Cell-Based Assays in High-Throughput Screening for Drug Discovery

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Abstract: Drug screening is a long and costly process confronted with low productivity and challenges in using animals, which limit the discovery of new drugs. To improve drug screening efficacy and minimize animal testing, recent efforts have been dedicated to developing cell-based high throughput screening (HTS) platforms that can provide more relevant *in vivo* biological information than biochemical assays and thus reduce the number of animal tests and accelerate the drug discovery process. Today, cell-based assays are used in more than half of all high-throughput drug screenings for target validation and ADMET (absorption, distribution, metabolism, elimination and toxicity) in the early stage of drug discovery. In this review, we discuss the uses of different types of cells and cell culture systems, including 2D, 3D and perfusion cell cultures, in cell-based HTS for drug discovery. Optical and electrochemical methods for online, non-invasive detection and quantification of cells or cellular activities are discussed. Recent progresses and applications of 3D cultures and microfluidic systems for cell-based HTS are also discussed, followed with several successful examples of using cell-based HTS in commercial development of new drugs. Finally, a brief discussion on potential applications of cell-based HTS for screening phytochemicals and herbal medicines is provided in this review.

Keywords: Cell-based high throughput screening, drug discovery, cell culture.

1. INTRODUCTION

The journey from drug discovery to its commercialization is complex, lengthy and expensive. It usually takes 10-12 years and well over $775 million for the research and development of a new drug. On average, approximately 250 lead compounds screened from a million compounds enter pre-clinical testing, 10 of them proceed to clinical trials, and only one will be approved by Food and Drug Administration (FDA) [1]. Historically, drug screening extensively relies on animal models as proxies for human beings in drug target validation and ADMET (absorption, distribution, metabolism, elimination and toxicity). Although these animal models have the capacity to provide a wealth of useful information for drug screening, they are relatively expensive, low throughput and present moral issues. With the development of combinatorial chemistry and biology, the number and diversity of compounds that need to be tested for activity against targets has rapidly expanded in recent years. In addition, with the enormous amount of new data generated from the Human Genome Project, the number of proteins that can be targeted for drug screening has also significantly increased with better understanding of the mechanisms underlying human diseases [2]. These trends have spurred the development of increasingly rapid, selective and reliable high-throughput screening (HTS) assays for the early-phase drug discovery.

HTS assays can be divided into two categories, namely biochemical assays and cell-based assays [3]. Target-based biochemical assays, mainly enzyme inhibition and receptor-ligand binding assays, have been the mainstay of HTS campaigns in the pharmaceutical industry. In a biochemical assay, the specific binding or affinity of tested compounds to the target of interest is carried out in homogeneous reactions that allow the miniaturization with low variations. However, the applications of biochemical assays are limited because not all targets can be purified or prepared in a manner suitable for biochemical measurement. Additionally, drug responses tested in biochemical assays cannot precisely represent tissue-specific responses since the activity of a small molecule in a biochemical assay is different from the activity in a cellular context [3]. Therefore, the toxicity testing market is gradually moving towards *in vitro* cell-based assays, as they provide an early indication of the toxicity characteristics of the drug candidates.

Cell-based assays for HTS include mainly three types: second messenger assays, reporter gene assays, and cell proliferation assays (see Table **1**) [4].

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Table 1: Major Types of Cell-Based Assays Used in HTS for Drug Screening

Assay type	Mechanism or method	Examples
Second messenger	monitor signal transduction following activation of cell-surface receptors	Using fluorescent molecules that respond to changes in intracellular Ca^{2+} concentration, membrane potential, pH, etc. to assay receptor stimulation and ion channel activation [5, 6]
Reporter gene	monitor cellular responses at the transcription/translation level	Coexpression of luciferase to catalyze the light-emitting luciferin reaction for detection of protein kinase C inhibitors [7, 8] Quantification of G-protein coupled receptor (GPCR) internalization using a GPCR-green fluorescent protein hybrid [9]
Cell proliferation/cytotoxicity	monitor the overall cell growth or death in response to external stimuli or stress	Virus-induced cytopathic effects on cell proliferation monitored by following the reduction of tetrazolium salt to formazan quantified by absorbance at 410 nm [10]

They are extensively applied in the majority of compound screening programs performed by the biopharmaceutical industry. The global market for cell-based assays in drug discovery was valued at $6.2 billion in 2010, and is expected to increase at an annual growth rate of 11.6% to reach nearly $10.8 billion in 2015 [11]. Cell-based assays can distinguish between agonists and antagonists, identify allosteric modulators, and provide direct information on compounds with regards to cell permeability and stability inside cells, and acute cytotoxicity associated with the compounds [12]. Furthermore, cell-based assays can be performed in a more biological relevant microenvironment and thus represent a good compromise between whole organisms and *in vitro* biochemical systems. These assays can provide representative tissue specific responses, and have been used throughout early drug discovery, from target identification and validation to primary screening, lead identification and optimization, and safety and toxicology screening. They have been successfully used for early drug discovery in identifying high-quality leads.

The main components of a cell-based HTS assay include cells, device for culturing the cells, and detection system for quantification of cells or cellular activities. This review provides an overview on different types of cells, cell culture systems, and *in situ* detection methods currently used or amenable to use in cell-based HTS assays. We highlight reporter gene techniques, which involve the expression of the reporter gene product such as an enzyme or green fluorescent protein that is used as part of a colorimetric, luminescent, or fluorescent read-out of gene activation in the cell or cellular responses to a stimulus, and recent developments of 3D cell-based fluorescent assays and microfluidic systems for HTS. Examples and perspectives of using cell-based HTS assays in early-stage drug discovery are also provided in this review article.

2. CELL SOURCES AND TYPES

Cells used in cell-based assays should be amenable to the assays, faithfully represent the system and express the necessary factors and signaling intermediates. Various types and sources of cells have been used in cell-based assays. Table **2** presents a summary of different cell types and sources with their most advantageous and disadvantageous features.

Immortalized cell lines are widely used for drug screening assays because they are cheap, easy to grow, reliable and reproducible. For example, HEK293 cells, derived from human kidney, are an easy, amenable experimental system [20, 21]. However, immortalized cell lines, derived from a well-known tissue type, have undergone significant mutations and their biological characteristics might be altered in the immortalization process and different from those of the native or normal cells [22]. ***Primary cells*** may provide representative responses; however, they have a limited life span in culture and are difficult to grow and transfect [22]. Human ***cancer cell lines*** represent the cancer of origin and are widely used for anticancer drug screening in pharmaceutical research. However, they can contain mutations that might affect the experimental results. For instance, the breast cancer cell line MCF-7 lacks a functional caspase-3 gene product [23]. Furthermore, cell-based assays employing primary cells or immortalized and cancer cell lines have been insufficient in developing effective therapeutics for cancers. Although the initial therapy

Table 2: Sources and Types of Cells Available for Drug Screening

Cell types	Examples	Comments
Immortalized cell lines	-Myocardial cell lines for screening adrenergic drugs [13] -Skeletal muscle lines for testing cholinergic compounds [13] -Nerve cell lines for screening neurotransmitter agonists [13]	-Low cost in growth and maintenance -Homogenous cell population -Lack in important aspects of native cellular function -Not representative of normal cells
Primary cells	Human T cell for screening HIV-1 inhibitors [14]	-Fully differentiated cell types -Close approximation of native function -Physiological response -Not easily accessible or available for all cell types -Need fresh preparation -Questionable reproducibility
Human cancer cell lines	NCI60, a panel of 60 human tumor cell lines (NCI60) representing 9 tissue types, for screening potential new anticancer agents [15]	-Easy to use -Cancer specific -Containing mutations that might affect the experimental outcome
Cancer stem cells	Phase II screening of new drugs in ovarian cancer and malignant melanoma [16]	-Self renewal -Multi-potency -Difficulty in maintaining as pure population in culture
Mesenchymal stem cells (MSCs)	Human mesenchymal stem cells derived osteoblasts for testing purmorphamine [17]	-Easy to obtain and in high quantity -Safe maintenance and propagation -No ethical issues -Multi-differentiation -Limited differentiation capacity
Embryonic stem cells (ESCs)	Human embryonic stem cell derived cardiomyocytes for electrophysiological drug testing [18] -Mouse ES cell derived neurons for screening glutamate receptor agonists [19]	-High quantity -Readily available source of all cell types -Capacity to fully differentiated into all cell types -Close approximation of native function -Growth and maintenance with high cost -Difficult to obtain fully differentiated cell types -Difficult to achieve purified populations -Moral issues
Induced pluripotent stem cells (iPSCs)		-Same advantages as ESCs -Can be derived from specific diseases -Growth and maintenance with high cost -Difficult to obtain fully differentiated cell types -Difficult to achieve purified populations -Difficult to generate iPSCs -Low efficiency of generating clones

Note: NCI 60: The US National Cancer Institute (NCI) 60.

and recovery is successful, the rate to a relapse of the disease is very high [24].

Recent advances in stem cell research have revolutionized the drug discovery process. ***Cancer stem cells***, which might be genetically arising from oncogenic transformation of either stem cells or progenitor cells, can be isolated from tumors and used as an effective platform for cancer drug screening. Cancer stem cells can self-renew, differentiate and regenerate a phenocopy of the original tumor [25]. Therefore, they are promising cell models for specific human cancers. However, they are rare and difficult to find or isolate from tumors. ***Mesenchymal stem cells*** (MSCs) isolated from bone marrow or umbilical cord blood can be differentiated into different somatic cells, such as osteoblasts, for testing drugs. However, MSCs have limited potency and are slow-growing and difficult to expand *in vitro*. ***Embryonic stem cells*** (ESCs) and ***induced pluripotent stem cells*** (iPSCs) can provide an unlimited source of normal human cells that can be

expanded for drug screening and toxicological studies. ESCs are isolated from embryo and have unlimited capacity to self renew and can be differentiated into any cell type *in vivo*. These cells can serve as better cell models for both drug efficacy and toxicity screening than primary cells or immortalized cell lines. However, ESCs present some moral issues since the embryos are destroyed during the procurement of the cells. More recently, the development of iPSCs has revolutionized the stem cell field. IPSCs are pluripotent cells artificially derived from somatic cells (e.g., fibroblasts and other adult cell types) by inducing a small set of powerful pluripotency genes. These cells lose their previous somatic cell properties and are similar to human ESCs in terms of morphology, growth properties, gene-expression profiles and differentiation potential. As iPSCs can be derived from patients with specific diseases, they have been considered as a new tool in drug discovery.

3. CELL CULTURE SYSTEMS

The majority of cell-based HTS assays are carried out in multi-well plates as they can be easily miniaturized to increase the number of wells per plate for high throughput rates, on the order of 10,000 compounds per assay per day, and handled with a robotic system for automation [4]. More recently, there are increasing interests in developing microfluidic devices for perfusion cultures that allow for the evaluation of long-term drug effects as well as studying interactions among different cell types in a biological system like the whole animal [26]. There is also increased awareness of that conventional 2D cell cultures in multiwell plates cannot represent *in vivo* tissues that are present in 3D environments [27]. The different culture systems used in cell-based assays are discussed and compared for their advantages and disadvantages or limitations below.

3.1. 3D vs. 2D Cultures

In general, cell culture modes include single cells, monolayer cells on a two-dimensional (2D) surface, multilayer cells or aggregate clusters in a 3D scaffold [28]. 2D cell-based assays in multiwell plates together with automated operation are widely used in drug screening because of their low costs and easy operation. However, 2D cell cultures can result in errors in predicting tissue-specific responses due to the loss of native morphology and limited cell-cell and cell-matrix interactions. As can be seen in Figure **1**, cells cultured in 3D scaffolds generally show similar *in vivo* morphology with intimate cell-cell and cell-ECM (extracellular matrix) interactions, which are absent in 2D cultures. It is known that cells grown on 2D flat surfaces behave differently from those in 3D scaffolds or *in vivo* tissues in their morphology, cell-matrix interactions and differentiation [29]. The third dimension in the 3D scaffold provides another direction for cell-cell interactions, cell migration, and cell morphogenesis, which are critical in regulating cell cycle and tissue functions. Differences in spatial organization and distribution contribute to the difference in cell growth. In addition, 3D cell cultures provide not only the templates for cells to adhere and grow, but also the interconnectivity within the 3D constructs to allow nutrients and metabolites to be transported in and out of the engineered tissues. Consequently, 3D cell cultures can support a higher cell density than 2D cell cultures [29]. The high specific surface areas offered by 3D also allow for a long-term cell culture *in vitro* [30]. The cellular performance between 2D and 3D cell cultures are also different in many studies. For example, the malignant phenotype of human breast epithelial cells cultured in 3D Matrigel based scaffolds can be reverted to normal morphology *via* the inhibition of β1-integrin and epidermal growth factor receptor (EGFR), but not in 2D cell cultures [31]. In addition, tyrosine phosphorylation, which plays a role

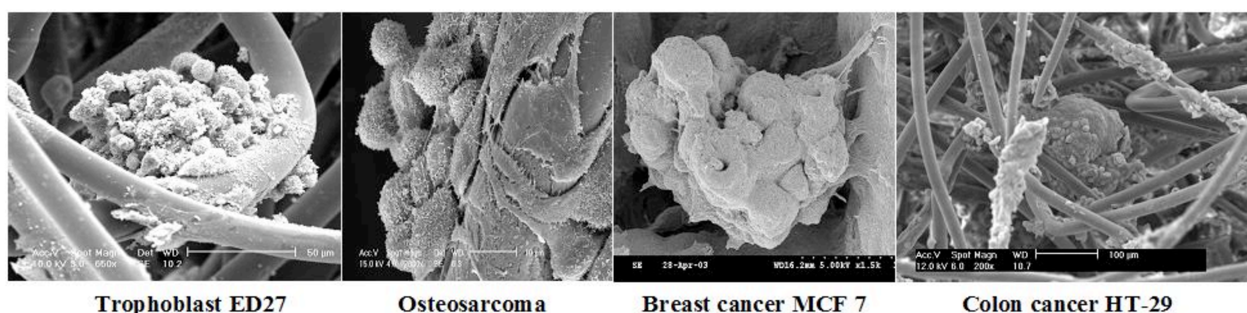

Figure 1: SEM photos of some human cells grown in nonwoven fibrous PET matrices showing 3D morphologies similar to those found in *in vivo* tissues.

in signaling of focal adhesion kinase (FAK), is down-regulated in 3D culture [32]. Furthermore, cow luteal cells (primary cells) cultured in a 3D bioreactor environment could better maintain its normal function, progesterone secretion in response to luteinizing hormone (LH) stimulation, for a longer period as compared to cells cultured in the 2D T-flasks (see Figure **2**). The ability for cells to maintain their normal function and response to environmental stimuli is critical in the development of cell-based HTS for drug discovery.

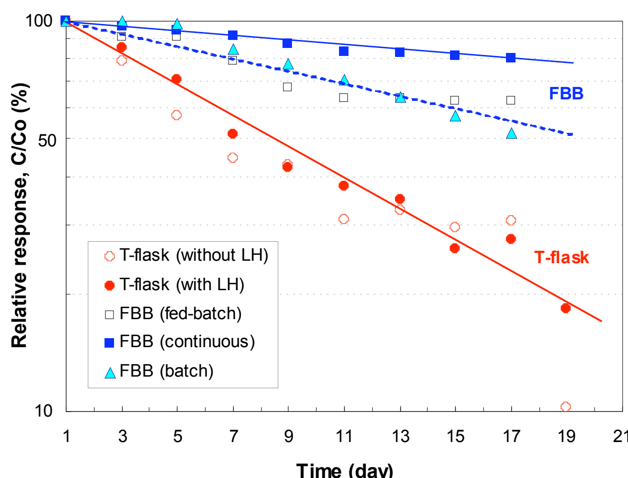

Figure 2: Decay of luteal cell's ability in response to LH stimulation in 2D T-flask and 3D fibrous bed bioreactor (FBB) cultures. The 2D T-flask culture lost almost all its response in 19 days, while 3D FBB cultures still maintained more than 60% of the initial activity.

Numerous studies have shown that cell responses to drugs in 3D cultures are distinct from those in 2D cultures, which highlights the advantages of using 3D-based models. For example, colon cancer cells in 3D cell cultures showed an increase in drug resistance of up to 180-fold as compared to 2D cell cultures [33]. Colon and ovarian cancer cells in 3D cell cultures exhibited a 1000-fold decrease in cytotoxicity responses to gemcitabine, while 2D cultures erroneously predicted gemcitabine to be an effective proliferation inhibitor [33]. Tumor cells cultured under 2D conditions are more sensitive to PI3-kinase inhibitor than those within engineered 3D poly(lactide-co-glycolide) tumor microenvironments [34]. These results support the important effects of physiological microenvironments within tumors on the effectiveness of chemotherapy and suggest that 3D engineered tumor models can offer more useful information in anticancer drug screening. Furthermore, coculture of endothelial, stromal, and/or epithelial cells within 3D systems allows the study of the side effects of a drug on neighboring stromal cells [35]. Moreover, human skin cells (keratinocytes, dermal fibroblasts and endothelial cells) in 3D cocultures can stand more oxidative stresses (hydrogen peroxide) and a potentially toxic heavy metal (silver) than in 2D cultures [36]. These results suggest that 3D culture systems outperform their counterpart 2D culture systems in studying physiological responses to xenobiotic materials.

In summary, 3D cultures can better recapitulate *in vivo* cellular responses to drug treatment and has potential to be a superior platform for drug development. Therefore, there is a great need for *in vitro* 3D cell culture assays, which would bridge the 2D monolayer cell culture systems and the animal models. However, 3D cultures create a non-homogeneous environment that is difficult to monitor for changes in cells or cellular activities using conventional detection methods, which will be discussed later in this article.

3.2. Perfusion *vs*. Static Cultures

Widely used HTS platforms (e.g. 96-, 384-, 1536-well plates) offer static microenvironments, with the medium supplied in a batch-wise manner. Although automation using robots allows the static cultures to be used as a feasible HTS platform for drug screening, static cultures cannot support a long-term cell culture due to the risk of contamination caused by repeated interventions. In addition, the intermittent medium replacement process would result in large fluctuations in the culture microenvironments [37, 38]. Although further miniaturization of these systems holds great potentials to increase throughputs, the relatively high surface to volume ratio in microscale wells results in uncontrolled liquid evaporation and leads to undesirable culture conditions [39]. Modified multiwell plates have thus been proposed with the integration of microfluidic systems, which has been reported with high throughput for drug screening [40] and cytotoxicity evaluation of anticancer drugs [41]. Such systems, where a perfusion cell culture is achieved to compensate liquid evaporation, can maintain a cell culture for an extended period for testing long-term effects of drugs.

In addition to continuously providing nutrients and waste removal and thus keeping the cell culture system stable, perfusion can also be used to generate gradients of drug concentrations, creating a specific physical microenvironment (e.g. shear stress or interstitial fluid flow) and constructing a circulatory

system to better mimic the *in vivo* conditions [39]. Compared to the static cell culture, perfusion increased cell content and matrix synthesis in a 3D chondrocyte culture [42]. Furthermore, cellular responses to the perfusion and static conditions were quite different for a human hepatocarcinoma cell line [43]. Although there was a time period, wherein the cell physiology was comparable, outside this period, the cultured cells behaved differently in the two culture systems. The different culture behaviors might be attributed to the uncontrollable difference in some unknown biochemical or biophysical factors in these two culture systems.

4. METHODS FOR QUANTIFICATION OF CELLS AND CELLULAR ACTIVITIES

Cell-based assays are well established and widely used to analyze the effects of compounds on cellular activities, including nuclear size, mitochondrial membrane potential, intracellular calcium levels, membrane permeability, and cell number. The failure of early identification of toxic side effects of a compound has resulted in about 30% of the attrition of new drug candidates [44]. Therefore, cytotoxicity testing, which generally relies on the quantification of cell number and viability, has become one of the most critical steps in early-phase drug discovery. Conventional methods for cell number counting use hemacytometer, Coulter counter or flow cytometry are labor-intensive and time consuming, while Trypan blue exclusion and neutral red uptake methods for determining cell viability require the use of invasive chemicals. Furthermore, these methods have a relatively low throughput, and thus are not good choices for HTS. As off-line sampling during the cell culture process is limited by the small amount of medium used in HTS assays, from several μl to several ml [45], online detection is required. Detection methods used in cell-based HTS assays can be divided mainly into two groups: electrochemical and optical techniques. In general, optical sensing is easier for miniaturization than electrochemical sensing [46]. These detection methods are discussed below.

4.1. Electrochemical Methods

Various electrochemical biosensors, which integrate biological recognition elements and electrochemical transduction units, based on (a) cellular activity and function; (b) cellular barrier behavior; and (c) recording/stimulation of electric potential of electrogenic cells have been developed [47]. They can be used to achieve noninvasive online monitoring of drug toxicity.

4.1.1. Electrochemical Method Based on Cellular Activity and Function

A living cell can be considered as an electrochemical system [48]. Electron generation and charge transfer caused by redox reactions and the changes of ionic composition and concentration in living cells can be used to characterize cell viability in a homogenous solution [49]. For example, when the tumor cells are attached to a gold nanoparticle-modified carbon paste electrode, with platinum wire as auxiliary and saturated calomel electrode as reference electrodes, the cells exhibit an irreversible voltammetric response, which is related to the oxidation of guanine. The oxidation peak can be used to investigate the exogenous effect, which provides an electrochemical approach for studying antitumor drug sensitivity [50].

In addition, metabolism in cells leads to changes in metabolic products (e.g., lactic acid and carbon dioxide) or substrates (e.g., glucose and dissolved oxygen [DO]). A variety of electrochemical biosensors based on metabolic changes have been fabricated [51-54]. Electrochemical methods based on cellular activities include potentiometry and amperometry.

Conventional potentiometry cell-based sensors include an ion-selective electrode (ISE) or gas-sensing electrode (GSE) coated with a layer of cells. An ISE has been developed for screening of toxins by integrating cells with a K^+ selective film. In such systems, a potential change caused by the ion accumulation or depletion on the electrode surface can be used to monitor metabolic products during cell growth [54]. However, this method requires a very stable reference electrode, which limits the application of potentiometry sensors.

Amperometric electrochemical methods using a specific enzyme electrode are widely used for the determination of pH, DO or glucose. The acidification rate in the vicinity of cells can be quantified using a microphysiometer [55]. Cellular biochemical responses resulting from the accumulation of lactic acid and carbon dioxide can be approximately monitored using the pH value in pH-sensing chambers. Furthermore, heterogeneous pO_2 distributions around tissues could be detected using a miniaturized system [56]. However, metabolic activities can be affected by many uncontrollable environmental factors, which limit the applications of this approach in high-throughput cell-based assays.

4.1.2. Electrochemical Method Based on Barrier Behavior

The local ionic environment at the electrode/solution interface changes in the presence of cells. In general, cells with insulating properties would significantly increase the electrode impedance [57, 58]. Thus, biological status of cells, including cellular viability, morphology, cell number, and cell apoptosis, and cell adhesion can be monitored using electrochemical impedance spectroscopic techniques. For example, a novel electrical impedance sensor array integrated into the bottom of a microtiter plate has been developed for the quantitative detection of living cells. Real-time assessment of cytotoxicity and acute toxicity can be achieved using this device [58].

4.1.3. Electrochemical Method Based on the Recording/Stimulating of Cellular Electrical Potential

Electrogenic cells and tissues, such as heart muscle, pancreas beta and nerve cells, are able to generate bioelectrical signals resulting from the orchestrated activities of ion channels embedded within cell membrane [47]. These bioelectrical signals can be used to test drugs against critical diseases such as cardiac arrhythmia, hypertension, Parkinson's disease, diabetes, depression, and neuropathic pain [59]. A nanoelectronic biosensor was developed based on single-wall carbon nanotubes (SWCNTs). Nerve cells were grown on a SWCNT field-effect transistor, and changes in the membrane potential influenced the measurable capacity between the microelectrode and axon [60]. This method can be used to non-invasively detect cellular activities for electrogenic cells with high throughput, high sensitivity, easy use, and the capacity of long-term cell culture.

Although, electrochemical methods of detection are noninvasive and offer an appropriate temporal resolution, they cannot provide information on specific cellular activities that are directly related to certain cell functions, biomarkers or signaling pathways, which are important to better understanding of cytotoxicity effects and mechanisms of drugs. Moreover, electrochemical methods are not amenable to 3D cell cultures as cell direct contact with the electrode is required. This limits the application of electrochemical methods to 2D cultures, which suffer from many limitations for cell-based assays as discussed earlier.

4.2. Optical Methods

Optical detection in cell-based HTS assays usually is carried out with colorimetric, luminescent, or fluorescent methods, which are discussed below.

4.2.1. Colorimetric Method

Colorimetric methods are based on color change of the growth medium after cell metabolites react with chemical agents. Colorimetric assays using ruthenium dye [61] and Alamar Blue [62] have been developed. However, the low sensitivity of ruthenium dye and the poor reliability of Alamar Blue for the measurement of kinetics limit their applications in the development of HTS platforms. A spectrum of assays using tetrazolium salts such as MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [63], MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) [64, 65], and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) [66] are commercially available. These methods are based on the reduction of a tetrazolium salt by actively growing cells to a colored formazan product that can be quantified with a spectrophotometer.

However, these colorimetric methods require multiple additions of chemicals at prescheduled time points, which interfere or can disrupt the targeted cells. In addition to the invasiveness, these methods are time consuming and laborious. Furthermore, they usually can only provide endpoint data, an intrinsic drawback for *in vitro* cytotoxicity tests. Dynamic data, which can provide more details about the effects of drugs on cells than the endpoint data, is preferred. In addition, in order to realize automation for HTS, conventional endpoint assays require the aids of expensive robotic arms. Also, miniaturization, which is an important factor to achieve HTS, is not compatible with colorimetric methods because of their relatively low sensitivity.

4.2.2. Luminescent Methods

Many organisms, including fireflies and some marine organisms, regulate their light production using luciferase in a variety of light-emitting reactions with emission color ranging from yellow-green to red. In luminescent assays, the oxidation of luciferin catalyzed by luciferase produces light that can be detected by a light sensitive apparatus such as an illuminometer or optical microscope, allowing observation of biological processes [67]. Some luminescent reactions are

mediated by ATP or calcium ions. Luciferase has been widely used as a reporter in cells expressing a luciferase gene under the control of a promoter of interest to assess its transcriptional activity [68]. Many commercial cell-based kinase activity assays use luciferase as the reporter. Luciferase can also be used to detect the level of cellular ATP in cell viability assays. In addition, some enzymes, such as caspase and cytochrome P450, can convert proluminescent molecules to luciferin, and their activities can thus be detected in a coupled or two-step luciferase assay [69].

Although firefly luciferase is widely used in cell-based assays, its application in HTS is usually limited to endpoint assays because the requirements of cell lysis and addition of luciferase substrates. Unlike firefly luciferase, the luciferase (MetLuc) derived from the marine copepod *Metridia longa* is naturally secreted, which allows the development of live cell assays and multiple assays on the same cells using a no-lysis protocol [70]. However, in addition to the amount of luciferase, the bioluminescence intensity can also be affected by many other factors such as luciferin absorption, availability of co-factors, pH, and transparency of culture media or buffer, causing discrepancies between detected bioluminescence signals and actual changes in cellular activity [71].

4.2.3. Fluorescent Methods

Compared to luminescent methods, fluorescent methods have higher sensitivity and are easier to be miniaturized for large-scale or high throughput measurements of cell activities, pathway activation, toxicity, and phenotypic cellular responses of exogenous stimuli [72]. Fluorescent methods for cell-based assays were initially developed using small, highly-fluorescent, organic molecules, monitoring ion concentrations, membrane potential and as intracellular substrates for reporter genes. However, conventional fluorescent molecules usually have narrow excitation spectrum and broad emission spectrum, and are difficult to use for simultaneous excitation and quantification of multi-targets with different emission peaks. In addition, they are photo-unstable and can be quenched by continuous excitation. More recently, nanoparticles such as quantum dots (QDs) have been widely used as labels in cell-based assays. These semiconductor nanocrystals are photo-chemically stable, can provide a narrow and adjustable emission, and can be excited by light of any wavelength shorter than that of the emission peak. Thus, various emission colors can be simultaneously obtained using nanoparticles of different sizes excited with a single-wavelength light [73]. Although QDs can be readily internalized into cells and used as fluorescent labels in cell-based assays [74], they can be toxic to cells. Also, like the luminescence-based and colorimetric methods, they can only be used to quantify cells or cellular activities at the endpoint, which cannot provide dynamic cell proliferation or death kinetics.

The development of reporter gene techniques using green fluorescent protein (GFP) has enabled online, non-invasive detection and quantification of cell proliferation and specific cellular functions. GFP, which was first discovered in the jellyfish, and its mutants have been developed with emission light colors ranging from blue to yellow [28]. GFP is species-independent and generally non-toxic to cells. Its detection can be performed in living samples. Therefore, cell-based assays using GFPs are amenable to real-time, automated, and non-invasive assessment of both chronic and acute cellular events [28]. In addition, GFP can be coupled with Disco soma species red fluorescent protein (dsRed) for two-color or multiplex assays [75].

Cell-based assays using cDNA encoding a fluorescent protein provide an HT platform for non-invasive analysis of cell proliferation and death kinetics. Table **3** shows some cell-specific promoters and reporter genes that can be used in drug screening. Since a specific cellular event or function can be monitored based on the regulatory DNA sequence or promoter used in controlling the expression of the reporter gene, the assay is responsive to targeted effects, such as activation of signal transduction pathways, and is suitable for use in disease-relevant assays. In addition, two fluorescent proteins fused with a peptide linker comprising a caspase-3 cleavage site can be used to study the activation of caspase-3 or apoptosis in live cells [105, 106] based on changes in emission wavelength due to energy transfer between two close fluorophores, a phenomenon called fluorescence resonance energy transfer (FRET).

In general, whole-cell autofluorescence-based systems are non-invasive, fast, and simple for HTS applications. It can provide dynamic data and be used as high-content assays as well. Current commercial HTS systems [107-109] use laser scanning imaging systems with fluorescence microscopy and quantitative image analysis to perform live-cell kinetic assays with high spatial and temporal resolution. They can be used to examine the context of living cells, quantify

Table 3: Some Cell-Specific Promoters and Reporter Genes Used in Drug Screening

Specific lineage	Gene cassette (promoter and related reporter gene)	References
	Heart	
Cardiomyocytes	α-cardiac MHC-G418	[76, 77]
	α-cardiac MHC-LacZ	[78]
	α-cardiac MHC-EGFP	[79]
	MHC-SEAP	[80]
	MLC-2v-EGFP	[81-84]
	MLC-2v-β-galactosidase	[85]
	Cardiac α-actin-GFP, G418	[86, 87]
	Cardiac α-actin-GFP	[88]
	HSVtk/GCV	[89]
	GFP-IRES-PAC	[89]
Chamber myocardium	Cardiac-specific distant upstream part of the Na+/Ca2+ exchanger-EGFP	[90]
	Neural	
Neurons	A necdin promoter-PAC	[91]
Neuroepithelial cell	Sox-1-GFP	[92, 93]
Neural precursor	Thymidine kinase promoter/nestin second intron-EGFP	[94]
Dopaminergic neuron	TH-EGFP	[95]
	TH-GFP	[96]
	Others	
Glioma	p53-Luc	[97]
	p63-Luc	
	p73-Luc	
Vascular endothelium	Tie-1-EGFP	[98]
Endothelium	Flk-1-GFP	[99]
Epithelial	CYP7A1-GFP	[100]
Melanoblast	Dct-LacZ	[101]
Renal progenitor	LacZ/T/GFP	[102]
ESCs (undifferentiated)	OCT4-EGFP	[93, 103]
	Survivin-EGFP	[104]
	Rex-1-GFP	

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

intracellular proteins, and monitor the trafficking of proteins fused with fluorescent reporters and some subcellular structures [110]. However, their high costs and relatively low capacity limit their uses to the late-phase compound characterization [111]. Moreover, these imaging systems are limited to read planar images of cells cultured on 2D surfaces and are not suitable for 3D cell cultures.

Although advanced high-throughput flow cytometry has also been developed for cell sorting and detection [112], it is not amenable for use in cell-based HTS assays because cell sampling from culture wells is intrusive and difficult to implement on a large scale. On the other hand, a fluorescent plate reader or spectrofluorometer commonly used in fluorescence-based assays may not have the sensitivity high enough for use in cell-based HTS assays because of the high background noise from media and relatively weak fluorescent signals from cells cultured in conventional multiwell plates [113]. To overcome this problem, a

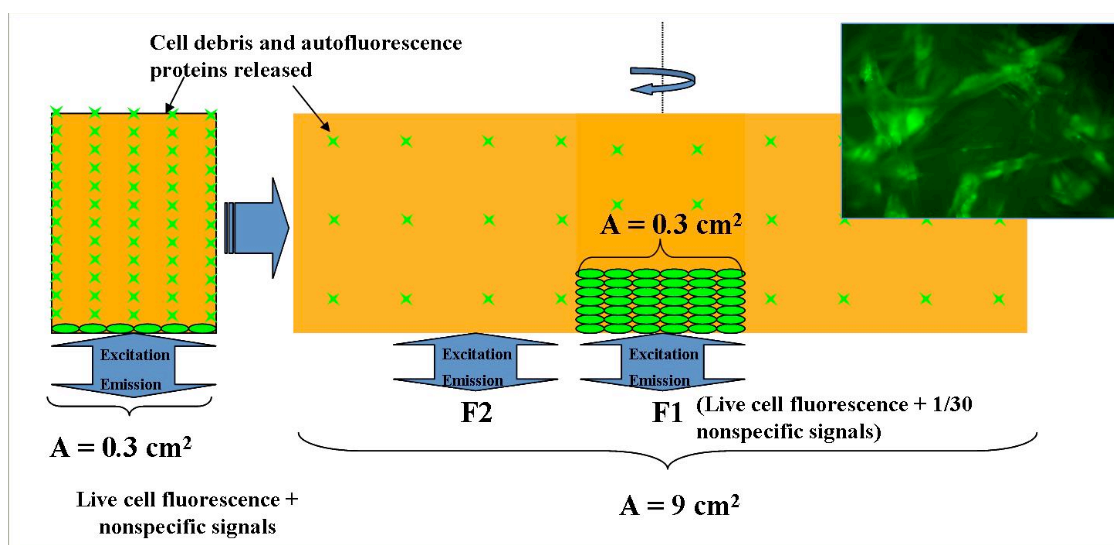

Figure 3: Schematic illustration of culture fluorescence from cells grown on 2D surface and in 3D scaffold in conventional and modified 96-well plates, respectively. Cells in the 3D culture (well dimension: 3 cm × 3 cm; area: 9 cm^2) are concentrated in the scaffold (diameter: 6.2 mm; area: 0.3 cm^2) at the center of the well, whereas in the 2D culture, cells spread uniformly on the bottom surface of the well. Consequently, the noise level in the 3D culture is reduced by a factor of 30 (9/0.3), and thus greatly increasing the signal to noise ratio. The microscopic picture shows GFP-cells in the PET matrix. (Adopted from [113]).

novel 3D culture platform with cells cultured in fibrous polyethylene terephthalate (PET) matrices in modified multiwells was developed, which is discussed in the following section.

5. DEVELOPMENT OF 3D CELL-BASED HTS ASSAYS FOR DRUG DISCOVERY

5.1. 3D Cell-Based Fluorescence Assays

Real-time analysis of cell proliferation based on fluorescence read-outs from a fluorometer was first demonstrated with Chinese Hamster Ovary (CHO) cells expressing GFP under the control of a human cytomegalovirus (CMV) promoter [114]. However, the weak fluorescence signals generated from cells cultured in conventional 96-well plates are hardly accurate by using a fluorometer for quantifying cells because of changes in the culture environment, including pH and other auto-fluorescent components present in the culture medium, including GFP released from dead cells [115]. High, fluctuating background signals from these non-specific effects could effectively mask live-cell GFP signal and render it unreliable or useless for assessing cytotoxicity or cell proliferation.

The aforementioned limitations can be overcome by culturing GFP-expressing cells in a PET scaffold in a modified well (see Figure **3**), which significantly increases the total cell number per unit area and reduces the background noises [113]. Such a 3D culture can provide a 20-fold higher cellular fluorescence and significantly improve signal to noise ratio because cells are concentrated in the scaffold at the center of the well and the background fluorescence can be measured separately and subtracted to give the true live cell signal. This new 3D fluorescent culture platform can give highly reproducible growth kinetic data, which can be more reliably used to assess drug effects on cell proliferation. It has been successfully used to study cytotoxicity effects of chemicals, cancer drugs (see Figure **4**) and Chinese herbal medicines, demonstrating its potential application in early-stage drug discovery.

5.2. Microfluidic Cell-Based Assays

Microfluidcs has emerged as a promising technology with widespread applications in engineering, biology and medicine. It has the potential to revolutionize the way we approach cell biology research. Microfluidics refers to the science and technology that allows one to manipulate tiny amounts (10^{-9} to 10^{-6} liter) of fluids using microstructures with characteristic dimensions on the order of tens to hundreds of micometers. The controllable processing of microfluidic devices at dimensions close to cells and biomolecules enable their biological applications at the cellular level. In addition, the scale of microchannels corresponds well with the native cellular microenvironments, in which the ratio of cell volume to extracellular fluid volume can be greater than one [39]. This paves the way to create a more *in vivo*-like cellular

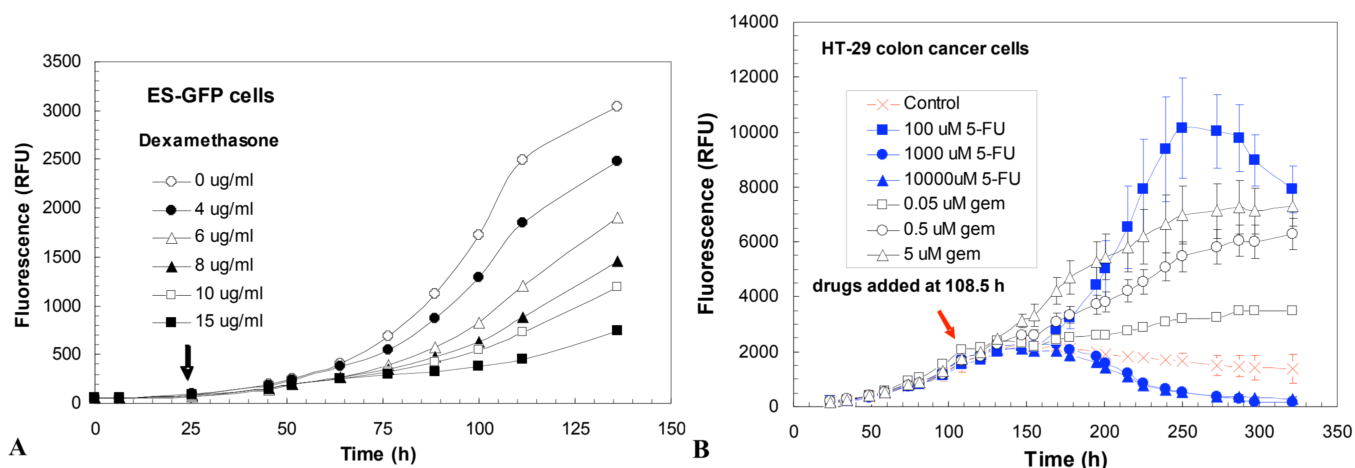

Figure 4: Cytotoxicity effect of drugs assayed with GFP fluorescence in 3D cell cultures. (**A**) Fluorescence kinetics of ES-GFP cells cultured with different doses of dexamethasone added one day after inoculation. (**B**) Effects of 5-fluorouracil (5-FU) and gemcitabine (gem) on HT-29-GFP cells. (Adopted from [113]).

microenvironment *in vitro*. Furthermore, microfluidic systems have the advantages of minute consumption of reagents, short diffusion path for quick reaction and fast analysis, highly paralleled operation, and versatile and precise controls for fluid transport, mixing and concentration manipulations [26]. Equipped with external physical control and online detection mechanisms, microfluidic systems can be fully automated for HTS assays with improved data quality and reduced assay time and cost.

Microfabricated cell culture devices have previously been demonstrated on silicon and polydimethylsiloxane (PDMS) substrates with hepatocytes [116], lung [117], and insect cells [118], but never for the purpose of realizing an integrated assay system. Hung *et al*. for the first time developed a microfluidic system integrated with concentration gradient generator capable of long-term cellular monitoring [119]. The integrated device consists of a concentration generator and a 10×10 array of microchambers (see Figure **5**), which enables performing 100 experiments in parallel with a unique condition in each chamber. The array can be applied to characterize the effect of culture medium components, pH, cell density, and perfusion rate on protein expression.

Most of microfluidic platforms are fabricated using PDMS, which is optically transparent, gas permeable and biocompatible [26, 39]. However, it can undesirably absorb hydrophobic drug molecules due to its hydrophobic property. Su *et al*. (2011) tested cytotoxicity using HEK cells using microchannels made from 2 different plastics, polystyrene (PS) and cyclo-olefin polymer (COP), and silicone elastomer. Their results showed that PS and COP might be more appropriate than PDMS devices when used in hydrophobic drugs selection [120].

The conventional format of microfluidics is not an ideal match for complete cell culture, because all of the reagents and cells are positioned in an interconnected network of enclosed microchannels, making it difficult to establish fresh, sterile sites for seeding new generations of cells. Recently, digital microfluidics (DMF) has emerged as an alternative to the conventional format of enclosed microchannels. DMF is a technique, in which nanoliter-sized droplets are manipulated on an open surface of an array of electrodes. For example, Barbulovic-Nad *et al*. introduced the first lab-on-a-chip platform capable of implementing all of the steps required for mammalian cell culture: cell seeding, growth, detachment, and re-seeding on a fresh surface for complete mammalian cell culture [121].

In addition, conventional mammalian cell culture protocols usually stipulate elevated carbon dioxide and relative humidity. These parameters for microfluidic systems are maintained by placing the whole microfluidic system inside an environmental chamber and using gas permeable materials such as PDMS. Forry *et al*. developed a microfluidic system that allowed on-chip control of the carbon dioxide partial pressure by flowing pre-equilibrated aqueous solution through control channel across the device. The system enabled long-term microfluidic culture of mammalian cells without requiring a cell culture incubator or CO_2-independent media [122]. These pioneering research projects are excellent examples that highlight the

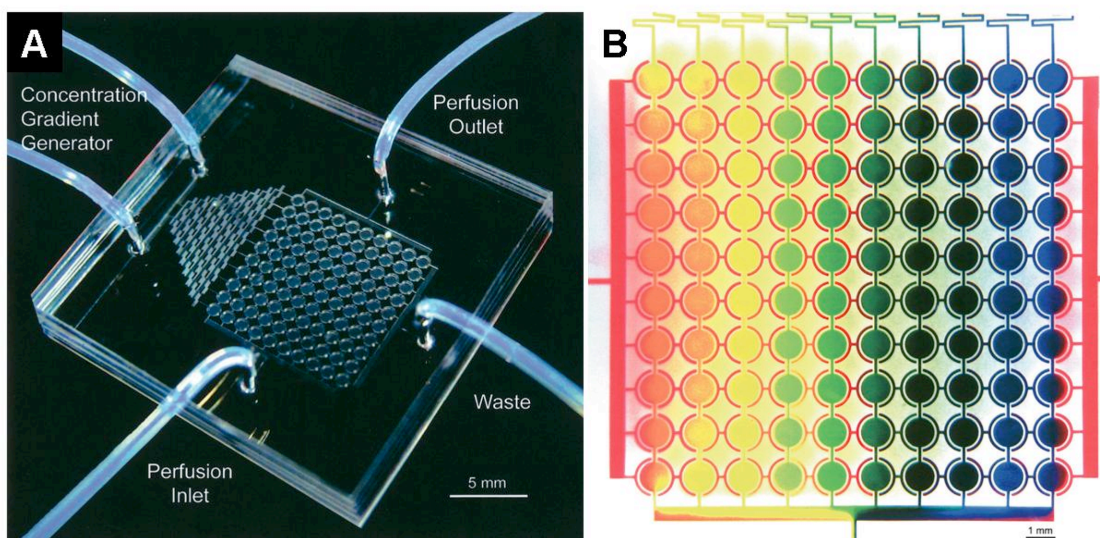

Figure 5: A perfusion microfluidic cell culture array for high-throughput cell-based assays. (**A**) Photograph of the microfluidic cell culture array with concentration gradient generator and 10 × 10 microchambers on a 2 cm × 2 cm device. (**B**) Concentration gradient across 10 columns generated from the concentration gradient generator at the top of the device. All chambers were initially filled with a red dye. Blue and yellow dyes were then loaded from two separate ports at the top of the gradient generator. This array can be used to test drugs at various dosages. (Adopted from [119]).

capacity of microfluidic techniques for improving HTS *via* well-controlled fluid handling and without the need for complex robotics.

Most of the previous studied microfluidic systems, however, rely on 2D cell cultures, which may limit their applications in situations where 3D cell culture is necessary to demonstrate authentic *in vivo* physiology for certain cell types, such as tumor cells, hepatocytes, chondrocytes, neural cells, and ESCs. Drug testing models based on conventional cell culture technique continue to give misleading and non-predictive data for *in vivo* responses. This failure is mainly a result of 2D culture's lack of capacity to mimic *in vivo* microenvironments and unable to preserve their phenotypic characteristics. As discussed earlier, 3D cultures exhibit profound differences from conventional 2-D cultures in cellular functions, morphology, and proliferation and drug responses.

5.3. 3D Microfluidic Cell Cultures

3D microfluidic cell culture systems offer a biologically relevant model to conduct micro-scale cell-based research and applications in drug screening. Various natural and synthetic hydrogels have been incorporated into microfluidic cell culture systems to support cells in 3D. A variety of 3-D microfluidic cell culture models have been developed [123-125]. More recent advances in modeling of the cell microenvironment have focused on the generation of *in vivo*-like ECM constructs for supporting 3D cell growth and examining cell migration. For example, Vickerman *et al*. developed a microfluidic platform capable of mimicking the *in vivo* microenvironments by integrating fluidic microenvironments and 3D microenvironments using microinjection of gel solution containing cells [123]. An open lumen-like structure was formed when human adult dermal micro-vascular endothelial cells were cultured in this microfluidic platform for up to 7 days. In another example, Toh and his group showed that cells could be maintained in 3D structures in a microfluidic channel perfusion microbioreactor with an array of micropillars [124]. Parallel microfluidic channels can be used for multicellular 3D cultures to study cell-cell and cell-ECM interactions in spatially well-defined geometries. Huang *et al*. studied the behaviors of metastatic breast cancer cells and tumor-derived macrophages cultured in adjacent ECM gels *via* real-time imaging [125].

However, real-time quantification of cell proliferation, which can be valuable information in assessing time-dependent cellular responses to drug treatments, could not be easily quantified in the aforementioned systems. Wen *et al*. developed a microbioreactor array (see Figure **6**) that has the capability of perfusion high-density 3D cell culture in modular and low-cost PET fibrous scaffolds [126]. Non-invasive and time-series cell proliferation and cytotoxicity assays can be achieved using a plate reader to monitor the fluorescence emitted from the

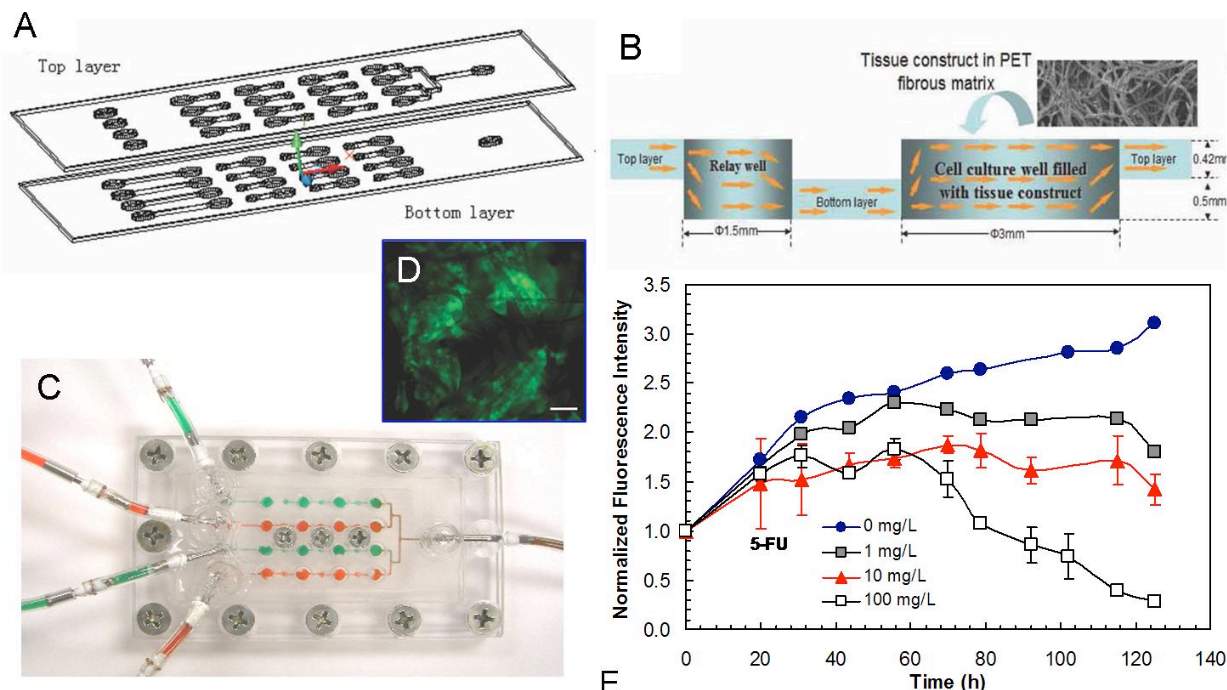

Figure 6. A microfluidic system for perfusion 3D cell culture. (**A**) Schematic drawing of the device composed of top and bottom layers; (**B**) Design of individual cell culture well and relay well formed by two layers, with cell culture well filled with fibrous PET scaffold; (**C**) photograph of assembled device at work with each inlet connected to an external tubing through a flexible connector. Food dyes were used to indicate proper fluid segregation for each line of flow; (**D**) fluorescence microscopic image of live HT-29 cells in the PET fibrous matrix (scale bar indicates 60 μm); (**E**) Kinetics of 3D perfusion cultures of HT-29 cells exposed to various 5-FU concentrations. Fluorescence intensity was normalized against the initial reading of each curve, respectively. (Adopted from [126]).

EGFP-expressing HT-29 cells cultured in the device. With continuous perfusion, cells could be maintained for an extended period, reaching a cell density as high as 6×10^7 cells/mL matrix. Such a perfusion culture system would be useful in assessing long-term drug effects on cells in a 3D environment.

6. CELL-BASED HTS IN COMMERCIAL DRUG DEVELOPMENT

The rapid progress in combinatorial chemistry, genomics, proteomics, and bioinformatics has led to a significant increase in the number of potential therapeutics, which has also spurred the development of HTS for lead identification and optimization. Over the past two decades, HTS has emerged and maturated as a platform in the early stage of drug discovery in the pharmaceutical industry. There has been a growing trend in drug discovery to perform lead identification and optimization using cell-based assays because they can provide more relevant physiological information than biochemical assays. Cell-based assays are emerging as the preferred tools for screening potential drug compounds. Pharmaceutical and biotechnology companies are gradually replacing other *in vitro* and biochemical assays with cell-based assays in drug discovery. Today, cell-based HTS assays represent approximately more than half of all high throughput screens currently performed, and their applications have been an integral component of drug discovery.

One successful example of cell-based HTS in drug discovery is the commercialization of Eltrombopag (Promacta/Revolade; GlaxoSmithKline), which is a thrombopoietin (TPO) receptor agonist approved by the FDA in 2008 [127]. This compound was selected out of about 260,000 compounds using a cell-based luciferase reporter system. This screening system was based on a stable TPO-responsive cell line, BAF-3/TPO-Rluc, which was obtained by transfecting murine hematopoietic progenitor cells (BAF-3) with a human TPO receptor (hTPOr) cDNA and a luciferase reporter gene [128]. Luciferase expression was controlled under a synthetic STAT-responsive promoter, which can be regulated by TPO. Therefore, this luciferase system can be used for high throughput screening of TPO modulators. SB-497115 (Eltrombopag) was selected using this system as a candidate for clinical studies and then was commercialized in 2008 for its maximal efficacy of TPO in the proliferation of BAF-3/TPO-R cells and the

increase of the amount of $CD41^+$ (marker of megakaryocyte differentiation) cells [129-132].

Another successful example is BMS-790052 hepatitis C virus (HCV) NS5A (Bristol-Myers Squibb), a clinical candidate for the inhibition of hepatitis C virus replication [127]. The original cell-based HTS system was developed by O'Boyle II *et al*. in 2005 [133]. This system utilized a mixture of HCV and bovine viral diarrhea virus (BVDV) cell lines, isolated from human hepatocarcinoma Huh-7. In such a system, parameters including cytotoxicity, the replication of HCV replicons and the amount of active luciferase expressed from BVDV replicon cells were used to evaluate the potency and specificity of the investigated HCV replicon inhibitor [133]. Cytotoxicity was determined using Alamar blue dye, while the replication of HCV replicons was estimated using the amount of NS3 protease activity. Lemm *et al*. employed this cell-based replicon screening method and targeted a potential candidate named BMS-824 among a group of HCV inhibitors [134]. Finally, BMS-790052 was developed and selected as a clinical candidate for the inhibition of hepatitis C virus replication [135].

Cell-based HTS has also made contributions to cancer chemotherapy. One example is the development of Bortezomib (Velcade®, formerly known as PS-341), which was approved by FDA in 2003 for the treatment of myeloma. It took only 8 years from initial screening to FDA approval, making Bortezomib the most rapidly developed new anticancer drug in recent history. Bortezomib along with other related peptide boronic acids were screened using the NCI60 cell line. Parameters such as GI_{50} (50% growth inhibition, relative to no compound), TGI (total growth inhibition), and LC_{50} (50% lethality) were used as cellular responses to drugs for the evaluation of drug efficacy [136].

7. CELL-BASED ASSAYS FOR SCREENING PHYTOCHEMICALS

Cell-based HTS can also be applied to evaluate cellular responses to the stimuli from toxic or growth promoting phytochemicals. Like a gift from Father Nature, numerous natural products from plant food and traditional medicine have been found to be health promoting. Phytochemicals, as nonnutrient plant chemicals that contain protective, disease-preventing compounds, have become an important focus in plant research. It is necessary, however, to understand how phytochemicals can be involved in various biological activities before the full beneficial potential can be reached with any well characterized recipe for either food supplements or clinical treatments. In general, plant chemicals can be categorized into several major types: terpenoids, phenolics, alkaloids, fiber and other nitrogen-containing plant constituents [137, 138]. Phytochemicals can provide many health benefits as: (1) substrates for biochemical reactions; (2) cofactors of enzyme reactions; (3) inhibitors of enzymatic reactions; (4) absorbents/sequestrants that bind to and eliminate undesirable constituents in the intestine; (5) ligands that agonize or antagonize cell surface or intracellular receptors; (6) scavengers of reactive or toxic chemicals; (7) compounds that enhance the absorption and or stability of essential nutrients; (8) selective growth factors for beneficial gastrointestinal bacteria; (9) fermentation substrates for beneficial oral, gastric or intestinal bacteria; and (10) selective inhibitors of deleterious intestinal bacteria [138]. Specifically, ample research evidence indicates that the health benefits of phytochemicals can be shown in their roles against various major health-threatening diseases, including cancers, coronary heart disease, diabetes, hypertension, inflammation, microbial, viral and parasitic infections, psychotic diseases, spasmodic conditions, and ulcers, etc.

Take cancer prevention for example, Steinmetz and Potter [139] reviewed 206 human epidemiological studies and 22 animal studies to show the positive relation between vegetable and fruit consumption and risk reduction of cancer. The benefits have already been evidenced for cancers of the stomach, esophagus, breast, lung, oral cavity and pharynx, endometrium, prostate, pancreas and colon. Different mechanisms may be involved in the efficacy of different phytochemicals in preventing cancers. Although many of them are still largely a black box, we may glean some knowledge of the representative mechanisms unveiled in the literature. The known mechanisms related to inhibition of mutagenesis include antioxidant activity, alteration of biotransformation enzyme activity, and antibacterial and antiviral effects [140]. Mechanisms that affect the cancer proliferation and progression include alteration of immune function, reduction of inflammation, modulation of steroid hormone concentrations and hormone metabolism, arrest in cell cycle progression, and stimulation of apoptosis. For instance, cell cycle arrest is an effective and important way of inhibiting cancer proliferation. In one study, terpenoids γ-tocotrienol, mixed isoprenoid, and β-ionone, suppressed the growth of both human

and murine tumor cell lines *via* initiating apoptosis and arrest of cells in the G1 phase in the cell cycle [141]. Soy isoflavones, e.g. genstein, genistin, daidzein and biochanin A, in another study, induced G1/M phase cell cycle arrest in murine (MB49 and MBT2) and human (HT1376, UMUC3, RT4, J82 and TCCSUP) bladder cancer cell lines, evaluated by flow cytometry [142]. Triterpenoid saponins extracted from soybeans, at physiologically relevant doses, could suppress HCT-15 colon cancer cell proliferation through S-phase cell-cycle delay [143]. In another study, starting from the findings that antitumor activities of various wheat cultivars were significantly different, even when the wheat fiber content was equal [144], Qu *et al*. [145] found that lignans, a group of diphenolic compounds present in the outer layers of grains, were capable of arresting colon cancer SW480 cells at S phase. Resveratrol, a triphenolic stilbene present in grapes and other plants, was used to treat six human cancer cell lines (MCF7, SW480, HCE7, Seg-1, Bic-1, and HL60), and most of the cells were arrested in the S phase of the cell cycle [146]. Another phytochemical of considerable interest is butyric acid [138], which is a short chain fatty acid from the digestion of fibers. Butyric acid was found to induce cell cycle arrest, differentiation and apoptosis of colonic epithelial cells and tumor cells *in vitro*.

However, there are some traditional tonic and herbal medicine plants, e.g. ginseng, found to exhibit ambiguous effects on cancer patients. Much testing has been done in humans to explore ginseng's purported antifatigue properties, but this area remains controversial [147]. One *in vitro* study [148], where cell-based reporter gene system was established, indicated that ginseng alone promoted the expression of CYP1A1, one P450 enzyme, which may profoundly influence drug-drug interactions, carcinogen activation and drug detoxification, and the high expression of this enzyme was also observed when ginseng was applied in the presence of TCDD, one tumor promoting dioxin, in sharp contrast to kava, which was a potent antagonist to the gene expression induced by the dioxin. In addition, there are ~13,000 medicinal substances used in China and Japan, and over 100,000 medicinal recipes recorded in the ancient literature [149, 150]. The compositions and concentrations of chemical compounds present in traditional Chinese medicines (TCM) vary with plant species, geographic area, harvest time, and storage [151], making the screening of TCM a very complex and laborious task using conventional drug screening methods.

In summary, the rapidly expanding discovery of phytochemicals with protective and disease-preventing and therapeutic effects has increasingly important impacts on human health and food and pharmaceutical industries. However, important mechanistic questions of biological activities need to be answered before the full beneficial potential can be reached with any well characterized recipe for either food supplements or clinical treatments. Phytochemicals have been extensively reported to have an effect on at least four of the leading causes of death in the United States: cancer, diabetes, cardiovascular disease, and hypertension [152]. Toxicity, one of the most important properties of any chemical that is used by humans, also needs to be examined for phytochemicals. This is not only true for toxicity in general; it may also be the working feature for certain phytochemicals to be effective in treating cancers. However, on one hand, *in vivo* toxicity examinations rely on animal experiments, which are expensive, ethically provoking, devoid of interaction mechanism, and most importantly, difficult to extrapolate to human responses. On the other hand, *in vitro* experiments, especially cell culture systems, have been extensively used for cytotoxicity studies. Nevertheless, there are some major problems that a static multiwell plate culture can hardly overcome. As discussed earlier in this review article, cells cultured on 2D surfaces may be a cause large enough to camouflage the authentic responses *in vivo*, where cells exhibit morphology and interact with each other in a 3D milieu. In addition, a relatively long-term response to phytochemicals can rarely be measured in a consistent medium due to its metabolic-waste accumulation in the static culture. Another disadvantage is that the growth inhibition is usually judged at an arbitrary point of time, lacking a dynamic long-term monitoring. Although this may be explored with multiple parallel wells for the same culture, variations between individual wells and waste accumulation may interfere with the results. In this context, 3D fluorescent cell-based assays using microfluidics can provide a better HTS platform for evaluating and discovering phytochemicals with scientific data to support their health claims.

8. CONCLUSIONS AND PERSPECTIVES

Conventional animal tests for studying the biological effects of drugs and phytochemicals including toxins and herbal compounds are expensive and may be obscured with the actual effects by various other factors. The animal experiment is also time consuming and may impose a barrier for social acceptance. A fast,

sensitive and reliable method that can be used to quickly and reliably screen potential biological effects of the vast amount and variety of drug candidates and phytochemicals is thus needed in the development of new health-promoting compounds and cancer-fighting drugs. Conventional 2D static cell culture systems widely used in current drug discovery campaign have many inherent limitations for proliferation and cytotoxicity studies. The newly developed fluorescent cell-based 3D culture systems are fast, sensitive, and physiologically relevant, and can be used more effectively to bridge the gap between biochemical assays and animal tests. It can save time and cost in the drug screening process. Furthermore, microfluidic microbioreactor array system, incorporating recent advances in tissue engineering, microfabrication and microfluidics, can be used to culture stem cells and carcinoma cells in 3D fibrous or microfabricated scaffolds. Such microfluidic systems operated with continuous perfusion can be used for long-term study of drugs in an *in vivo* like 3D environment and flow fields. The use of fluorescent cells in the assay also allows for real-time, non-invasive monitoring of cellular responses to drugs and dynamically changing environments. A microfluidic system can also provide on-chip serial dilutions to generate various concentrations and combinations of multiple drugs to be tested simultaneously on a single chip. In addition, it is also possible to culture multiple cell types in different but interconnected chambers or channels to evaluate cell-cell and cell-environment interactions on a microfluidic chip, providing the biosystem-level drug responses that can only obtained in animal tests so far. Microfluidic 3D cell-based HTS assays thus can enable the development of *in vitro* models for studying specific diseases. Such *in vitro* models may replace animal models and be used more effectively in the exploration of new drugs including phytochemicals for their therapeutic and health benefits. This could revolutionize the drug discovery process in the near future.

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