that from 3-D high-density HT-29-GFP cultures. This indicates that the 3-D high-density culture models could replicate *in vivo* human biology accurately and provide a better predictive value, whereas assays based on 2-D cultures were errorneous in determining effective drug dosages.

Both mESC and HT-29 cells were very sensitive to gemcitabine (IC_{50}: 0.001 and 0.0043 μM) in 2-D monolayer cultures, but showed a high resistance when present in the 3-D high-density cultures with multicellular structures (Figure 5.5B).

Rf of sodium butyrate is the lowest for both ES-GFP (Rf = 3.5) and HT-29-GFP cells (Rf = 25). This could be because sodium butyrate is not an antimetabolite. Higher IC_{50} of

sodium butyrate to high-density HT-29 cells could be partially explained by the higher survivability of tumor cells than normal cells (Figure 5.5C).

Figure 5.5D shows that in comparison to gemcitabine, 5-FU had a lower efficacy on monolayer HT-29, but a higher efficacy on 3-D multicellular structures. Considering the low performance of gemcitabine and the greatest impact of 5-FU on colon cancer treatment, the prediction using 3-D multicellular structures instead of monolayer cultures agree with clinical results.

In treatment of colorectal cancers, both sodium butyrate and gemcitabine have conflicting data between the exciting experimental results in conventional cell-based assays and poor effects in clinical trials [29; 30]. For sodium butyrate, the confusing data is because of organoleptic problem and rapid blood elimination, but the resistance of gemcitabine is mainly due to formation of multicellular structures in human. The different mechanisms leading to the same conflicting result can be demonstrated with the help of multicellular models developed in this work. In the 3-D models, they showed significantly different effectiveness in 3-D tissue-like models, in which one was 30 times less sensitive and one lost all its activity.

5.4.6. Microbioreactor array to confirm the activity of 5-FU and gemcitabine on high-density HT-29

A microbioreactor array with 40 microbioreactors was also developed and used to confirm the result from toxicity tests of 5-FU and gemcitabine on high-density HT-29-GFP cells (Figure 5.6). Doses used for 5-FU and gemcitabine were 63, 630, 6300 times

and 12, 120, 1200 times of IC_{50} in monolayer cultures, respectively. As can be seen in Figure 5.6, culture fluorescence increased at a low dose (100 μM) of 5-FU, but decreased significantly (P<5%) at high doses (1000 μM and 10,000 μM). However, no fluorescence decrease was found at all gemcitabine doses studied. This finding agreed with the previous results that high-density HT-29-GFP was highly resistant to gemcitabine, while 5-FU showed significant cytotoxicity against high-density HT-29 cells.

Figure 5.6C and 5.6D show metabolism of the culture. The decrease of glucose consumption and lactic acid production was obvious in a dose-dependent manner with 5-FU. On the other hand, both glucose consumption and lactic acid production maintained at the same level for different gemcitabine doses, although glucose consumption was a little lower than the control. It was also noticed that although the culture fluorescence increased at a low dose of 5-FU, glucose consumption and lactic acid production actually decreased. This indicated that the increased culture fluorescence was due to the increased cell specific fluorescence even when the total cell biomass had decreased.

It is clear that the microbioreactor array would allow us to study a small number of cells while maintaining enough cell density to replicate *in vivo* biology. The 3-D high-density cultures on the microbioreactor array can provide us a much better predictive value than 2-D monolayer cultures.

5.5. Discussion

Because most chemotherapeutic drugs target rapidly dividing cells rather than only cancer cells, 2-D monolayer cultures supporting rapid proliferation make all cells targets

of chemotherapy no matter cancer cells or normal cells. Furthermore, tumor cells come from the mutations which could activate oncogenes or repress tumor suppressor. These mutations could often impair cell ability of apoptosis and prevent cells to destroy themselves when cellular DNA is damaged severely. Therefore, tumor cells could be even more resistant to genotoxic substances such as 5-FU and gemcitabine compared with normal cells when cultured as monolayer. On the other hand, although chemotherapy has the potential to harm healthy tissues in the body, most normal tissues except those tissues with a high replacement rate are more resistant than tumor because tumor cell are more likely to be dividing *in vivo*. Using conventional 2-D proliferating assays, the selectivity of these toxic agents to solid tumor could be difficult to discover or even worse, in contrast. In this case, assays with multicellular structures are desired in drug screening and discovery with a better predictive value than monolayer cultures. Our case studies supporte this hypothesis. The high density model of ES could be considered as healthy tissues in comparison of HT-29 due to its normal genotype. Since 5-FU is the most effective for colon cancer treatment, it is assumed to have higher activity against HT-29 cells instead of normal tissues. However, HT-29 is less chemosensitive to antimetabolites than ES cells in monolayer cultures, partly because of more rapid proliferation in ES cells or inability of apoptosis in colon cancer cells. Selectivity of 5-FU to colon cancer can only be demonstrated by our tissue-like models.

In our study, solid tumor cells are more resistant to some toxic agents such as sodium butyrate in both monolayer and multicellular structures than normal cells (ES cells). However, antimetabolites showed selectivity for tumor-like structures over high-density

normal cells, although they might have similar or even lower activity against tumor cells in monolayer. In this case, our microbioreactor array using 3-D high cell density model stands a better chance to screen for chemicals with the selectivity to tumors.

Not only prediction for chemical activity, our system also provides the time to take effects for each chemical. For example, sodium butyrate showed the potency of cytotoxicity within one day after exposure to high density HT-29-GFP, but four days were required for 5-FU. This kinetics study would be very help to understand the mechanism of compound actions.

Conventional drug discovery and pharmaceutical research usually involve monolayer cell-based assays and animal experiments. A big gap exists between these two phases of study. Cell-based assays with standard monolayer cultures reveal toxicity assuming drugs reach individual cells easily and cells behave the same as *in vivo*, while animal experiments show the effect of drug infusion into the whole bodies. More and more drug candidates could be obtained by increasing the high-throughput capability of *in vitro* assays, but *in vivo* experiments are difficult to speed up. High-throughput multi-cellular assays could serve as a bridge between monolayers and animals to remove poor candidates and increase pipeline efficiency in drug discovery and at the same time discover the mechanism of drug actions in pharmacological study.

Although CMV promoter was widely believed as a constitutive promoter [31], the activity of it is still affected by some external stimuli including some genotoxic agent (such as 5-FU and gemcitabine) and enhancers of production such as sodium butyrate.

Thus, confounding effects were found to be an impediment in 2-D fluorescence toxicity tests. 3-D cultures were better, partly because of their compacted structures. However, more stable controls of reporter expression needs to be considered [32].

Our preliminary studies showed that the responses to drugs were different between monolayers and multicellular structures. The application of our system to target specific cellular functional units would help to reveal the mechanisms which result in these discrepancies and have an impact on pharmacological studies.

In summary, high-throughput cell-based assays have brought a renaissance to drug screening and greatly speed up the whole process of drug discovery recently. However, almost all of these assays are based on monolayer cultures. The responses of 2-D monolayer cells to drugs are not always in agreement with those *in vivo*. Our 3-D real-time micro-bioreactor array accurately resembles *in vivo* tissue status and provides a highly automated way to measure temporal responses in a high-throughput format. Eventually, the array proposed is anticipated to have a much better predictive value to decide which leads or hits will or will not enter *in vivo* tests compared to monolayer. Expensive and time-consuming animal experiments will be reduced and partly replaced by these arrays with cells cultured in tissue-like morphology in the future drug discovery process.

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	Dose	Cell number (10^4)	Cell size (μm)	Cell specific fluorescence[a]
Control	0	133	11.19	1
5-FU	0.096 μM	120	11.85	1.09
	0.384 μM	30	13.98	4.29
	1.73 μM	16	13.78	6.55
Gemcitabine	0.001 μM	45	12.10	0.82
	0.01 μM	20	13.48	3.37
	0.1 μM	2	14.11	-
Sodium Butyrate	2 mM	8	12.99	5.58
	8 mM	15	12.11	36.55
	32 mM	0	-	-

[a] nomalized with control as 1.

Table 5.1. Effects of chemicals on ES-GFP cell cultures in cell number, size, and specific fluorescence after culturing for three days.

Drug	Dose	Cell number (10^4)	Cell size (μm)	Cell specific fluorescence[a]
5-FU	0 μM	320	13.91	1
	2 μM	145	18.95	4.81
	10 μM	103	20.0	7.33
	50 μM	18	20.51	11.43
Gemcitabine	0 μM	294	13.62	1
	0.001 μM	254	13.94	0.89
	0.005 μM	140	14.59	2.03
	0.025 μM	14	17.87	-
Sodium Butyrate	0 mM	260	15.01	1
	0.5 mM	259	15.29	2
	2 mM	58	17.48	4.09
	8 mM	16	17.6	6.24

[a] nomalized with control (dose = 0) as 1.

Table 5.2. Effects of chemicals on HT-29-GFP colon cancer cell cultures in cell number, size, and specific fluorescence after culturing for six days.

	5-FU (μM)	**Gemcitabine** (μM)	**Sodium butyrate** (mM)
ES cells (IC_{50})			
2-D cultures	0.2	0.001	1
3-D low-density cultures	0.27	0.006	0.5
3-D high-density cultures	>770[a]	>100[a]	3.5
Resistance factor (Rf)[b]	>3850	$>10^5$	3.5
Colon cancer cells (IC_{50})			
2-D cultures	1.6	0.0043	1.2
3-D low-density cultures	160	0.0017	5.3
3-D high-density cultures	300	>1000 [a]	30
Resistance factor (Rf) [b]	187	$>2 \times 10^5$	25

[a] no significant difference in fluorescence kinetics was found between the control (no drug) and the culture subjected to even the highest dose studied.

[b] $Rf = IC_{50,3\text{-D high-density}} / IC_{50,2\text{-D}}$

Table 5.3. Chemosensitivity (IC_{50}) of ES-GFP and HT-29-GFP cells to 5-FU, gemcitabine, and sodium butyrate under different culturing conditions.

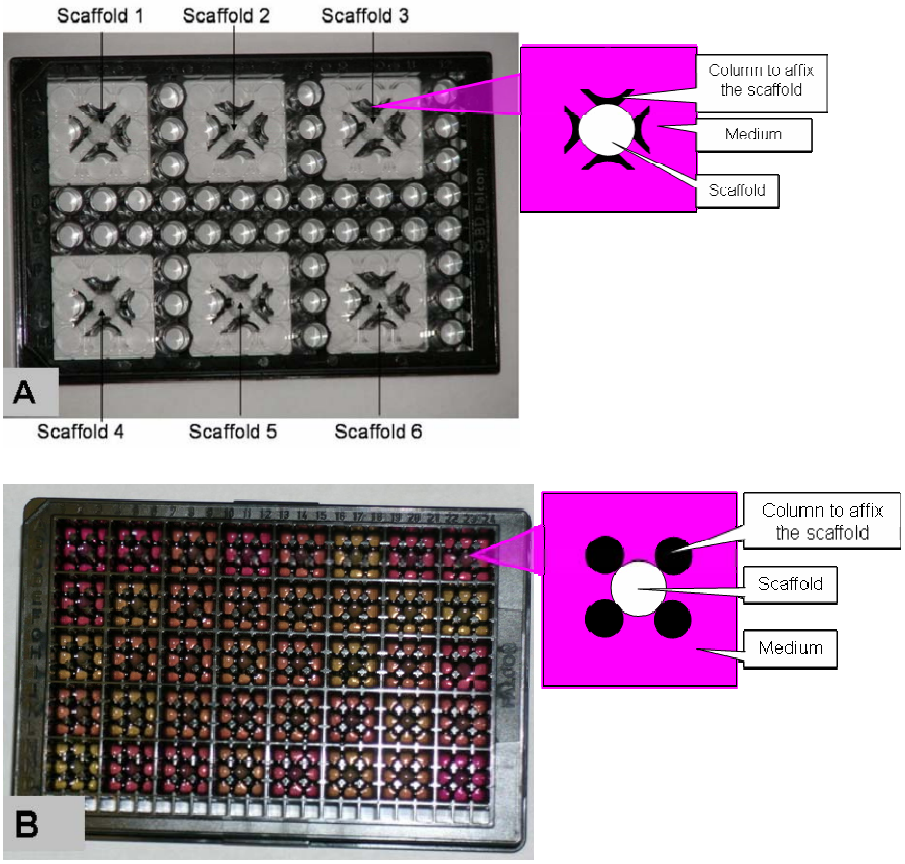


Figure 5.1. Different configurations of bioreactor array. A. a 6-chamber bioreactor array with larger cake-shape scaffolds (0.1cm height, 0.3 cm2 bottom; working volume 3 ml). B. 40-chamber bioreactor array with smaller cake-shape scaffolds (0.1cm height 0.12 cm2 bottom; working volume 1ml).

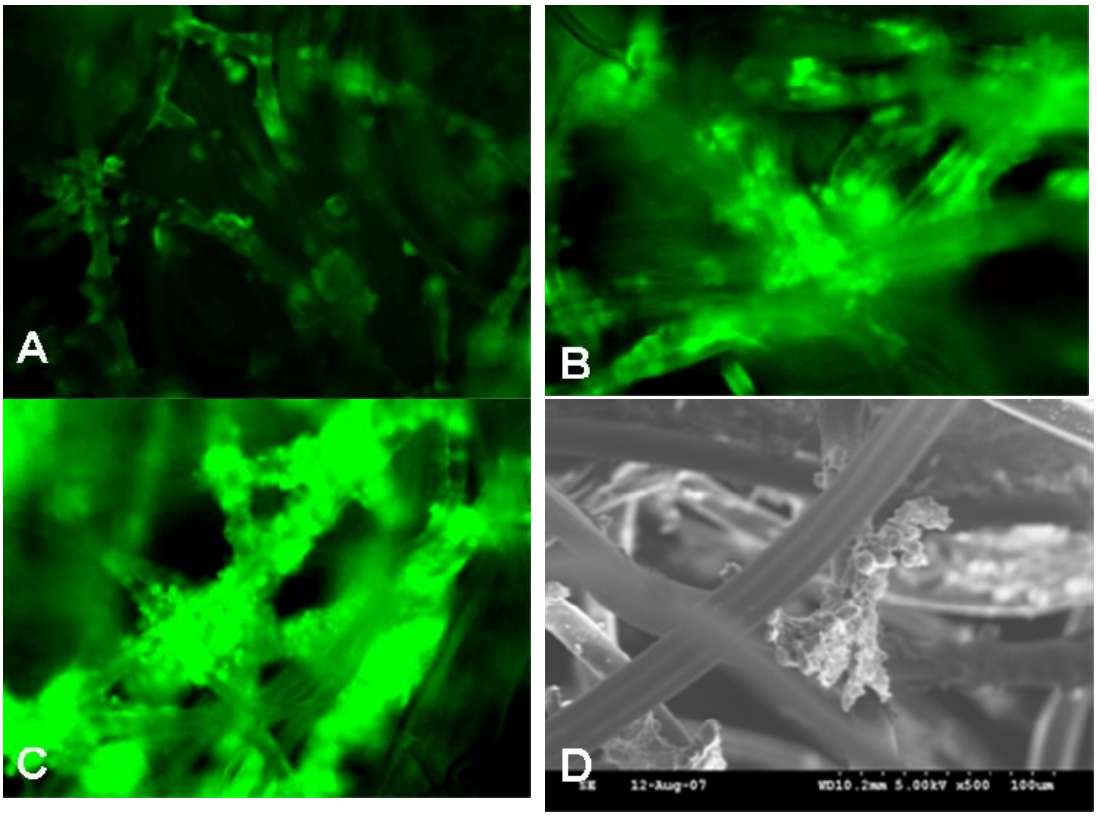
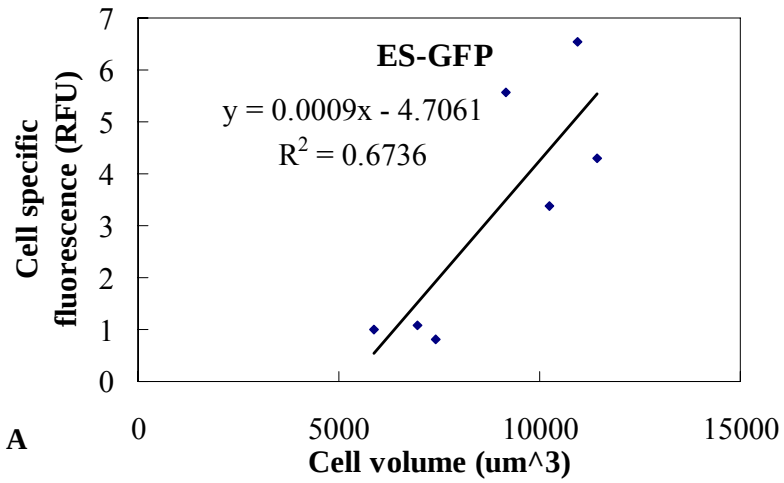
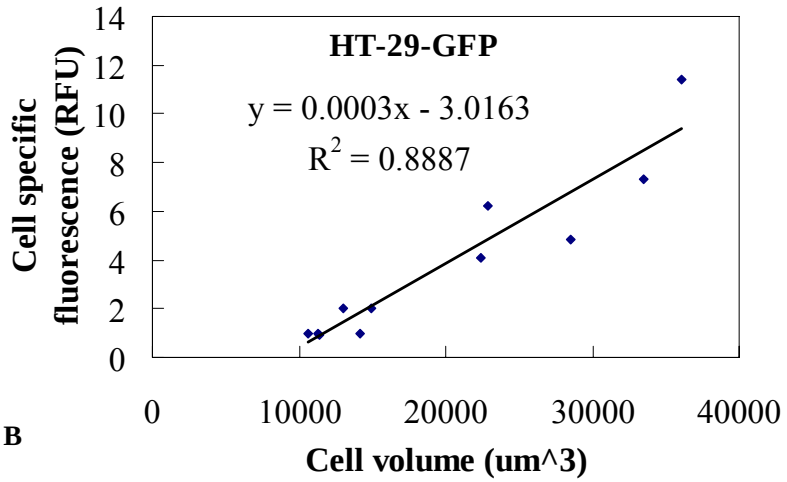


Figure 5.2. Fluorescent microscopic images of HT-29-GFP cells in 3-D PET scaffolds after culturing for A. 1 day, B. 5 days, and C. 8 days. An inverted fluorescent microscope (Nikon Ecllipse TE2000-U) was used for detection. D. Scanning electron micrographs of HT-29-GFP after 8 day culture.



A



B

Note: In Table 1, cell specific fluorescence with 8 mM of butyrate was considered as an outlier and not included in Figure 5.3A

Figure 5.3. Correlation between cell volume and cell specific fluorescence of ES-GFP (A) and HT-29-GFP (B).

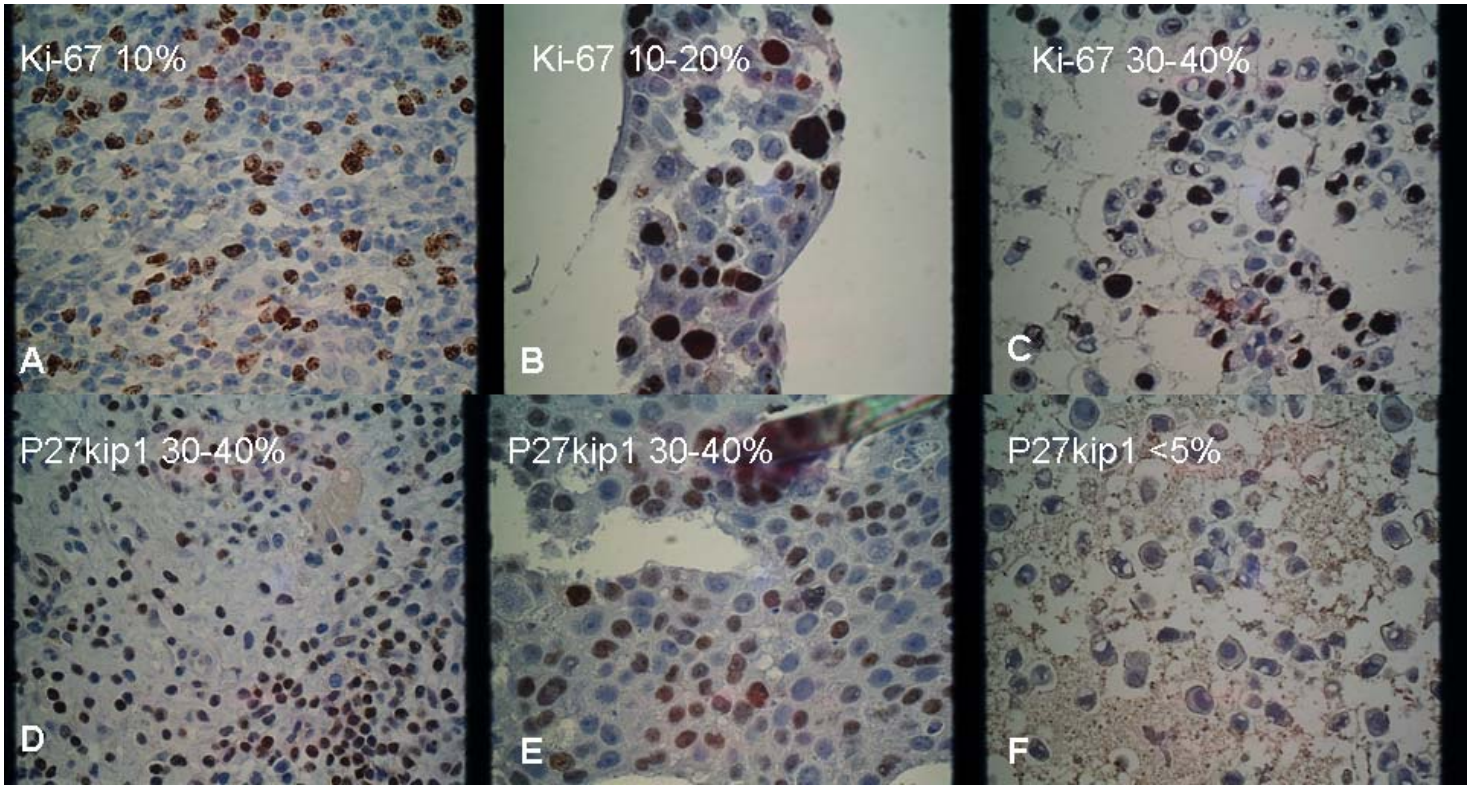


Figure 5.4. Immunohistochemical staining of proliferation marker Ki-67 and quiescence marker p27kip1. A. fresh human tonsil, B. 3-D tissue culture and C. agarose-embedded cell pellets were stained with Ki-67; D. fresh human tonsil, E. 3-D tissue culture and F. agarose-embedded cell pellets were stained with p27kip1

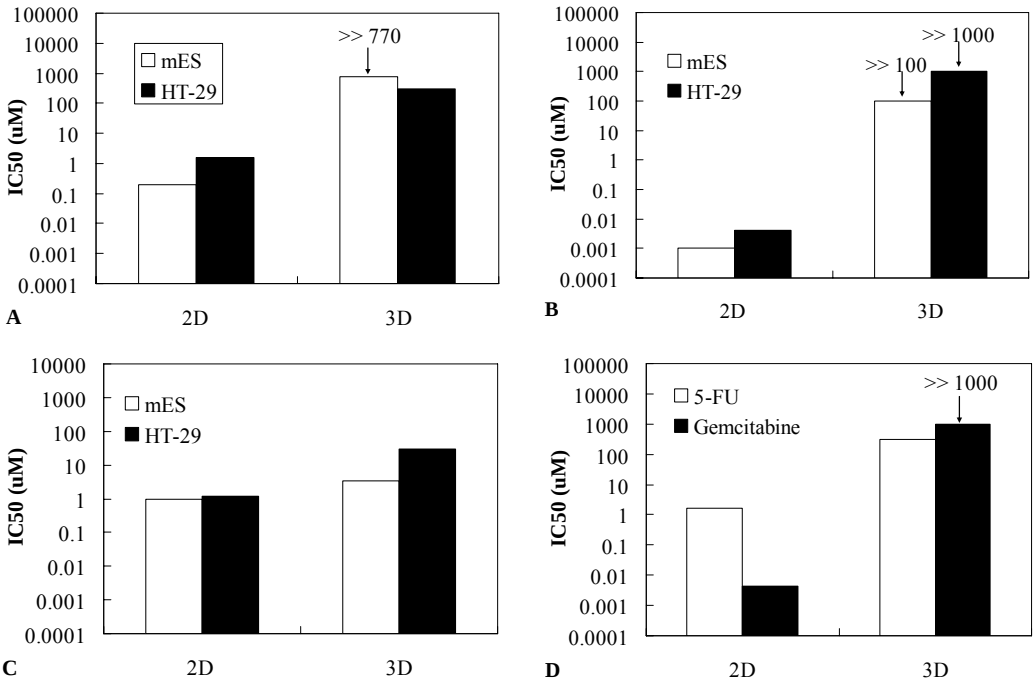


Figure 5.5. Comparison of cytotoxicity. Cytotoxicity of A. 5-FU, B. gemcitabine, and C. sodium butyrate on low and high density of ES-GFP or HT-29-GFP. D. Cytotoxicity of 5-FU and gemcitabine on low and high density of HT-29. Arrows indicate that IC50 of drugs to 3-D high density cells are much higher than corresponding values instead of equal to.

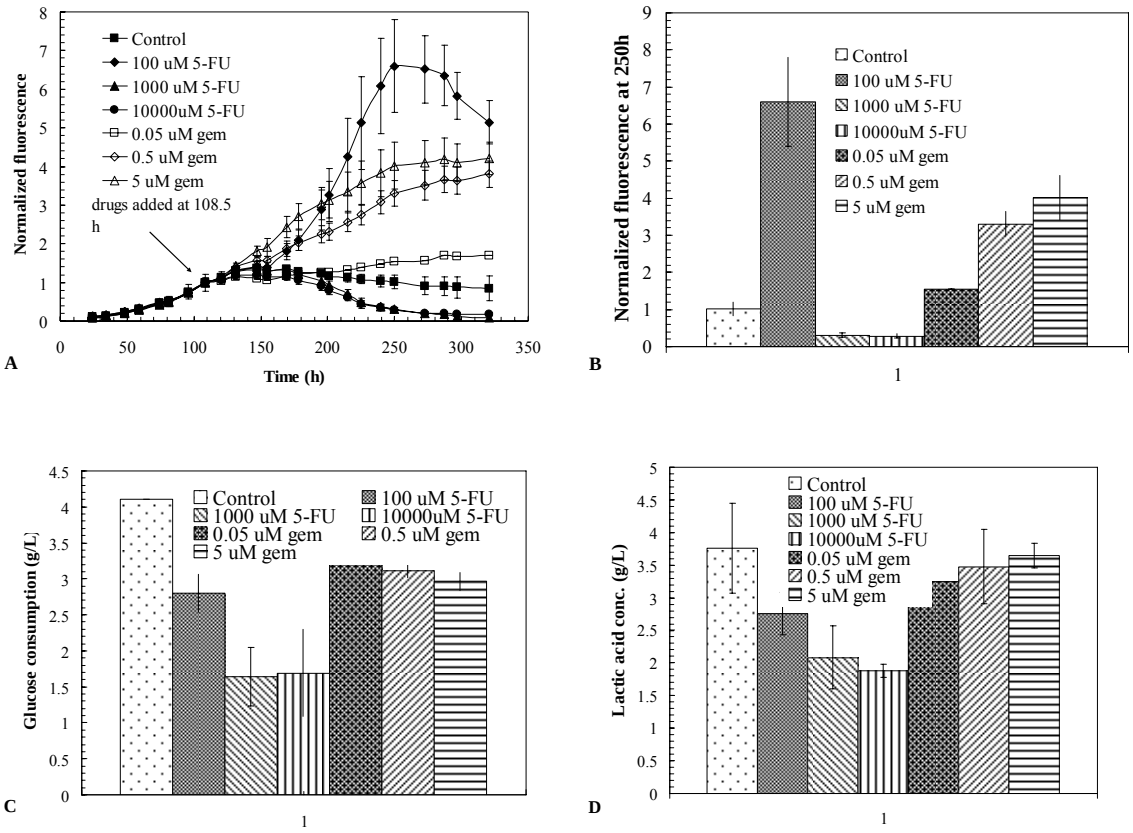


Figure 5.6. Comparison of 5-FU and gemcitabine toxic effects on high-density HT-29-GFP using a 40-unit microbioreactor array. A. Fluorescence kinetics with different doses of chemicals. Mean values were derived from triplicate repeats for each dose except 0.05 μM gemcitabine. B. Fluorescence responses to different doses of 5-FU or gemcitabine when the control reached the maximal signal (250h). C. Glucose consumption and D. lactic acid production after 2 weeks.

CHAPTER 6

GREEN FLUORESCENCE PROTEIN IN CELL CULTURE PROCESS DEVELOPMENT: FROM MICROBIOREACTOR ARRAY TO LAB-SCALE OPERATION

6.1. Abstract

An online monitoring system based on the expression of green fluorescent protein (GFP) by cells was developed for high-throughput screening and process development. The system consisted of a novel 3-D microbioreactor array for early-stage high-throughput screening (HTS) and an online fluorescent biosensor ready to use in bioreactors. Both of them allowed continuous and quantitative detection of the expression of GFP, which could reflect cell growth and predict the production of recombinant protein. After transformation, recombinant CHO cells with stable GFP expression were obtained through cell sorting using flow cytometry. The performance of the system was demonstrated with a case study involving sodium butyrate treatment to enhance MAb production by CHO cells. Results showed that the non-invasive fluorescence detection method provided good quantification of MAb production by cells immobilized in a fibrous bed bioreactor. The system proved to be a rapid, reliable and robust approach for cell culture process development to optimize target protein productivity without using disruptive cell counting or protein analysis.

6.2. Introduction

Some of the most complex systems in the world are biological systems such as proteins and whole cells. Because the precise prediction of these systems is extremely difficult, biological studies are mainly based on virtual detection. This complexity is further increased when bioprocesses using these systems are of interest, because the measurement of parameters during the process becomes more critical [1].

Mammalian cell microbioreactors offer a potential to reduce medium cost and intensity of labor and time for various cell cultivations in process development by highly parallel evaluation of process options. They have been widely applied in the fields of medium development, strain selection and process optimization, increasing industry productivity to bring new drugs to market [2-5]. It is desired that microbioreactors could mimic larger scale operations in order to provide useful information which would be further tested in bench-top bioreactors followed by pilot-scale. Besides cell culture process development, mammalian cell microbioreactors have also demonstrated their power in various areas including tissue engineering and cell-based screening [6; 7].

The attractive futures of microbioreactors has brought a commercial high-throughput system SimCell microbioreactor array from Bioprocessors, which has the ability to control and operate several hundreds of cultures at the same time. Multiwell plate-based systems have become the majority of these miniature systems because their standardization eases the integration with currently existing automated, robotic liquid handling platforms to give the highest throughput capability [2; 8; 9].

Off line sampling during the culture process is limited due to the small amount of medium used in a microbioreactor (ranging from several μl to several ml), so the expense of HT capability is quality and quantity of information [2]. In this case, on line detections are always required. Among these integrated measurement devices, optical sensors are becoming more and more popular compared to electrochemical sensors because of their easy miniaturization [3]. A number of different microbioreactors used fluorescent dyes to monitor pH and dissolved oxygen in cultures [9-14]. One advanced detection approach is a mass spectrometer probe with a membrane inlet [15]. However, in addition to its complex design and high cost, its measurement is only good for volatile inorganic compounds and not ready for experimental high-throughput, limiting its application. Although current microbioreactors could feature online pH and DO measurements, it is always impractical to non-invasively monitor protein production in addition to the difficulty in tracking nutrition, metabolite and cell growth as a function of time. Betts and Baganz (2006) suggested that incorporation of biomarkers such as GFP could help to solve this problem because GFP was considered to be not harmful to cell activity [2]. However, high and fluctuant background fluorescence and relatively low signals from live cells limited this application in mammalian cell cultures [16]. Taking advantage of high cell density by immobilization, our group reported that signal to noise ratio increased more than one magnitude in a microplate-based small-bioreactor array than monolayer cell cultures. In addition to exciting applications in drug screening and discovery, this array can also be used in HT cell culture process development to study antibody production by immobilized CHO cells. In this study, fluorescence was used to quantify cell growth and indicate protein production.

After the initial HT study in microbioreactors, optimized conditions would be further confirmed in laboratory-scale and pilot scale bioreactors and finally used in production operation. Besides commonly used online pH, DO and temperature probes, people show great interest in real time detection of viable cell density. There is a common sense that online quantification of viable cell density is one of the focuses in the future development of monitoring and control regime in bioprocessing. It is often necessary to improve bioprocess quality and address optimization by modeling cell population dynamics, because protein productivity has a positive relationship with specific growth rate [17; 18]. Many different approaches have been developed for online growth quantification in bioreactors, but more effort is necessary to find a robust and reliable online detection system, which could be easily adapted to most cultivations. Optical density and medium capacitance are not linearly responsive to cell mass and have low sensitivity due to high noise, although they are probably the easiest to detect. Sensitivity is improved with turbidity probing, but values are subject to changes in cell viability and aeration. The acoustic system and advanced spectroscopy such as NIR and NMR are still under development and not available for common usage even for academic uses [19; 20]. Newly developed *in situ* microscopes (ISM) have proven suitable for cell mass measurement, but their application is limited to suspension cultures [21]. To date, none of these methods has proven suitable for immobilized cell cultures.

Besides viable cell density, attention has been drawn to real-time, non-invasive assessment of cell physiological status and productivity. Due to the requirement of reactions or chromatography, reliable *in situ* probes for direct protein production

measurement are virtually impossible currently. The conventional method consists of collection, dilution and further analysis at different time points, which is time-consuming and entails a high risk of contamination. Flow injection analysis (FIA) measuring nutrients and metabolites provides another solution by automatic online sampling and allows on-line quantification of mAbs with integrated HPLC [22; 23], but it is still limited by the issues of enzyme instability, interferences with other similar components and risk of contamination during sampling in addition to their complexity and expense [19]. Green fluorescent protein (GFP) is normally used as a non-invasive reporter in mammalian cells without interference with other cell activities. The application of it to reflect promoter activity, recombinant protein production and location has been widely studied [24; 25]. In comparison with synthetic indicators, all manipulation steps and toxicity caused by reagent addition are eliminated in GFP technology, so the approach provides a great potential to monitor dynamic properties of cultures such as cell mass, cell physiology, and even protein productivity [26-30]. Randers-Eichhorn et al. (1997) was the first to realize online fluorescent detection of GFP in bioreactors with LED excitation [31]. Hisiger and Jolicoeur (2005) applied GFP as a non-invasive indicator of the physiological state of cells to monitor culture progress [27]. In this study, a non-invasive real-time fluorescence probe was developed and tested in spinner flasks and bioreactors with glass observation points to track immobilized cell growth through different process phases and predict the concentration of mAb.

A fibrous bed bioreactor (FBB) using polyethylene terephthalate (PET) fibrous matrices has been developed for cultivation of various cell lines, such as CHO, hybridoma, human

osteosarcoma, human cytotrophoblast, mouse embryonic stem cells, and human embryonic stem cells. It provides high porosity, high surface to volume ratio, high permeability, low pressure drop, easy downstream process, low material cost and an *in vivo* mimicking microenvironment. When FBB is used in perfusion culture, filter clogging can be prevented because all cells are immobilized within 3-D scaffolds. Previous results showed that perfusion FBB was able to allow high density cell culture (3×10^{8} cells/ml) and give high productivity for a long time (up to months) [32-36]. Compared to other immobilized cell culture systems, FBB is more likely to be applied to industrial production because of these advantages. The reason that it has not been well commercialized is partially because cell growth on fibrous scaffolds could not be well quantified during culture, which made the study of cell seeding and growth in 3-D matrices very difficult. The work presented in this paper is dedicated to addressing this critical issue.

6.3. Material and methods

6.3.1.Cells and chemicals

CHO 6E6 (ATCC number CRL-11398) producing 23F2G, a humanized anti-CD18 IgG, was used in this study. Both heavy and light chain expression gene cassettes contained a CMV promoter. The basal medium consisted of DMEM/F12 (1:1) supplemented with 5% FBS, 5 g/L glucose, 2 mM L-glutamine and 100 nM methotrexate. Cells were propagated in T-flasks in a 37°C incubator with 5% CO_2. Cells were subcultured every four days.

Sodium butyrate (Sigma Chemical Company, St. Louis, MO) was stocked as 1 M solution in 4 °C and sterilized with 0.22 μm filter prior to use.

6.3.2.Transfection and selection of recombinant cells

CHO 6E6 cells were transfected using Lipofectamine 2000 (Invitrogen) with pEGFP-N3 (Clontech, Palo Alto, CA), in which EGFP was under the control of a CMV promoter. This same promoter also controlled the expression of anti-CD18 IgG. All cells were collected for each subculture and 100% confluence was obtained in 4 T-75 flasks after two weeks. All cells were subjected to FACS Aria (BD, CA) sorting and 1% with the highest fluorescence were collected (more than 10^6 cells). The culture consisted of over 90% fluorescent positive cells after two months of consecutive subculturing. Cells could be stocked in a liquid nitrogen tank and the fluorescent positive percentage was the same after recovery.

6.3.3. Analytical methods

Samples were centrifuged at 12,000 rpm for 2 min and diluted to an appropriate concentration. Concentrations of glucose and lactate were then measured with a YSI Biochemistry Select Analyzer (Yellow Spring, Ohio). Enzyme-linked immuno-sorbent assay (ELISA) was used to analyze IgG concentration. Briefly, 96-well EIA/RIA Easy Wash plates (Corning Lowell, MA) were coated at room temperature for 2 hours with diluted primary antibody, a goat anti-human IgG (Jacksonimmuno, West Grove, PA). Diluted human IgG standard (Sigma, St. Louis, MO) and samples were added into plates in duplicate and incubated for another 2 hours. The second antibody, goat HRP-anti

human IgG (H+L), was then applied to each well for 1 hour. A fluorescence plate reader (GENios Pro, Tecan, Durham, NC) was used to detect fluorescence at excitation 320nm and emission 405nm after reaction with 3-(p-hydroxyphenyl) propionic acid.

6.3.4. Effects of butyrate on culture fluorescence, cell number and cell specific fluorescence in 2-D cultures

5000 cells were inoculated in each well of 96-well plate. Four days after inoculation, cells (at late exponential growth phase) were exposed to 0, 0.5, 1, 2, 4, 8, 16 and 32 mM butyrate. Fluorescence was detected every day after replacing medium with the same volume of PBS to remove background fluorescence. In order to detect the effects on cell specific fluorescence and cell number, different doses of butyrate were added to cultures one day after inoculation. Four days later, cell specific fluorescence was measured with a flow cytometer (BD FACS Calibur, Franklin Lakes, NJ) and cell number was counted by a hemocytometer with trypan blue exclusion.

6.3.5. HT study of butyrate treatment on immobilized CHO cells using 3-D microbioreactor array

The fluorescence intensity was proportional to the number of cells seeded in PET scaffolds (Figure 6.1). This indicated that GFP fluorescence could be related to cell number linearly within a growth phase. In other words, cell growth kinetics could be quantified directly by fluorescence either in the exponential growth phase or in the stationary phase.

Briefly, 25,000 cells in 11 µl medium were inoculated into each heat-compressed PET scaffold in 96-well plates, which had been pretreated with 1M sodium carbonate, 1M sodium hydroxide and culture medium. Cells were allowed to attach to scaffolds for 6 h after inoculation before the addition of 180 µl medium. One day after inoculation, each scaffold was transferred and fixed to the center of each microbioreactor with 1 ml working medium containing different doses of butyrate in the array (Figure 6.2). The microbioreactor array was then put onto a rotational shaker with a constant rotation speed of 90 rpm in a cell culture incubator. Fluorescent detection was performed using a plate reader (Tecan GENios). Wet paper was put under the plate, which had significant effects to prevent excessive evaporation. After 10 days of culture, volume of evaporation varied from 0.13-0.15 ml.

The average signal of 8 wells surrounding a scaffold plus a constant background from the scaffold itself was subtracted from the central reading to give fluorescent signals for live cells in each microbioreactor. Cells were exposed to different doses of butyrate 7 days after inoculation. Samples were collected for IgG measurement 3 days and 6 days after butyrate addition. Glucose and lactate concentrations were quantified at the end of the batch (13^{th} day). For performance evaluation, assignment of dose was done randomly and there were 2-3 repeats for each dose.

6.3.6. Fluorescent probe development and installation

GFP fluorescence from cells in spinner flasks and bioreactors was monitored by an *in situ* fluorescent sensor system under the control of a computer. A 50 watt tungsten-halogen

lamp provided the fluorescent excitation light ranging from 320 nm – 700 nm. The light passed through an excitation filter and was directed by a bifurcated fiber-optic cable into a 3.0 mm quartz probe. Emission radiation from samples was collected by the probe and detected by a 9781 photomultiplier tube detector after an emission filter. The set of optical filters used in the study had a 485 nm excitation with a bandwidth of 20 nm and 530 nm emission with a bandwidth of 25 nm, but can be easily changed according to different fluorescent samples. All of these were from Applied Biosystems (Foster City, CA). Holders enabled fast fluorescent detection of multiple fixed points on spinner flasks or bioreactors with simple alignment (Figure 6.3). In this study, all fluorescence values from spinner flasks or bioreactors were the average fluorescence from 5-7 points using this probe.

6.3.7. Spinner flask culture and perfusion bioreactor system

Spinner flasks (100 ml with adjustable hanging bar from Bellco Biotechnology, Vineland, NJ) with modified paddles (3 cm × 3.6 cm) were used to culture cells. A stainless steel mesh (17 cm × 6 cm) was used to affix a PET scaffold (2 cm × 9 cm) around the inside wall of the spinner flask. The flasks were autoclaved at 121ºC for 15 min before use. 1.2×10^7 cells were inoculated into each spinner flask containing 70 ml medium. The spinner flask culture was maintained at 37°C in an incubator with 5% CO_2 and agitated at 90 rpm.

Batch cultures were then performed in two spinner flasks using PET scaffolds with the same size. Cells in one spinner flask were exposed to 2 mM butyrate 7 days after

inoculation, the same addition time as in the microbioreactor array. The other spinner flask did not receive any butyrate and was considered as the control. Daily measurement of fluorescent signals from multiple points on each scaffold could be completed within 2 minutes and samples were taken at the same time for IgG quantification.

The perfusion bioreactor system consisted of two reactors (Figure 6.4). The first reactor, a 450 ml bioreactor with a magnetic bar and a water jacket, was used as the media tank. A New Brunsiwick Bioflow 3000® control system was used to control its pH and DO at 7.0 and 80%, respectively, by surface aeration and base solution. It contained 220 ml medium. The second reactor, a spinner flask, same as described above but with a larger scaffold (16 cm × 6.25 cm), was used to house the cells. After inoculation with 7×10^7 cells in 110 ml medium, it was incubated in a cell culture incubator for the first day to allow cell immobilization. Then, it was put into a holder on a hot plate with thermal control and connected to the bioreactor. Medium circulation between these two reactors was maintained at 13 ml/min. The continuous perfusion began on the 5th day with a constant dilution rate of 140 ml/day (0.4 v/v/day). At 384 h, concentrated butyrate was pumped in and the concentration of butyrate reached 2 mM within two hours. This was followed by continuously providing fresh medium with 2 mM butyrate. At 696 h, medium containing no butyrate was used for washout. Glucose, lactate and MAb were measured every day.

6.4. Results

6.4.1. Effects of butyrate on culture fluorescence, cell number and cell specific fluorescence in 2-D

Monolayer cell growth was inhibited by butyrate in a dose dependent manner (Figure 6.5A). Culture fluorescence and IgG production with different doses of butyrate are shown in Figure 6.5B and 6.5C. In order to minimize the inhibitory effect on cell growth, butyrate was added at late exponential growth phase (102 h after inoculation). It was clear that the higher the dose, the earlier the fluorescence reached the maximum. All doses could increase fluorescence at the beginning, but the fluorescence with higher doses decreased to a level lower than the control earlier. 1 mM butyrate had the best improvement (20%) compared with the control. The doubling time of the control fluorescence (T_D) at the exponential growth phase was about 21 hours.

6.4.2. HT study on immobilized culture with butyrate treatment using a microbioreactor array

In addition to its application in high-throughput toxicity screening, the microbioreactor array was also applied to a high-throughput process study with butyrate treatment. Figure 6.6A shows that low doses of butyrate (1 mM and 2.5 mM) increased fluorescent signals one day after exposure, while high doses reduced signals immediately. Fluorescent signals and IgG concentration were analyzed together 3 days (Figure 6.6B) and 6 days (Figure 6.6C) after butyrate addition. It was obvious that similar to fluorescence, IgG titer

increased with low doses of butyrate but decreased when doses increased to a certain level. Compared to 2-D, butyrate demonstrated a much better performance (35% enhancement) with a higher optimal dose (2.5 mM) on day 6. It suggested that immobilized cell culture in PET scaffolds with higher density had a higher tolerance to adverse conditions [37]. In other words, inhibition of cell growth by additives could be reduced by immobilized cell culture. For all normalized fluorescence or IgG concentration, the value at the time of exposure was 1.

Metabolism of CHO cells with different doses of butyrate was also studied (Figure 6.6D). Both glucose consumption and lactate production decreased continuously as butyrate dose increased. This result also agreed with the effect of butyrate on cell numbers in 2-D culture. Because of the growth inhibition by butyrate, less amount of nutrient was used to maintain cell growth and less amount of metabolite was produced. However, the total protein productivity could increase with appropriate doses of butyrate.

Cell number, glucose consumption and lactate production were often considered as parameters to predict the productivity of therapeutic proteins, whereas in this study the dose dependent manner by which butyrate affected IgG productivity can be predicted only by fluorescence instead of cell number or metabolite rate.

6.4.3. Follow-up confirmation in spinner flask equipped with a fluorescent probe

In order to confirm the results from the microbioreactor array, experimentation was carried out in spinner flasks and a set of fluorescent probe and holder was specifically designed for this purpose. A 3 mm thick glass (the same thickness as the glass wall of the

spinner flask) reduced fluorescence detection by 56%, but fluorescence intensity was still linearly related to concentration of fluorescein (Figure 6.7), a chemical with fluorescence characteristics very similar to GFP. Therefore, the probe could allow online fluorescence quantification to monitor cell growth in spinner flasks.

Figure 6.8 shows that after 2 mM butyrate addition, both fluorescent signal and IgG concentration were significantly increased. The fact that a low dose of butyrate could increase IgG production was confirmed by fluorescence again.

6.4.4. Action of butyrate revealed in perfusion culture

Because in the previous study butyrate treatment experienced the transition from late exponential growth phase to death phase, the action of butyrate on IgG and GFP production was difficult to study. Treatment within a stationary state was considered critical to establish a guideline for usage of butyrate to increase protein production in batch or fed-batch cultures.

Perfusion culture with FBB was used to obtain a stationary growth phase (steady state) in order to investigate the responses to butyrate treatment. At 384 h, butyrate concentration was increased to be 2 mM and kept as a constant until butyrate removal started at 696 h by continuous feeding with fresh medium. The historical performance of the perfusion bioreactors is showed in Figure 6.9A. After cell mass reached the maximum as indicated by fluorescence, there was an increase of glucose concentration and a significant drop of lactate and IgG concentration, which suggested a washout effect. The production of IgG was growth-associated and 0.4 v/v/day dilution rate was so high that IgG was washed out

gradually. However, this dilution rate was used for the whole culture in order to maintain the same conditions. It should also be noted that the fluorescence signal dropped dramatically after it reached the first peak. Because CMV was reported as an S-phase specific promoter [38; 39] and the percentage of actively dividing cells reduced after exponential growth phase, the decrease of IgG and fluorescence was expected. This drop indicated that cells from exponential growth phase rapidly entered the stationary growth phase or cell cycle distribution changed from S to G0/G1. In despite of the same promoter, IgG had a larger drop compared to fluorescence after the first peak. This indicated that the genome location of gene cassettes also contributed to the expression level. We could also notice that even after most of the butyrate had been washed out by 840 h (less than 0.2mM), the fluorescence decreased continuously, suggesting that the action of butyrate on cell growth was irreversible.

In order to better study the performance of butyrate on cell culture, reaction rates of IgG, fluorescence, glucose and lactic acid were calculated. The concentration of all four components in an ideal perfusion bioreactor changes with time as shown in Eqn (1)

$$\frac{dC_i}{dt} = \frac{C_{i,f} - C_i}{\theta} + \left(Q_i - k_{i,D} \cdot C_i\right) \quad , \quad t=t_1,\ C_i=C_{i,1};\ t=t_2,\ C_i=C_{i,2} \tag{1}$$

where, C_i is the concentration of a specific component i (IgG, fluorescence, glucose or lactic acid) in the bioreactor, θ is the constant resident time 60h, $C_{i,f}$ is the feed concentration, Q_i is the volumetric reaction rate and $k_{i,D}$ is the degradation rate constant.

With a constant Q_i over a short time period from t_1 to t_2, the equation can be integrated to obtain the analytical solution for Q_i:

$$Q_{i,CSTR} = \frac{\left\{\left[\frac{C_{i,f}}{\theta} - C_{i,2}\cdot\left(\frac{1}{\theta}+k_{i,D}\right)\right]\cdot\exp\left[\left(t_2-t_1\right)\cdot\left(\frac{1}{\theta}+k_{i,D}\right)\right]\right\} - \left[\frac{C_{i,f}}{\theta} - C_{i,1}\cdot\left(\frac{1}{\theta}+k_{i,D}\right)\right]}{1-\exp\left[\left(t_2-t_1\right)\cdot\left(\frac{1}{\theta}+k_{i,D}\right)\right]} \quad (2)$$

Here, data points with the subscripts 1 and 2 correspond to t_1 and t_2. For the initial batch culture part (t<123h), $\theta = \infty$ and the solution can be written as:

$$Q_{i,BATCH} = \frac{k_{i,D}\cdot\left\{C_{i,1} - C_{i,2}\cdot\exp\left[k_{i,D}\cdot\left(t_2-t_1\right)\right]\right\}}{1-\exp\left[k_{i,D}\cdot\left(t_2-t_1\right)\right]}$$

Here, $k_{i,D}$ is 0.0267h^{-1} for GFP [40]. Glucose and lactic acid are much more stable than GFP and the same is for IgG, so $k_{i,D}$ for glucose, lactic acid and IgG were considered as zero.

Figure 6.9B is the kinetics of fluorescence and IgG production rate in the culture. The shape of curves for fluorescence and IgG are very similar in the exponential growth phase, in which both had the same doubling time of 25 h. The curve of IgG was about one day right-shifted compared to the fluorescence curve. This delay might be due to IgG secretion, though GFP detection did not require secretion. Therefore, fluorescence could be used to indicate IgG production within the exponential growth phase.

In the stationary growth phase, 2 mM butyrate started to increase IgG production rate two days after addition, reached a peak one day later and on the 8th day decreased to a level lower than that before addition. Considering the irreversible effect of butyrate, in both batch and fed-batch culture it was desirable to add 2 mM butyrate to culture no more than 7 days before the final collection in order to avoid the toxic effect and at the same time provide enough time for butyrate to take effect. A similar result was observed with fluorescence. The fluorescence production rate increased after butyrate addition, reached a peak between day 4 and 6 and subsequently decreased. Volumetric metabolite rate was also calculated in Figure 6.9C. However, unlike IgG production rate, both glucose consumption rate and lactate production rate decreased continuously after addition. In this case, combining with the results from the microbioreactor array, we concluded that fluorescence could be used to predict the effect of butyrate on IgG production in a dose dependent manner and as a function of time while metabolite rate or cell number could not.

6.5. Discussion and conclusion

Butyrate has pleiotropic stimulatory effects. It can enhance protein productivity by inducing histone hyperacetylation to increase the accessibility of DNAase to chromosome DNA. At the same time, it can also stop DNA synthesis, inhibit cell growth and cause apoptosis. Because of these opposing effects, butyrate is a popular enhancer for protein production in bioprocessing, though the overall effect on productivity varied and was unpredictable with different cell lines and different treatment strategies [41-44]. Our results from FACS showed that 2 mM butyrate treatment increased cell specific

fluorescence by three-fold but with significant cell growth inhibition. These agreed with the general comment on butyrate that it increases cell specific productivity while inhibits cell growth.

Jiang and Sharfstein (2007) indicated that proteins with lower expression would have greater enhancement by butyrate treatment than those with higher expression, so reporter gene cassettes should be at genome sites with similar activity as the sites for IgG components [45]. Considering that only cells with the highest fluorescence were collected by FACS and this cell line CHO 6E6 was designed specifically for IgG 23F2G production, all gene cassettes of GFP, light and heavy chains of IgG were in highly active sites of genome in addition to the same CMV control system. These suggested that although GFP was not conjugated together with the heavy chain or light chain of IgG, expression levels could be tightly related especially within the same growth phase. Therefore, theoretically GFP fluorescence could serve as a good parameter to predict the effects of external stimuli on mAb production. Additionally, all recombinant cells with GFP expression were from the same parent cell line and cell activity was generally not affected by GFP expression, so they had the same IgG expression and secretion pattern as their parents. In this case, although therapeutic proteins are not allowed to be produced by GFP expressing cells in manufacture processes, culture conditions optimized by using these GFP expressing cells could be directly applied to manufacture using their non-GFP parents.

Compared to intensive labor and time required to establish stable cell lines with biocistron structures directly connecting reporters with IgG components, the extra work

required in our approach was almost negligible - two weeks to obtain fluorescent positive cells with FACS. However, this extra work could bring us much convenience in immobilized culture process development. This simple, reliable and robust approach also has high adaptability to a variety of cell lines. If the promoter for the IgG chains is not CMV, the promoter/enhancer for GFP in the plasmid can be changed from CMV to the promoter/enhancer of the IgG chains. A similar platform for suspension culture is also under development.

Besides viable cell density, attention has been drawn on real time, non-invasive detection to monitor bioprocesses by identifying cell physiological state, because people believe that a suitable on-line sensor could control cell growth in the most appropriate environmental conditions to produce the greatest amount of products with high fidelity. Market competition has led to more and more on-line measurement instrumentation for suspension cultures such as Nova Autosampler (www.novabiomedical.com). For non-suspension culture, process monitoring can only be realized by indirect measurement such as glucose and oxygen utilization [46]. However, these measurements seem useless to predict effects of butyrate from our previous results. This study demonstrates that the direct tracking of cell physiological state in immobilized cell culture could be automated by deploying autofluorescent technology, which made immobilized cell culture even easier to monitor than traditional suspension or monolayer culture. Two on-line fluorescent detection platforms were introduced here. Conceptually, both of them are very simple. The first is a microbioreactor array for highly parallel bioprocessing during the earliest stages. The second is a fluorescence probe, which could be installed in

spinner flasks or bioreactors for the following scale-up. In addition to quantifying cell growth, fluorescence signals detected could be used to predict IgG productivity at least within a specific growth phase (exponential phase or stationary phase). Our study also found that the effects of butyrate on protein productivity could only be recovered in a dose-dependent manner and as a function of time by fluorescence rather than either metabolite rate or cell number. The established platforms are ready for application in the screening of other additives such as peptones to increase protein expression.

Besides the production of therapeutic biologics and cell mass by immobilized cell culture, PET fibrous matrix closely resembles *in vivo* environmental conditions and has demonstrated many advantages for use in tissue engineering and regenerative medicine. At the same time, bioreactors were used to control all physiological conditions and hydrodynamics to regulate cellular differentiation and proliferation [47-49]. In addition to PET, more and more supporting scaffolds have been widely used to regenerate *in vivo* mimicking tissue using primary cells or established cell lines [50; 51]. End-point assays such as immunohistochemical staining are popular, but useful information related to tissue culture status is always poor in the middle of culture, which prevents insight for improving culture systems. High level auto-fluorescent signals given by high cell density inside bioreactors would allow us to study the dynamic formation of 3-D tissues with reporter genes conjugated to regulative units, which could give characteristic responses such as neural differentiation.

6.6. Reference

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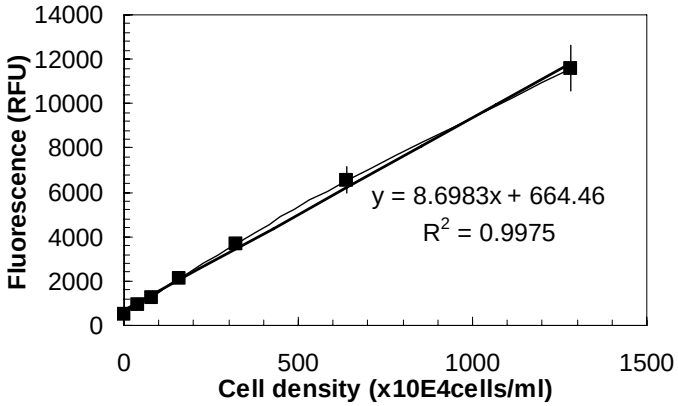

Figure 6.1. Fluorescent intensity is proportional to the number of cells.

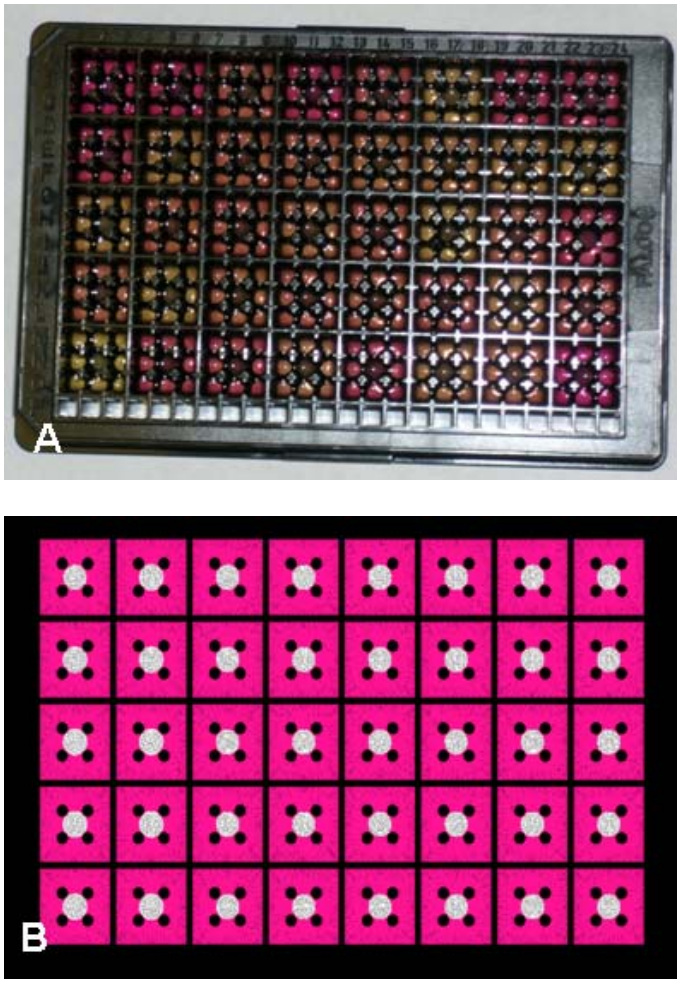

Figure 6.2. Microbioreactor array. (A) A microbioreactor array with 8*5 units in one plate. In each unit, a cake-shape scaffold (0.1cm height; 0.12 cm2 bottom) was affixed in the center with 1 ml working volume and all cell growth was concentrated within this area. (B) Sketch of the array. The white circle in the center of each pink square is a scaffold. Four black dots surrounding are columns to affix a scaffold in the center. Each pink square represents a unit with medium.

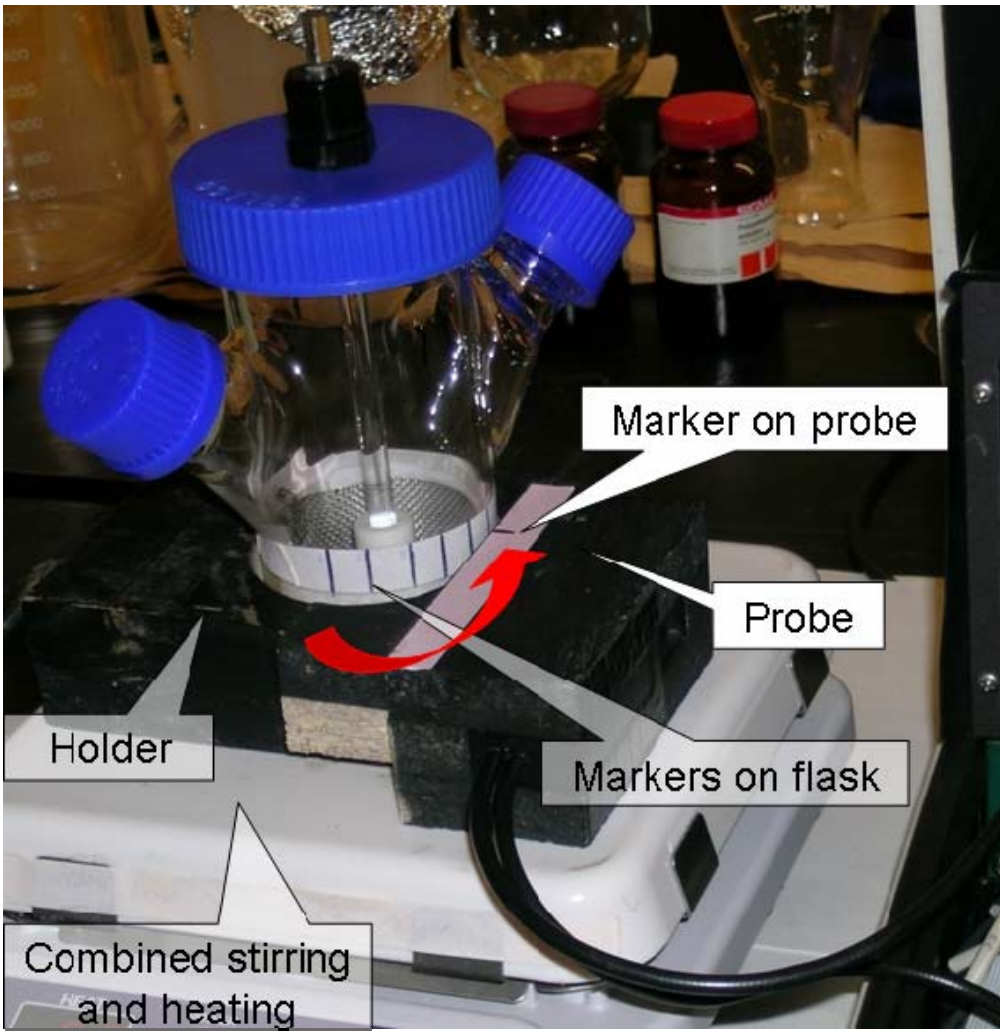

Figure 6.3. Probe and the holder. Points on the flask were pre-marked with corresponding short lines. The whole device could rotate in the direction as shown by the arrow while the flask was immobilized. To measure the fluorescence of each point, the marker on the probe was aimed at a corresponding line on the flask.

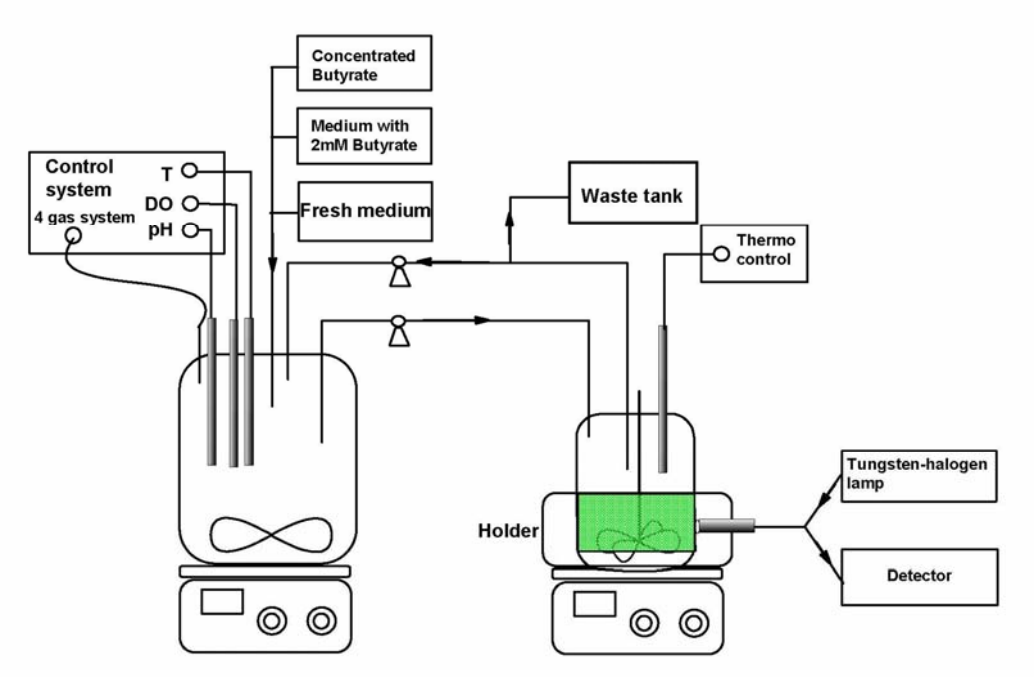

Figure 6.4. Experimental setup for the perfusion bioreactor.

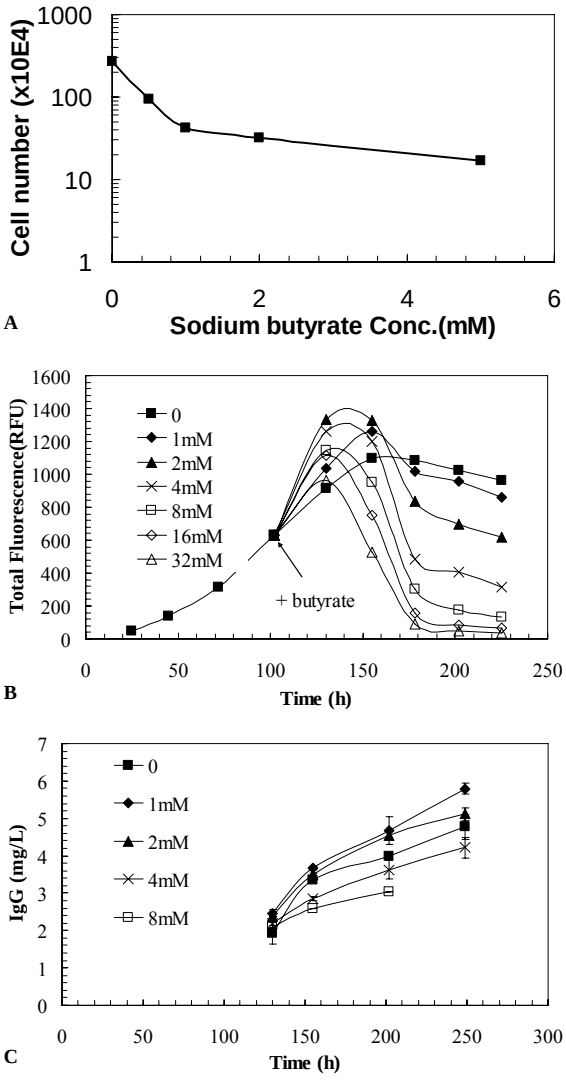

Figure 6.5. Effects of butyrate on cell number (A), fluorescent kinetics of culture (B) and IgG production (C) in 2-D.

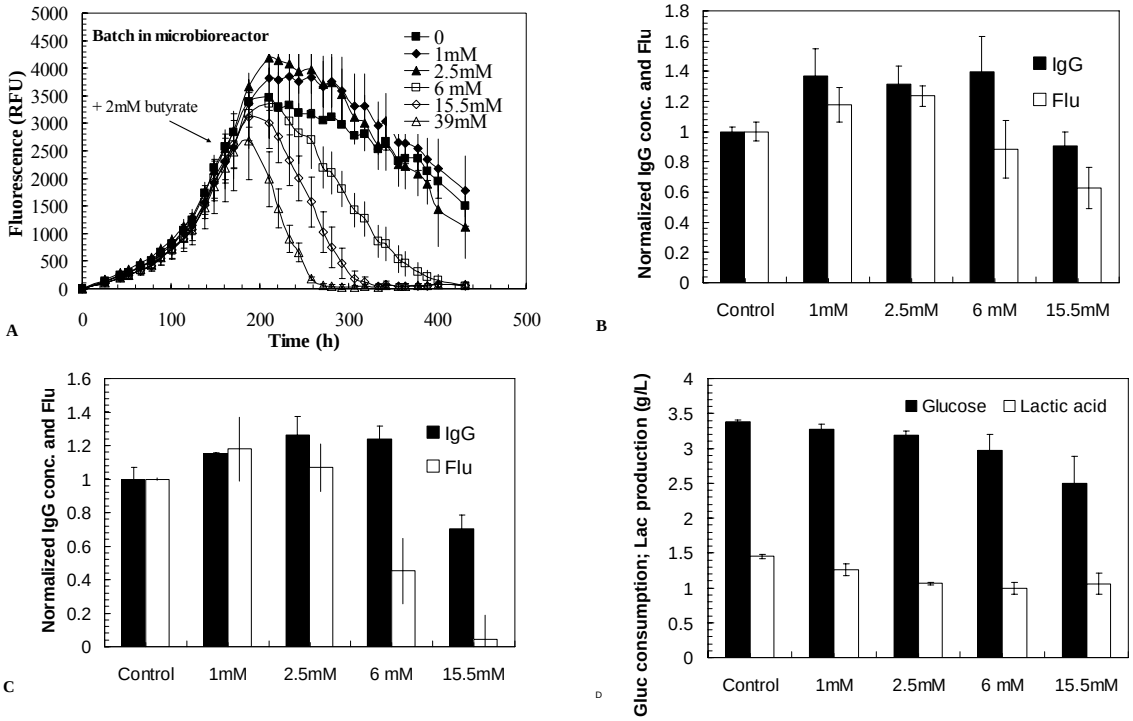

Figure 6.6. Effects of butyrate on 3-D immobilized CHO cell culture in the microbioreactor array. Fluorescent kinetics (A); fluorescent signals and IgG concentrations at different doses 3 days (B) and 6 days (C) after butyrate addition; glucose consumption and lactate production (D).

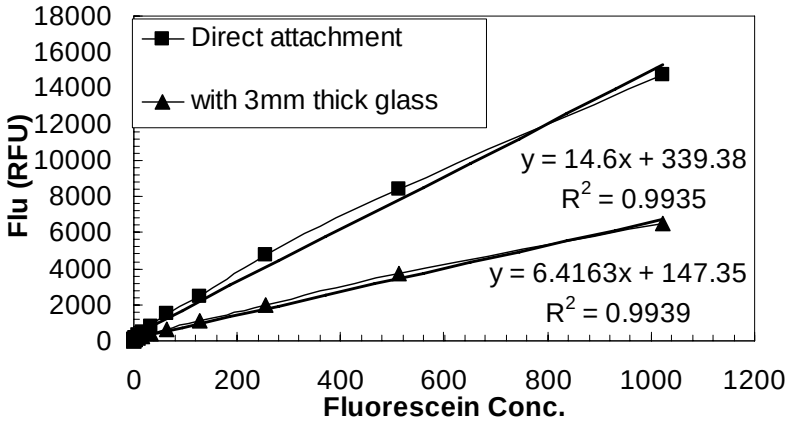

Figure 6.7. Fluorescent signal is proportional to fluorescein concentration with or without 3 mm-thick glass between the samples and the probe.

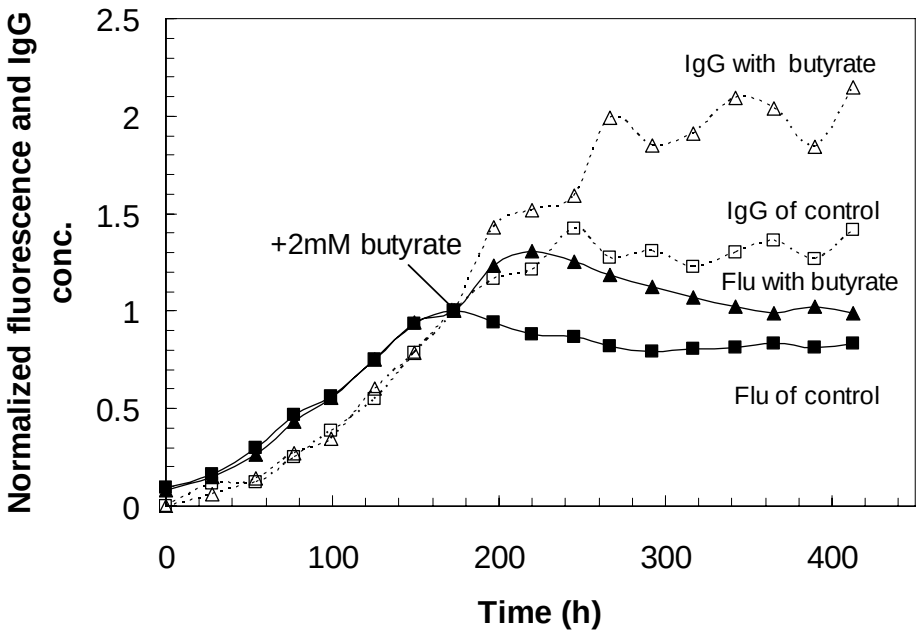

Figure 6.8. Effects of butyrate on fluorescence and IgG concentration in spinner flasks.

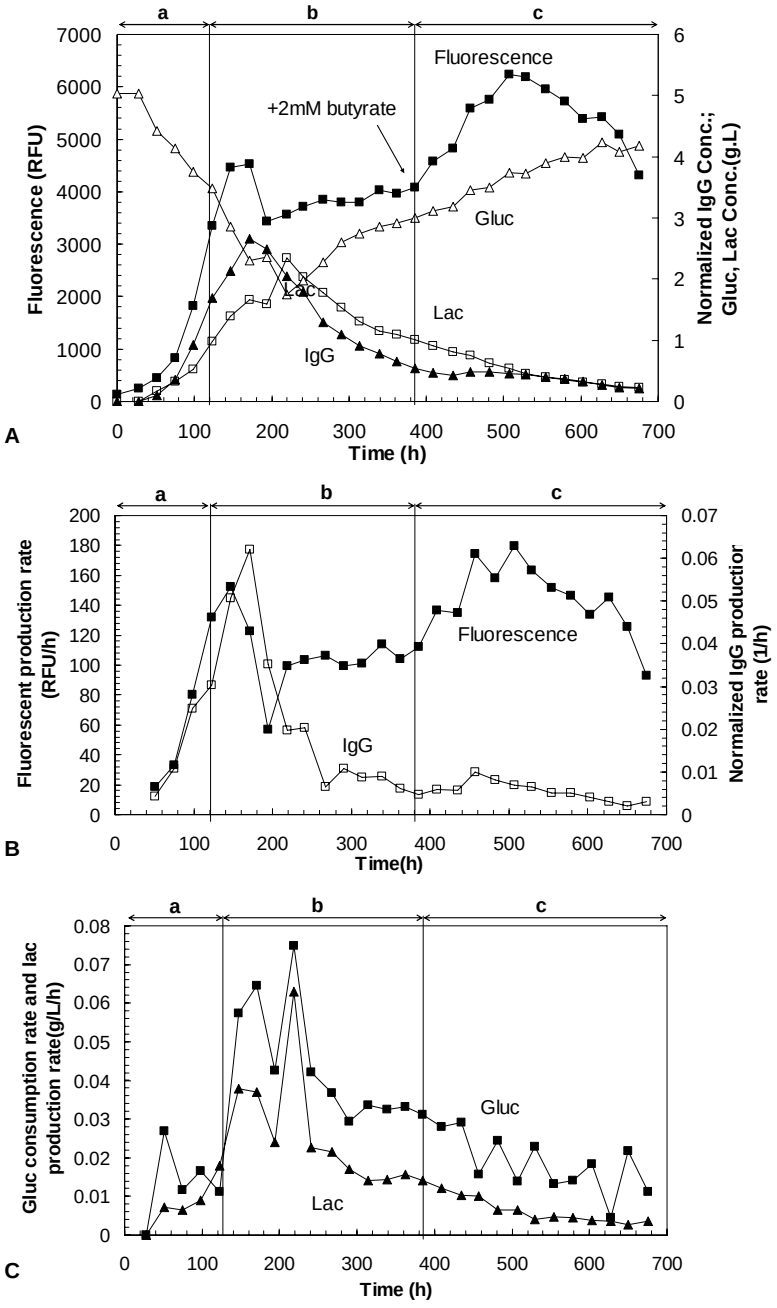

Figure 6.9. Historical Performance of the perfusion bioreactor. a: Batch (0-123 h); b: Perfusion without butyrate (123-384 h); c: Perfusion (384-696 h)with 2 mM butyrate. Fluorescence, IgG, glucose, and lactate concentration (A); production rate of fluorescence and IgG (B); glucose consumption rate and lactate production rate during the culture (C).

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

7.1.1 Microplate-Based High-Throughput Platform with 3-D Culture

Using fluorescence signals to monitor cell growth in a microplate format suffered from high background signals and noises. This critical issue could be solved by introducing 3-D tissue culture, which could increase signal to noise ratio by at least one order of magnitude as compared to conventional 2-D culture systems. Based on this principle, we established a microplate-based system, combining 3-D tissue culture and autofluorescent technique. The effects of fetal bovine serum and fibronectin coating on 3-D ESC culture were evaluated in the system. The increase of growth rate between 0% and 1% FBS was significant, but little influence was found above 1%. Fibronectin coating benefited homogeneous distribution of cells and thus increased cell specific growth rate. Real-time detection could discover acute toxicity of Triton X-100 in a dose dependent manner. The system also enabled the high-throughput study of drug toxicity.

7.1.2 Chemical Toxicity on 2-D and 3-D ESC Cultures

Histological study and theoretical modeling revealed that cells in our 3D models grew without diffusion limitation even in long term cultures, which resembled the microenvironment of most cells *in vivo*. ESCs treated by S-phase specific chemicals 5-fluorouracil and gemcitabine could increase cell-specific fluorescence, which agreed with the previous results that the GFP expression controlled by a CMV promoter is S-phase specific. Cells were a little more susceptible to DM, 5-FU, and penicillin G in 3D proliferation assays than in 2D proliferation assays, while cells showed different levels of resistance in the tissue-like response assays. Compared to proliferation assays, 3D multi-cellular organizations were 5.7, 15.4 and 30.7 times more resistant to DM, DPH and Penicillin G, respectively, while 5-FU was almost non-effective. These results suggested that our recently developed 3D culture system was able to reveal physiological responses of chemicals and enabled toxicological studies in a high-throughput manner.

7.1.3 High-Throughput Anti-Cancer Drug Study

Based on the study using a proliferation maker Ki-67 and a quiescence marker $p27^{kip1}$, we concluded that 3-D tissue-like structures had a similar cell cycle progression as *in vivo* tissues, but monolayer culture not. Three commonly used drugs 5-Fluorouracil (5-FU), gemcitabine and sodium butyrate were tested on a mouse ESC line and a colon cancer cell line at low cell density or high cell density (multicellular structures). The results show that our 3-D cell-based assay provided a much better predictive value especially for those S-phase specific drugs than monolayer cultures.

7.1.4 Online Biosensor in Bioprocess Development

We present a system based on online monitoring of green fluorescent protein (GFP) for fast process development of immobilized cell culture. The system consists of a novel 3-D microbioreactor array for early-stage high-throughput screening and an online fluorescent biosensor ready to use in bioreactors. Both of them allowed continuously and quantitatively monitoring the expression of GFP, which could reflect cell growth and predict therapeutic protein production. Sodium butyrate treatment was used as a case study to demonstrate the performance of both platforms. Results show that non-invasive fluorescent detection provided a much better predictivity on mAb productivity with butyrate treatment in a dose dependent manner and as a function of time than either cell number or metabolite rate.

7.2 Recommendation

Using other particular regulators such as a p53-responsive element instead of a CMV promoter in our system to control the expression of reporter proteins will allow the application of 3-D principle in more functional assays. To better interpret the results of these functional assays, it is favorable to introduce an internal control to monitor live cell number. DsRed2 (Clontech, Palo Alto, CA) is a newly developed living color protein. Because its emission peak is clearly distinct from any other EGFP variants, it can be easily distinguished in fluorescent quantification using appropriate filters. This offers DsRed the ability to colabel with any other GFP variant, so that it could serve as an internal control under the control of a constitutive promoter to quantify cell number,

while specific cellular responses are quantified by other reporter GFPs. Here, the internal control can tell whether the detected fluorescent changes are due to specific responses of functional units or due to influences of cell number by the stimuli. For example, Gorbunov et al (2002) showed that using fluorescence to measure p53 transactivity was dose-dependent only within lower doses of agents. Beyond a dose limit, cytotoxic effect will blunt the signal indicating p53 transactivity. Therefore, coupling with assays to measure cell viability is necessary.

However, the introduction of internal controls is not easy. The expression of a fluorescent protein restrictedly related to cell number in a linear relationship even with external stimuli is very important to provide an internal control, while other mechanistic promoter-reporter structures give characteristic responses to stimuli. Factors affecting the expression of reporter genes include not only promoter/enhancers but also the "position effects" of gene cassettes inside of the genome. In this case, different recombinant cell lines established with the same plasmid might have different fluorescent levels and different behaviors upon the treatment of external factors. One stable recombinant CHO cell line was established from a colony in a 96-well plate, in which all cells were with approximately the same high level of fluorescence. The cell specific fluorescence of this cell line was not affected by butyrate. It was suspected that the fluorescent signal of this cell line would be constitutively proportional to cell number, but further confirmation is required. Integration via homologous recombination to a well-studied target position in genome might provide a solution to prevent the confounding effects due to position effect and make the system more controllable.

A similar perfusion culture system to mass-produce undifferentiated embryonic stem (ES) cells using conditioned medium provided by feeder cells was proposed in our group. In the work, one bioreactor for immobilized ESC growth was connected to the other bioreactor for immobilized feeder cell culture through a fast recirculation, so feeder cells could continuously produce growth factors and hormones to support ESC growth but did not contaminate ESCs. Although the system yielded an economical way for expansion of undifferentiated ESCs, this immobilized coculture made culture process development even more difficult. In addition to difficulty in cell sampling, metabolite data such as glucose and lactate concentration measured during culture were only related to the total cell growth instead of corresponding to the ESC growth. In this case, it was very difficult to obtain useful dynamic information to predict ESC growth. The fluorescent probe demonstrated previously was the exact solution to solve this critical issue in ESC culture process development. Future study will apply this fluorescent probe into large-scale production of human embryonic stem cells.

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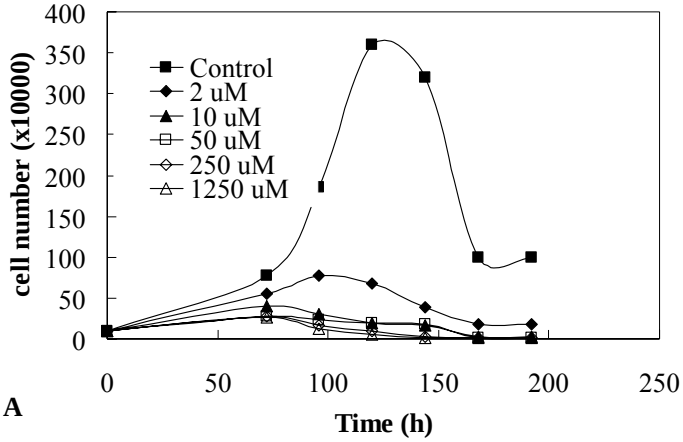
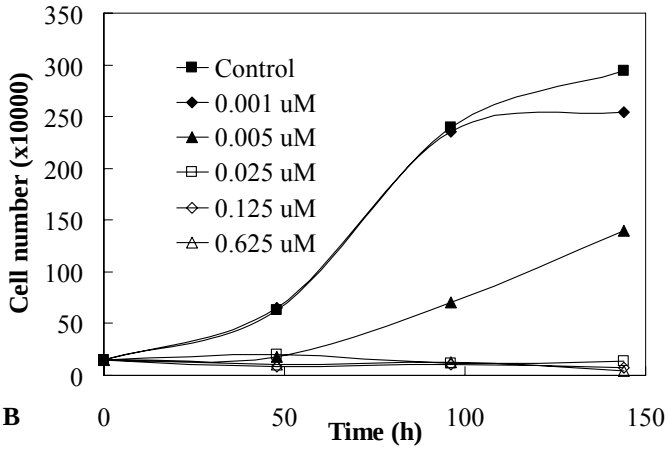
Appendix A Kinetics of 2-D and 3-D (Low-density and High-density) mESC and HT-29 Cultures with Three Drugs



A



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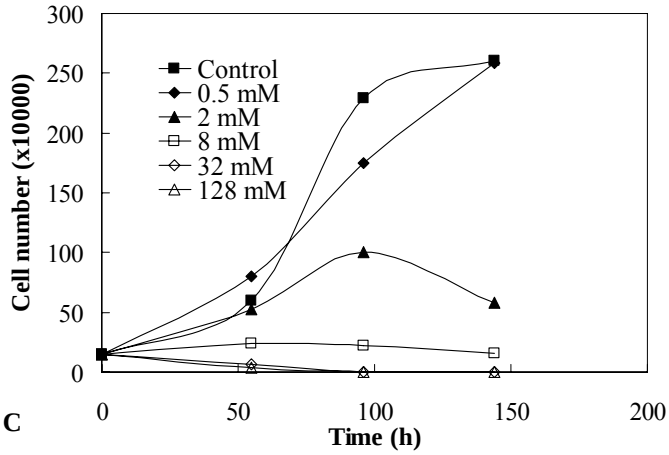
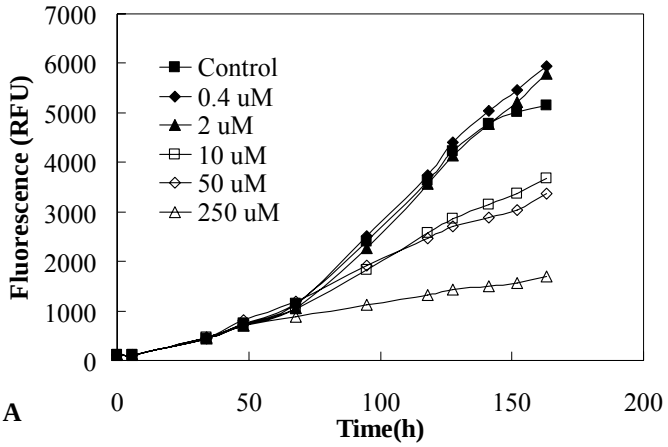


C

Growth kinetics of 2D cultures of HT-29-GFP upon the treatment of different drugs. Drugs were added one day after seeding. A. 5-FU; B. gemcitabine; C. sodium butyrate.



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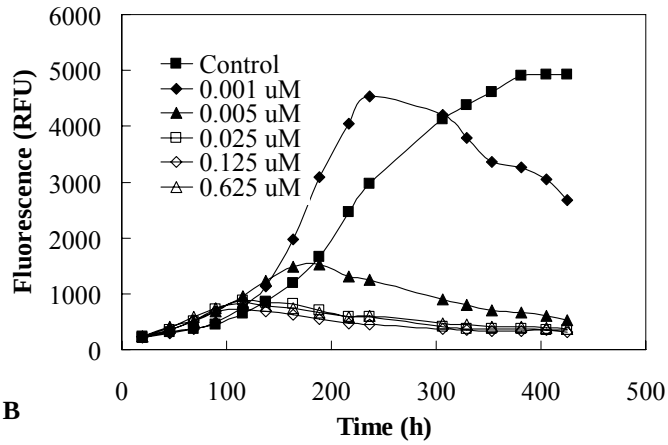

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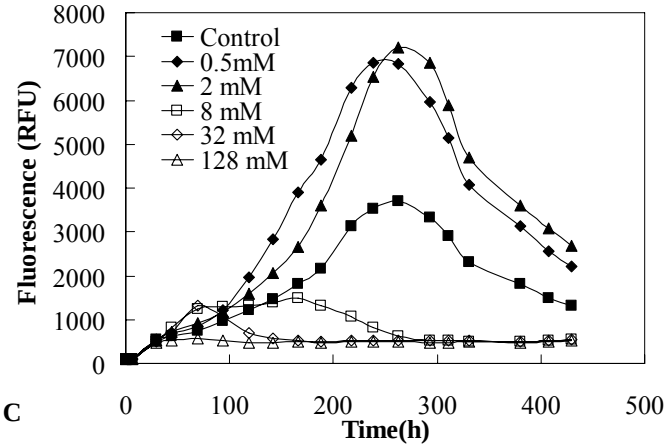
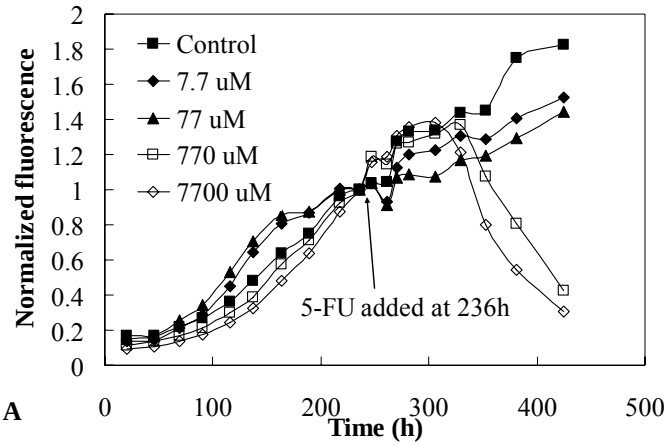


C

Growth kinetics of 3D cultures of HT-29-GFP upon the treatment of different drugs. Drugs were added one day after seeding. A. 5-FU; B. gemcitabine; C. sodium butyrate.



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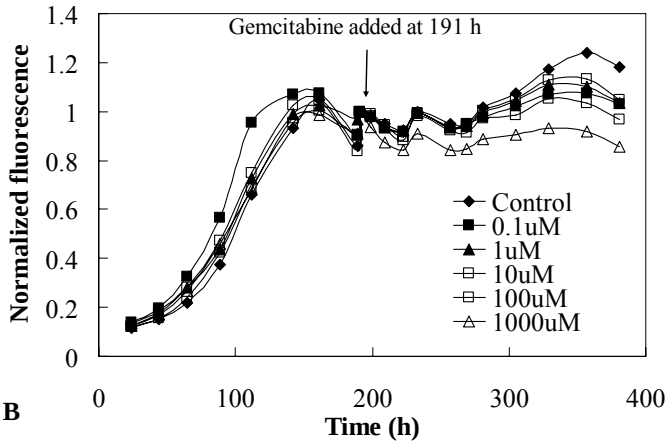

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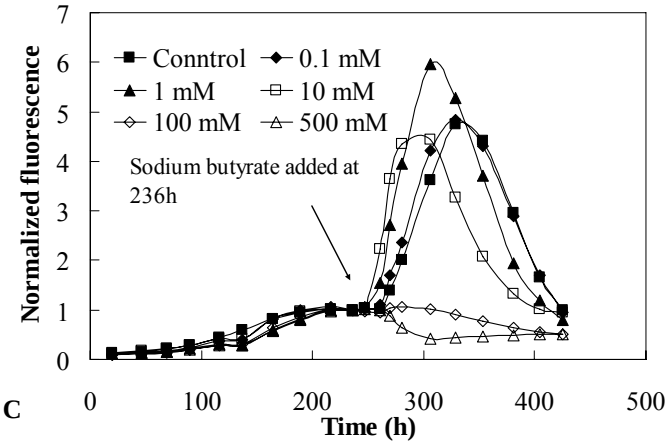


C

Growth kinetics of 3D cultures of HT-29-GFP upon the treatment of different drugs. Drugs were added 8 or 10 days after seeding. A. 5-FU; B. gemcitabine; C. sodium butyrate.

5-FU

Dose (uM)	0	0.096	0.384	1.73
Cell number (X10000)	133	120	30	16

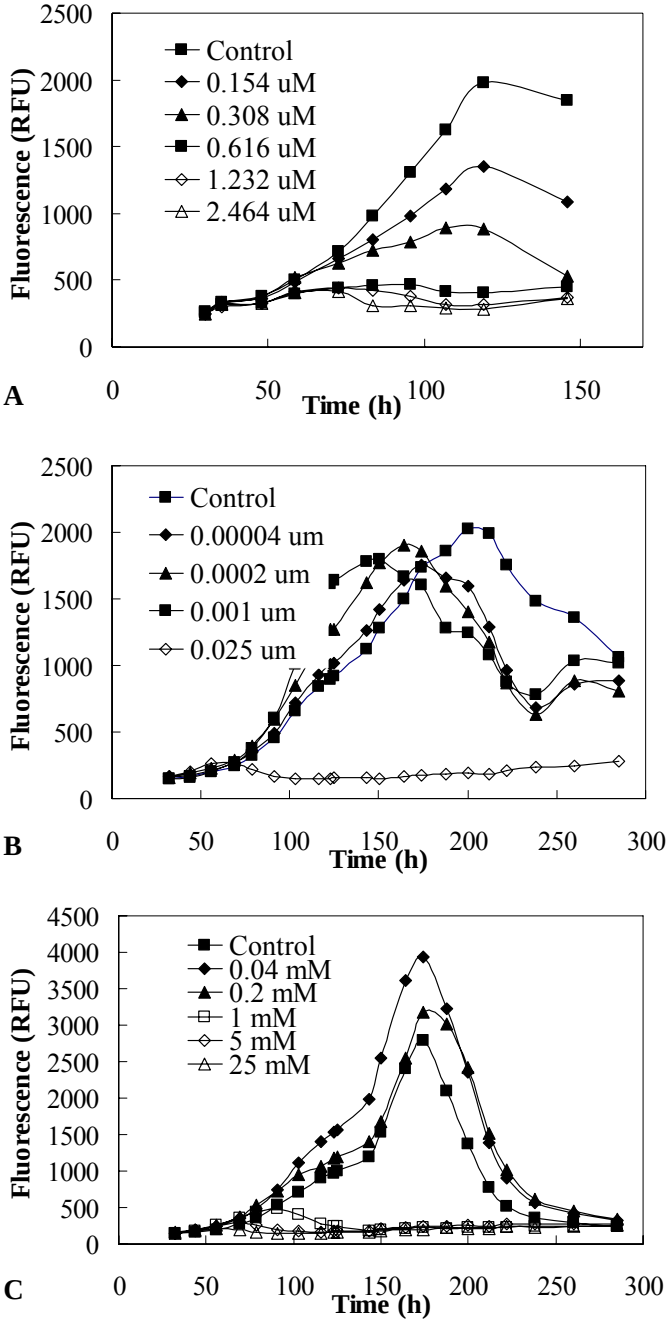
Gemcitabine

Dose (uM)	0	0.001	0.01	0.1
Cell number (X10000)	133	60	20	2

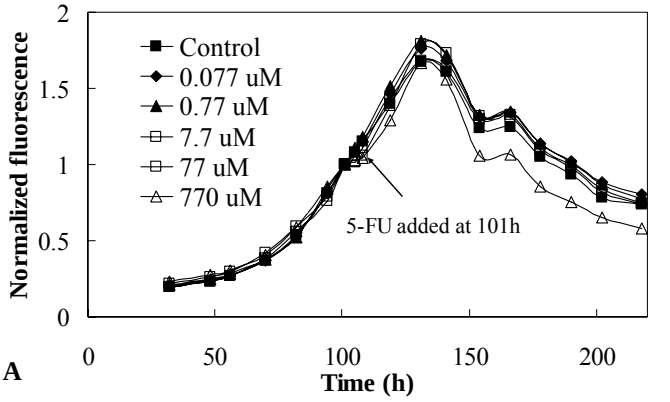
Sodium butyrate

Dose (mM)	0	2	8	32
Cell number (X10000)	133	8	15	N/A

50000 ES-GFP cells were inoculated into each well of 12-well plates. One day after seeding, drugs were added. Cell numbers were measured 2 days after drug addition.



Growth kinetics of 3D cultures of ES-GFP upon the treatment of different drugs. Drugs were added one day after seeding. A. 5-FU; B. gemcitabine; C. sodium butyrate.



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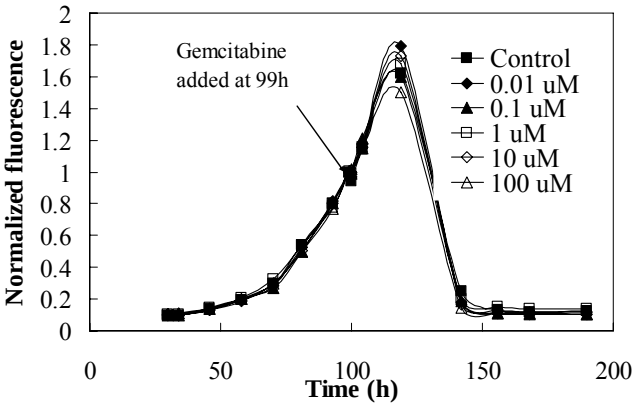

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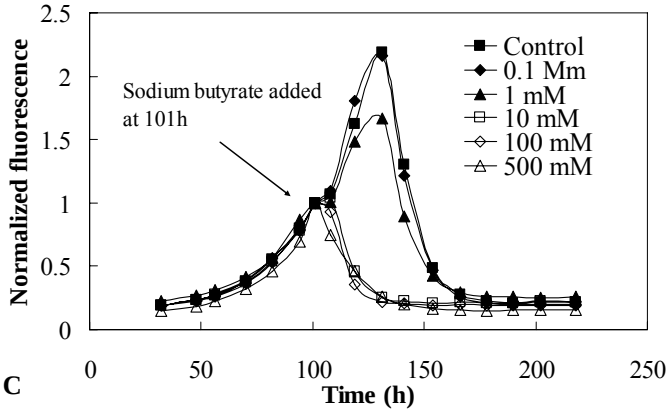

C

Growth kinetics of 3D cultures of ES-GFP upon the treatment of different drugs. Drugs were added four days after seeding. A. 5-FU; B. gemcitabine; C. sodium butyrate.