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Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices

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ABSTRACT

Culture of cells using various microfluidic devices is becoming more common within experimental cell biology. At the same time, a technological radiation of microfluidic cell culture device designs is currently in progress. Ultimately, the utility of microfluidic cell culture will be determined by its capacity to permit new insights into cellular function. Especially insights that would otherwise be difficult or impossible to obtain with macroscopic cell culture in traditional polystyrene dishes, flasks or well-plates. Many decades of heuristic optimization have gone into perfecting conventional cell culture devices and protocols. In comparison, even for the most commonly used microfluidic cell culture devices, such as those fabricated from polydimethylsiloxane (PDMS), collective understanding of the differences in cellular behavior between microfluidic and macroscopic culture is still developing. Moving in vitro culture from macroscopic culture to PDMS based devices can come with unforeseen challenges. Changes in device material, surface coating, cell number per unit surface area or per unit media volume may all affect the outcome of otherwise standard protocols. In this review, we outline some of the advantages and challenges that may accompany a transition from macroscopic to microfluidic cell culture. We focus on decisive factors that distinguish macroscopic from microfluidic cell culture to encourage a reconsideration of how macroscopic cell culture principles might apply to microfluidic cell culture.

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1. Introduction

Microfluidics refers to a set of technologies for the manipulation of small fluid volumes (μL , nL , pL), within artificially fabricated microsystems (Whitesides, 2006). Microfluidic systems enable

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generic and consistent miniaturization, integration, automation and parallelization of (bio-)chemical processes (Mark et al., 2010). The application of microfluidics to biology and medicine has led to a diversity of new research directions (Melin and Quake, 2007; Yeo et al., 2011), some of which have had significant impact (Sackmann et al., 2014). Cell culture refers to the maintenance and growth of cells in a controlled laboratory environment. Such in vitro cell culture models are the mainstay of experimental cell biological research. Microfluidic cell culture attempts to develop devices and techniques for culturing, maintaining, analyzing and experimenting with cells in micro-scale volumes (Meyvantsson and Beebe, 2008).

Understanding the interplay between critical cell culture parameters and the microenvironmental conditions created by microfluidic devices will accelerate the development of microfluidic cell culture technology (Sackmann et al., 2014). Some important aspects of microfluidic cell culture systems have previously been reviewed, including the effect of surface modification on cellular behavior (Zhou et al., 2012), cell biology (Paguirigan and Beebe, 2008; Salieb-Beugelaar et al., 2010), cell culture models (Meyvantsson and Beebe, 2008), cellular analysis (Park and Shuler, 2003; Yeo et al., 2011), cellular microenvironment (Meyvantsson and Beebe, 2008; Young and Beebe, 2010), cell secretion (Huang et al., 2011), chemotaxis (Kim and Wu, 2012), apoptosis (Wlodkovic et al., 2011), vascular function (Wong and Chan, 2012), neuroscience in general (Soe et al., 2012), in particular neuron culture (Millet and Gillette, 2012) and development (Millet and Gillette, 2012), single cell resolution metabolomics (Rubakhin et al., 2011), population transcriptomics (Plessy et al., 2013), lab-on-chip platforms (Mark et al., 2010; Ni et al., 2009), large-scale integration and biological automation (Melin and Quake, 2007), micro total analysis systems (Kovarik et al., 2012), drug research (Wu et al., 2010), cellular separations (Bhagat et al.,

2010), stem cell biology (Wu et al., 2011), system biology (Breslauer et al., 2006), bioreactors (Pasirayi et al., 2011), three dimensional cell culture (Haycock, 2011), tissue engineering (Inamdar and Borenstein, 2011), and efforts toward organs-on-chip (Huh et al., 2011).

Complementing the aforementioned reviews, the present review is aimed at researchers familiar with conventional/macroscopic cell culture, who are considering microfluidic cell culture for the first time. This review focuses on the practicalities of microfluidic cell culture and some advantages it may hold over macroscopic cell culture, but also the challenges that may accompany the culture of cells using a microfluidic device. Decisive factors are discussed that distinguish macroscopic from microfluidic cell culture. The overall aim is to give the reader a better understanding of the rewards and challenges that microfluidic cell culture can bring.

2. Advantages of microfluidic cell culture

Microfluidic cell culture has significant advantages over macroscopic culture, that is, culture in flasks, dishes and well-plates. Fig. 1 describes the most significant advantages and challenges when using macroscopic versus microfluidic cell culture. There is great flexibility in the design of microfluidic devices, which can be tailored to the needs of individual cell types and cellular co-cultures can be implemented on the same chip (Yeo et al., 2011). The advantages of microfluidic cell culture include the ability to more closely mimic a cell's natural microenvironment, for example by continuous perfusion culture or by creating chemical gradients, and to study low numbers of cells or single cells in high temporal and/or spatial resolution via automation, parallelization, on-chip analysis or direct coupling to downstream analytical chemistry

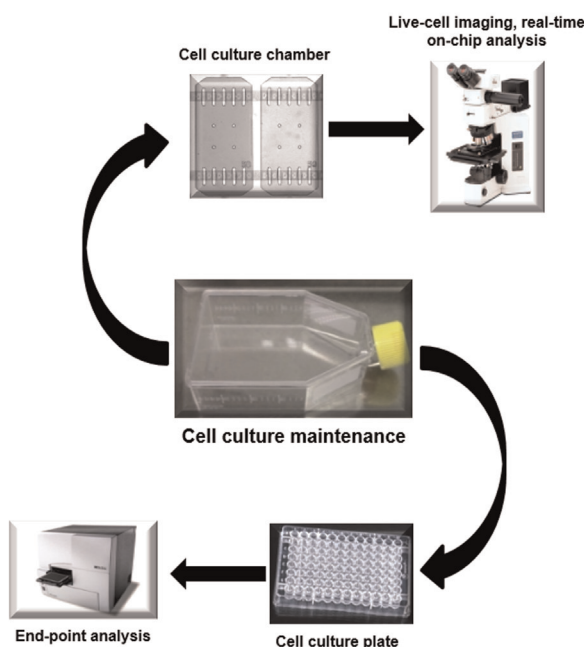
Macroscopic cell culture

Typical advantages

- Established culture material
- Standardized measurement of pH, CO₂, and O₂
- Established culture protocols
- Standardization and availability of assays
- Ability to scale up a single experiment

Typical challenges

- Rigid culture surface
- Fixed device architecture
- High reagent consumption
- Perfusions and chemical gradients are difficult to achieve
- Stagnant culture media
- Mainly end-point analysis



Microfluidic cell culture

Typical advantages

- Flexibility of device design
- Experimental flexibility & control
- A low number of cells is sufficient
- Single cell handling
- Real-time, on-chip analysis
- Automation
- Direct coupling to downstream analysis systems
- Ability to perform perfusion culture
- Controlled co-culture
- Reduced reagent consumption

Typical challenges

- Non-standard culture protocols
- Novel culture surface (e.g. PDMS)
- Small volumes, challenging subsequent analytical chemistry
- Complex operational control and chip design

Fig. 1. Overview of advantages and challenges of both macroscopic and microfluidic cell culture.

Table 1
Comparative analysis of advantages on reported studies using microfluidic cell culture (Poss=possible, N/C=Not Clear).

Advantages	Migration mechanisms		Biochemical stimulation		Metabolic analysis		Toxicity assay		High throughput		Diverse cell types		Cell-cell interactions		
	Huang et al. (2011)	Vedel et al. (2013)	Bianco et al. (2012)	Biffi et al. (2012)	Croushore et al. (2012)	Shintu et al. (2012)	Gao et al. (2012)	Cooksey et al. (2011)	Gomez-Sjoberg et al. (2007)	Lecault et al. (2011)	Grossmann et al. (2011)	Antia et al. (2007)	Zheng et al. (2012)	Hong et al. (2012)	Ramadan et al. (2013)
Low sample/reagent usage	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Experimental resolution	Yes	Yes	Yes	Yes	Yes	N/C	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Flexibility of device design	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Single cell handling	Yes	Poss	Poss	No	No	No	Poss	Poss	Poss	Poss	No	Poss	Poss	Yes	No
High experimental control	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Poss	Yes	Yes	Yes	Yes
On-chip analysis	Yes	Yes	Yes	Yes	N/C	Yes	Yes	Yes	Yes	Yes	Yes	Poss	Yes	Yes	Yes
Real-time data acquisition	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Culture under perfusion	N/C	Poss	No	Yes	Poss	Yes	Poss	Yes	Poss	Poss	Yes	Yes	Yes	Yes	Yes
Downstream analytical tools	N/C	Poss	Yes	Yes	Yes	N/C	Yes	Poss	Poss	N/C	Poss	N/C	N/C	N/C	N/C
Automation	Yes	Yes	Poss	Poss	Yes	Poss	Poss	Yes	Yes	Yes	Yes	Poss	Yes	Yes	Yes
High throughput capabilities	No	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Poss
Co-culture with other cells	No	Poss	Yes	No	No	No	No	No	Poss	No	No	Poss	Yes	Yes	Yes

platforms. At the same time, microfluidic cell culture offers reduced consumption of reagents, reduced contamination risk and efficient high throughput experimentation. Table 1 presents a comparative analysis of the advantages of microfluidic cell culture and selected publications that exploit those advantages.

Macroscopic cell cultures typically contain 10^4 – 10^7 cells, with fluid measurements representing the average over a large group of cells. This inevitably evens out some of the inherent heterogeneity within a cell population. Microfluidic cell culture devices bring the cell population down to a few hundred cells, or even a single cell, making it possible to capture perturbations to individual cells, increasing the spatial and temporal resolution for a given experimental setup. For instance, the thermodynamic, kinetic and mechanical characteristics of cell locomotion (protrusion, attachment and translocation) can be better understood with experiments performed at single cell resolution (Lauffenburger and Horwitz, 1996; Nishimura et al., 2009). Macroscopic methods used to study cell migration processes include the Boyden chamber and scratch or wound-healing assays (Liang et al., 2007). These methods lack the single cell resolution required to better understand the process of cell locomotion. These methods are relatively easy to set up and can somewhat reflect the migratory behavior of cells in vivo when performed within a live-cell imaging station. However, they are time consuming, larger amount of cells are required, and chemical gradients cannot be established (Liang et al., 2007; van der Meer et al., 2010).

Microfluidic cell culture offers an alternative to macroscopic methods to study cell migration processes and their mechanisms at single cell resolution. Taking into consideration the advantages of design flexibility, the ability to handle single cells for experimentation, real-time on-chip analysis via time-lapse microscopy and low reagent consumption. Huang et al. (2011) developed a compartmentalized microfluidic cell culture device which resembles the physiological environment of migrating cells. They characterized cellular locomotion mechanisms and cell morphology during brain tumor stem cell migration by resolving the behavior of individual cells. For instance, migrating stem cells display morphological polarization, membrane extension, formation and stabilization of attachments, contractile force and traction, and release of attachments. This platform can be tailored to study migration at the single cell level, providing superior experimental resolution over macroscopic cell migration assays, such as the wound-healing assay.

Microfluidic cell culture devices also make it feasible to study complex cellular behavior, like the relationship between single cell movements and collective cell migration. For example, Vedel et al. (2013) studied the role played by collective cellular interactions on cell motility at different cellular densities within a microfluidic device. Single cell locomotive behavior (straight lines, curved paths and short distances with no directionality), speed distribution and pseudopodia formation were quantified. By capturing sufficient data on the locomotion of individual cells within culture chambers with independently varied conditions, these authors developed a mathematical model to predict the role of social interaction in motility.

Microfluidic devices offer the advantages of precise control over experimental conditions via custom designed chip architectures, parallelization, automation, and direct coupling to miniaturized downstream analysis platforms. This, so-called lab-on-a-chip, versatility has been exploited in neuroscience research, mainly for studies concerning cellular and molecular neurobiology, cellular electrophysiology and neurodegenerative diseases (Soe et al., 2012). In comparison with macroscopic neuronal cell culture methodologies like hanging drop, Carrel flask, slide chamber, Campenot chamber and brain slice chamber, the chemiotemporal and spatial control over the cellular microenvironment is limited

(Millet and Gillette, 2012; Kovarik et al., 2012). Microfluidic cell culture can overcome some of these drawbacks as it is possible to culture networks formed by small numbers of neuronal and non-neuronal cells seeded in prescribed patterns. This allows for more control over the extracellular microenvironment, monitoring of communication between cells and spatiotemporally localized stimulation. For instance, Bianco et al. (2012) developed an overflow microfluidic network system, operated in open and closed configurations, to culture primary neurons. This system was used to study the influence of astrocytes, derived from different regions of the brain, on the viability of neurons precisely supplied with stimulant molecules. Immunocytochemical staining, quantitative intracellular calcium imaging and electrophysiological recording were also integrated into the system. In order to study biochemical stimulation of neuronal networks, Biffi et al. (2012) developed a microfluidic system consisting of a dual channel configuration with micro chambers for the culture and drug stimulation of spatially and temporally controlled neuronal networks. This device reduced the experimental variability and the time of experimentation, bringing considerable improvements over macroscopic methods. Recently, Robertson et al. (2014) developed a microfluidic device to culture primary hippocampal neurons in adjacent chambers that were individually fed through inlet and outlet ports and synaptically connected via microchannels through a barrier that prevented exchange of extracellular fluid between the two chambers. Using calcium imaging, they measured electrophysiological communication between neurons in separate chambers in response to stimulation of neurons in one chamber with KCL and glutamate, revealing how the activity of one hippocampal neuronal network is modulated by changes in the activity of a second network.

An advantage of microfluidic cell culture is the ability to incorporate analytical biosensors into the culture platform, thus combining living cells and sensors for detection of cellular physiological parameters and analysis of external stimuli in situ, in a non-invasive way (Liu et al., 2014). These biosensors can provide rapid and sensitive analysis based on a small number of cells and low reagent volumes. In metabolomics, highly reproducible quantification is desired (Verpoorte et al., 2010). Metabolomic protocols for macroscopic cell culture samples require several experimental steps that are usually completed as separate operations. Sample preparation requires efficient cell lysis and optimal analyte extraction with minimal dilution. Sample measurement requires high resolution separation techniques and sensitive detection. Microfluidic systems have the potential to integrate cell culture with the aforementioned analytical chemistry on a single device, thereby increasing reproducibility (Yeo et al., 2011; Rubakhin et al., 2011). For instance, Croushore et al. (2012) assembled a microfluidic system to culture bag cell neurons in a controlled microenvironment. Cells were stimulated with precise doses of potassium chloride and insulin, and released molecules were collected with minimal dilution. The system was coupled to an off-line mass spectrometer for neuropeptide characterization. Ges and Baudenbacher (2010) embedded lactate sensing electrodes within a microfluidic cell culture system, creating a biosensor to evaluate anaerobic respiration in living fibroblasts. Recently, Shintu et al. (2012) integrated microfluidic cell culture with metabolic profiling to investigate the potential of metabolic footprinting to characterize the response of “bio-artificial organs” to various small molecules, an approach which may have the potential to be used for the testing of toxicological responses in vitro.

Some microfluidic cell culture devices incorporate in situ separation columns, which are directly coupled to mass spectrometry, or use electrochemical sensors and other technologies as analytical tools. For instance, Chen et al. (2012) developed a microfluidic system combining cell culture with stable isotope

labeling-assisted electrospray ionization mass spectrometry. This system consists of a microfluidic network for reagent supply, cell culture chambers and on-chip separation micro columns for sample pre-treatment, preceding analyte detection using mass spectrometry. This system was used to study drug-induced apoptosis and perform quantitative measurements of cell metabolism in MCF-7 cells. A similar approach was used by Gao et al. (2012). This group created a microfluidic cell culture system to validate and perform studies on the absorption of methotrexate and its effects on HepG2 and Caco-2 cells (heterogeneous human epithelial colorectal adenocarcinoma cell line), using electrospray ionization quadrupole time-of-flight mass spectrometry. To evaluate the performance of a cell based toxicity assay using a microfluidic cell culture system, Cooksey et al. (2011) studied the repeatability with the same device, the reproducibility between devices, and the robustness of the microfluidic assay to variations in cell density. They used real time quantitative fluorescence imaging to measure green fluorescent protein decay due to inhibition of ribosome activity by cycloheximide. They found that assays performed in microfluidic devices showed comparable results to macroscopic culture assays and that microfluidic assays generally showed higher levels of confidence. In another study, Sugiura et al. (2010) used a serial dilution microfluidic network to characterize dose drug response of HeLa cells challenged with a mitotic inhibitor (paclitaxel) in a microfluidic system. Electrochemical methods have also been used in the detection of cellular responses in microfluidic cell culture. Ges et al. (2012) used chromaffin cells cultured in a microfluidic biosensor to quantify catecholamine release using iridium oxide films on platinum electrodes. Cao et al. (2014) created a microfluidic cell culture perfusion chip integrated with electrochemical sensing electrodes allowing non-invasive and accurate estimation of proliferation and apoptosis of HeLa cell in 3D culture in response to anticancer drugs. To evaluate the migratory properties of breast cancer cells, Nguyen et al. (2013) developed a microfluidic device integrated with cell-sensing impedance measuring electrodes. This device allowed rapid and sensitive detection of cell adhesion, cell spreading and cell migration at the single cell level in 2D and 3D cultures, eliminating the need for time consuming, large cell population based experiments such as the Boyden chamber. All these research endeavors exemplify some advantages in analytical control and the system design flexibility that microfluidic lab-on-a-chip offers when compared with macroscopic approaches to perform the same type of studies.

Most macroscopic high throughput cell culture settings depend on microtiter plates, together with liquid handling devices for the delivery of cells and reagents. This increases reagent consumption and the labor required for a given experiment. The possibility of fabricating miniature devices with complex fluidic architectures together with the flexibility of parallelization and automation, allows for implementation of high throughput cell culture, thus reducing the reagent consumption and labor costs. Considering these advantages, Zhou et al. (2012) developed a liquid pipet chip (microfluidic liquid handler system), for the automated delivery of nanoliter amounts of liquid to culture cells for high throughput screening applications. This device allowed the delivery and change of reagents for cell manipulation experiments in a 96 micro-well format and could be integrated to a microscope for real time data acquisition. Gomez-Sjoberg et al. (2007) developed a microfluidic cell culture system with 96 individually addressable chambers. This highly automated platform allows the surface coating treatment with extracellular matrix (ECM) proteins of each individual culture chamber in the microfluidic chip. A sequential cell loading mechanism permits control over the number of cells to load into each chamber, as well as the specific formulation of reagent composition for each individual chamber. It is possible to seed several cell lines without cross-contamination. Cell viability

and data acquisition via time-lapse microscopy can be maintained for weeks. This platform is ideal for use in cell culture experiments requiring a high number of different culture conditions. Going a step further on throughput capabilities, Lecault et al. (2011) developed an iso-osmotic perfusion microfluidic cell culture device, with a non-perturbing cell-capture mechanism that uses gravity to trap cells and automated medium exchange. This 1600 chamber microfluidic cell culture device is capable of keeping the desired osmolarity in the culture chamber. Furthermore, non-adherent cells are immobilized during medium exchange and viable cells are recovered. Such devices are envisaged to be of use in colony growth and variability studies, drug-response screens and other applications that require high throughput cell culture.

Utilizing the flexibility and ease of prototyping PDMS, it is possible to design, mold and fabricate microfluidic systems with several advantages over macroscopic systems. For instance, systems capable of sustaining a variety of cell types, under perfusion or statically, with control over environmental parameters and real-time data acquisition at single cell resolution. The study of root metabolism in plants has been limited by the availability of more versatile macroscopic root/cell culture methods, suitable experimental systems, the underground nature of this organ and reliable methods for data acquisition. Grossmann et al. (2011) developed a generic microfluidic platform integrated with a live-cell imaging station, "RootChip", to study root cell physiology, growth, nutrient uptake, root metabolism and signaling in *A. thaliana* roots. This microfluidic system was specifically designed for plant culture applications where cell-resolved assays can be performed. Perfusion culture at scales typical of microvasculature would be a challenge with macroscopic culture. Antia et al. (2007) replicated physiological flow conditions, within a microfluidic device, to study the cytoadherence and rheological responses of infected red blood cells to purified ICAM-1 and CD36. Golchin et al. (2012) cultured single mycobacterial cells in a microfluidic system to measure the molecular mechanisms of stochasticity of bacterial persistence using time-lapse microscopy and omics analyses.

Macroscopic co-culture of cells either relies on layering different cell types on top of each other or the use of a permeable membrane to keep cells physically separated, while allowing transmembrane communication (Miki et al., 2012; van Moorst and Dass, 2011; Burguera et al., 2010). These macroscopic methods of co-culture are being surpassed by more sophisticated microfluidic platforms, which allow experiments to be performed using different types of cells at the single cell level (Chiang et al., 2013). For instance, to quantify cell–cell interactions mediated by soluble factors, Zheng et al. (2012) developed a fully automated microfluidic cell co-culture system where migration of co-cultured HeLa and HUVEC cells was monitored at single cell resolution. Hong et al. (2012) achieved the co-culture of single-cells that were paired in single chambers, by using variable fluidic resistance and a sequential cell trapping mechanism. The microfluidic chip contains 340 single-cell culture chambers that provide a well-controlled physiological microenvironment to study intercellular communications at a single cell level.

While the physiological architecture of human organs currently exceeds the complexity of all in vitro culture systems, microfluidic cell culture devices can be fabricated that capture some of this architectural complexity. For instance, Ramadan et al. (2013) developed a gastrointestinally motivated microfluidic system capable of co-culture of Caco-2 cells and U937 cells (macrophage-like). This system allows monitoring the response of immune cells to pro-inflammatory stimuli under fluid flow conditions. Ramadan et al. envisage this to be used as an in vitro model to study the absorption of nutrients and immune-modulatory functions in the human gastrointestinal tract. Lee et al. (2013) recently developed a three dimensional microfluidic platform to study hepatocyte-

Table 2
Comparison of key parameters between macroscopic and microfluidic cell cultures.

Parameter	Macroscopic culture (Polystyrene)	Microfluidic culture (PDMS)
Physical properties	Transparent Stiff Low gas permeability	Transparent Soft, flexible Very high gas permeability
Cell numbers	Little or no absorption of molecules Few thousand (microtiter plate) to tens of millions (large culture flask).	Absorbs small hydrophobic molecules (Wang et al., 2012). Single cell (Zheng et al., 2012) to a few thousand.
Volume densities	Flasks, dishes and well plates generally have 2–4 μL of medium and 100–1000 cells per mm^2 of growth surface.	Varies between microfluidic devices but can be as high as 60 nL of medium and 200 cells per 1 mm^2 or approx. $50 \times$ higher than macroscopic culture (Gomez-Sjoberg et al., 2007).
Nutrient consumption	Nutrients in cell culture medium generally in great excess, medium exchange typically needed every 48 h to once a week (Freshney, 2010).	Medium turnover is faster and needs to be assessed for every cell line and device. Some reports indicate increased glucose consumption (Paguirigan and Beebe, 2009).
Proliferation	Doubling time of immortalized cell lines varies but is typically between 18 and 24 h.	Proliferation rates need to be re-evaluated when cells are cultured in microfluidic devices. Some studies report reduced proliferation (Paguirigan and Beebe, 2009).
pH regulation	Culture medium is buffered at pH 7.4 with bicarbonate if CO_2 levels are kept at 5%. HEPES is also a common buffer that is not as sensitive to CO_2 .	PDMS is more permeable to CO_2 than to O_2 or N_2 (Mark, 1999). Care must be taken to ensure that dissolved gas levels within chambers are as expected.

hepatic stellate cell interactions under continuous flow conditions. They reported that liver spheroids cultured in their co-culture system showed improved albumin and urea secretion as well as increased enzymatic activity over spheroids cultured in conventional monocultures. In addition to the devices mentioned above, microfluidic devices mimicking the organ specific architecture and functionality of lung (Long et al., 2012), heart (Grosberg et al., 2011), microvascular networks (Zheng et al., 2012) and a blood brain barrier (Booth and Kim, 2012) have been developed. Efforts to inter-connect some of the “organ-on-a-chip” systems to produce micro total bioassay systems for pharmacological studies are already under way (Imura et al., 2012). This promising technology will provide a valuable tool to predict whole-body responses to drugs and other pharmacological challenges (Sung et al., 2014).

Only a very limited selection of the literature on miniaturization, integration, automation and parallelization of cell culture processes in microfluidic devices has been treated in this section. However, from this selection it is clear that microfluidic cell culture devices can have significant advantages over macroscopic cell culture systems. As is evident from Table 1, different microfluidic cell culture devices have distinct benefits. As there is great versatility with regard to the possible designs of a microfluidic cell culture device, undoubtedly new advantages of these devices will emerge over macroscopic cell culture. Although microfluidic cell culture does have significant advantages, it is important to be clear about the remaining challenges in order to manage expectation among end users. In the next section we discuss what we perceive are the main challenges associated with microfluidic cell culture.

3. Challenges of microfluidic cell culture

Although microfluidic cell culture provides great flexibility with respect to experimental design, moving cells from a macroscopic culture environment of dishes, flasks and well-plates to microfluidic cell culture requires revision of culture protocols. Several unique factors distinguish microfluidic from macroscopic cell culture, such as different culture surfaces, reduced media volumes, and vastly different rates of, and methods for, medium exchange. Careful evaluation of these differences needs to be completed before experiments are translated between these platforms. Table 2 compares key parameters of macroscopic and microfluidic cell cultures. Despite a growing number of microfluidic cell culture devices, efforts to compare cellular behavior in microfluidic devices versus macroscopic cell culture have been few and

in some respects contradictory. This is likely a result of the large variety of device designs and parameters, as well as cell-specific responses to microfluidic culture. Table 3 summarizes some selected publications that contain general information on microfluidic cell culture systems, their parameters and, if possible, how those systems compare to macroscopic cultures on polystyrene or glass. In this section the focus is on some of the intrinsic differences between macroscopic cell culture in Petri dishes, flasks, and well-plates on one hand, and microfluidic culture platforms on the other. The intention is to aid researchers wishing to enter the growing field of microfluidic cell culture.

3.1. Culture materials: polydimethylsiloxane versus polystyrene

Silicone is a synthetic polymer whose backbone is a repeating chain of Si–O molecules with various organic groups attached to the silicon. The silicone termed polydimethylsiloxane $[(\text{CH}_3)_2\text{Si}-\text{O}]_n$, abbreviated PDMS, has two methyl groups attached to the silicon. As with all silicones, PDMS polymers will cross-link, with the addition of a curing agent containing a catalyst, usually platinum. PDMS has been widely used to produce microfluidic devices. It can be easily molded to create complex fluidic circuits, using soft lithography techniques, making prototyping relatively simple and cost effective. Some characteristics of PDMS, such as gas permeability, optical transparency, and flexibility also make it appealing for cell culture devices. In addition, it is generally regarded as inert, non-toxic, and fully bio-compatible. The fabrication of PDMS devices involves mixing the PDMS elastomer base with a curing agent, pouring it into a mold and heating to accelerate the curing process. The cured PDMS can then be permanently bonded to a glass or plastic slide by plasma or thermal bonding. The duration of this process depends on device design and PDMS type. Two types of PDMS are commonly used by researchers to fabricate microfluidic chips: RTV-615 from Momentive Materials² and Sylgard 184³ from Dow-Corning. Both manufacturers report the use of irritants such as ethylbenzene and

² Base: polyvinylsiloxane (60–90%), polyalkylalkenylsiloxane (10–30%), and ethylbenzene (< 1%). Curing agent: polyvinylsiloxane (30–60%), modified silica (SiH) (30–60%), octamethylcyclotetrasiloxane (< 1%) and toluene (< 1%).

³ Base: dimethylsiloxane oligomers with vinyl-terminated end groups (> 60%), silica filler (dimethylvinylated and trimethylated silica, 30–60%), tetra(trimethylsiloxy) silane (1–5%) and ethylbenzene (< 1%). Curing agent: a cross-linking agent (dimethyl methylhydrogen siloxane, 40–70%) and an inhibitor (tetramethyl tetra-vinyl cyclotetrasiloxane 1–5%).

Table 3
Key parameters used in selected microfluidic cell culture studies and cellular response. Abbreviations: NR, not reported; PDL, poly-D,L-lysine; PLL, poly-L-lysine.

Reference	Description	PDMS type	Culture surface	S _{APDMS} (mm ²)/vol (μL)	Cell type	Medium exchange	Coating	Cellular response
Gomez-Sjoberg et al. (2007)	μ-Chambers	RTV-615	PDMS	33	Human mesenchymal stem cells	30%–60% Every hour	fibronectin	Steady proliferation
Paguirigan and Beebe (2009)	μ-Wells	NR	Polystyrene	0.5	Mouse mammary fibroblasts	Every 48 h	NR	Slow proliferation
Paguirigan and Beebe (2009)	μ-Channels	NR	PDMS	2	Mouse mammary fibroblasts	Every 48 h	NR	No proliferation
Chung et al. (2005)	μ-Chambers	NR	Glass	10	Human neuronal stem cell	0.1 μL/min (perfusion)	PLL/laminin	Steady proliferation and differentiation
Milliet et al. (2007)	μ-Channels	Sylgard 184	Glass	33	Primary rat neurons	Gravity perfusion	PDL	Steady decline
Wlodkowic et al. (2009)	μ-Wells	Sylgard 184	Glass	0.12	Human tumor cell lines	every 48 h	NR	Steady proliferation, comparable to traditional
Chen et al. (2011)	μ-Channels	Sylgard 184	PDMS	26	A549 lung tumor cells	10 μL/min (perfusion)	Fibronectin	Steady proliferation, comparable to traditional
Nalayanda et al. (2007)	μ-Channels	Sylgard 184	Glass	NR	HMEC-1 (endothelial) and A549	10 μL/min (perfusion)	gelatin	Steady proliferation but slower than traditional
Wu et al. (2009)	μ-Chambers	NR	Glass	10	Human mesenchymal stem cells	1.7 μL/min (perfusion)	NR	Proliferation and differentiation comparable to macroscopic
Liu et al. (2010)	μ-Wells	Sylgard 184	PDMS	5.5	Human mesenchymal stem cells	48 h	gelatin	Little or no proliferation
Liu et al. (2010)	μ-Wells	Sylgard 184	Glass	4.3	Mouse embryonic fibroblasts	48 h	gelatin	Steady but slow proliferation
Liu et al. (2010)	μ-Wells	Sylgard 184	Polystyrene	4.3	Mouse embryonic fibroblasts	48 h	Gelatin	Steady proliferation, comparable to traditional
Liu et al. (2010)	μ-Wells	NR	Glass	33	HeLa (tumor)	0.2–0.4 μL/min (perfusion)	NR	Steady proliferation, comparable to traditional
Rhee et al. (2005)	μ-Channels	Sylgard 184	Glass	NR	E18 rat cortical neurons	NR	PLL	Culture was steady for 6 days

xylene in small amounts in their PDMS blends. If and how this may affect cells cultured on cured PDMS is unknown. Sylgard 184 is more commonly used for fabrication of cell culture devices.

Despite the wide range of PDMS based microfluidic cell culture devices that have been reported to date, few compare cellular proliferation in the device to macroscopic culture. Some consider PDMS to be bio-compatible and not cytotoxic, based on the use of medical grade PDMS within long term medical device implants (Hassler et al., 2011; Nag and Banerjee, 2012) and reports of growth of a variety of cell lines on various formulations of PDMS. However, this does not imply that all types of cells will grow on all formulations of PDMS in the same way that they do on macroscopic culture plastics, such as polystyrene. Others regard the often cited biocompatibility of PDMS to be “something of a misnomer” (Sackmann et al., 2014).

There have been reports of artifacts arising from chemical and physical interactions between in vitro cultures and certain formulations of PDMS. One of the first studies on cellular attachment and proliferation on PDMS (and many other synthetic surfaces) was performed by Ertel et al. in 1994. They found that culturing cells on an untreated PDMS (“NIH reference material”) surface induced rapid and high levels of cell death on two types of fibroblasts (3T3 and BHK). This effect could be blocked by treating the PDMS culture surface with serum or a concentrated protein solution. As PDMS is hydrophobic, cellular attachment to native PDMS surfaces may be increased with treatment to reduce its hydrophobicity, or coating the surface with proteins that facilitate cellular attachment. Lee et al. (2004) studied the compatibility of four mammalian cell lines (HUAEC primary endothelial cells; 3T3 fibroblast cell line; MC3T3-E1 osteoblast cell line; HeLa transformed epithelial cells) on PDMS (Sylgard 184) surfaces with different ratios of PDMS base and curing agent, with or without extraction of low molecular weight components with organic solvents, and with or without oxidizing the surface. All surfaces were coated with fibronectin to facilitate cellular attachment. All of the cell lines tested attached and proliferated on PDMS to some extent and sometimes at rates comparable to polystyrene. However, the cells tested seemed to have different preferences for PDMS formulations. For example, MC3T3-E1 osteoblasts attached and grew on native, fibronectin coated PDMS, at rates comparable to polystyrene. Solvent extraction of unbound PDMS oligomers and oxidation reduced attachment and severely impaired proliferation. Excess curing agent ($10_{\text{Base}}: 3_{\text{CA}}$ instead of $10_{\text{Base}}: 1_{\text{CA}}$) had little effect either on attachment or proliferation. However, HeLa cells showed poor attachment and proliferation on native PDMS, or PDMS with excess curing agent, but attachment to solvent extracted and oxidized PDMS was comparable to polystyrene. Wang et al. (2010) found that Caco-2 cells showed reduced attachment and little or no proliferation on PDMS with excess curing agent ($5_{\text{Base}}: 1_{\text{CA}}$ instead of $10_{\text{Base}}: 1_{\text{CA}}$). Taken together, the results from these studies show that PDMS is capable of sustaining growth of different cell lines, provided that the formulation and surface treatment are optimized for each cell line.

Incomplete curing of PDMS leaves uncross-linked oligomers within the material that can leach out and contaminate the culture medium. Indeed, when deionized ultra-filtered water was incubated within a PDMS-based microfluidic channel for 24 h and analyzed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, a continuous range of uncrosslinked PDMS was detected despite efforts to extract uncrosslinked PDMS with ethanol in a Soxhlet extractor overnight (Regehr et al., 2009). The same study also showed that PDMS oligomers were detectable in the membranes of mouse mammary fibroblasts cultured in PDMS (Sylgard 184) microfluidic channels for 24 h. The effect of PDMS incorporation into cellular membranes of cultured cells is as

yet unknown. To reduce the amount of uncrosslinked polymers left in the material, care must be taken to allow curing to run to completion. This should be done as per instructions provided by each manufacturer of PDMS as different formulations may cure at different rates. It should be noted that in this experiment the PDMS was cured for only 2 h, which may have increased the possibility of uncrosslinked PDMS in contact with the cells.

The surface area-to-volume (SA/V) ratio in macroscopic culture is roughly 0.5 mm^2 to $1 \mu\text{L}$ of medium and this ratio holds true from 96-well plates to larger culture flasks. In contrast, the surface area-to-volume ratio varies greatly from one microfluidic device to the next. The surface area in direct contact with culture medium in microfluidic culture devices can be fully or partially composed of PDMS. [Paguirigan and Beebe \(2009\)](#) published an extensive study on phenotypic changes of mouse mammary fibroblasts cultured in macroscopic polystyrene culture wells versus open unspecified PDMS microwells that had the same SA/V ratio as macroscopic culture, or closed PDMS microchannels that had 4 times higher SA/V. They found that proliferation was impaired in the microchannels, which could not be mitigated with increased glucose or serum supplementation. In addition, they observed several distinct cell cycle progression problems in cells cultured in the microchannels. Their conclusion was that PDMS surface area to media volume ($\text{SA}/V_{\text{PDMS}}$), a ratio that can be as high as $30 \text{ mm}^2 \text{ PDMS}/\mu\text{L}$ medium in some microfluidic cell culture devices ([Gomez-Sjoberg et al., 2007](#); [Millet et al., 2007](#); [Lee et al., 2006](#); [Chen et al., 2011](#)), is a determining factor for cellular proliferation and behavior. As this ratio increases, changes in baseline functions such as proliferation and metabolism become more pronounced.

[Millet et al. \(2007\)](#) examined primary mammalian neuron culture and differentiation at low densities in open versus closed microfluidic channels. They found that open channels, with large media volumes, could support neuron growth for at least 8 days, while neurons did not survive in closed channels made of native PDMS (Sylgard 184). Autoclaving the PDMS to drive polymerization to completion, or extraction of uncrosslinked polymers with organic solvents, improved neuron growth in the closed channels, although it did not reach the same level of growth as in the open channels. It should be noted that these channels were constructed of a PDMS roof and side-walls mounted onto a glass coverslip, so that the culture surface was poly-D-lysine coated glass, not PDMS. [Włodkowiec et al. \(2009\)](#) compared growth rates of three different cell lines (2 human leukemic cell lines, K562 and U937 and a human tumor cell line, U2OS), in a PDMS (Sylgard 184) based microfluidic cell culture device versus in macroscopic polystyrene culture plates. They found no adverse effects on cell viability or growth after 48 h of culture ([Włodkowiec et al., 2009](#)).

[Lopacinska et al. \(2013\)](#) studied the use of polymethyl methacrylate (PMMA) as a culture substrate. They compared the biocompatibility of PMMA alone, polystyrene alone or a layer of PDMS (Sylgard 184) underneath a perforated layer of PMMA (PMMA-PDMS), with respect to PC12 (adrenal pheochromocytoma) cell growth and PC12 differentiated into neuronal-like cells. They observed that for non-differentiated cells there was little difference in gene expression among polystyrene, PMMA and PMMA-PDMS, while there were significant differences between the gene expression profiles of PC12 cells differentiated on PMMA versus PMMA-PDMS surface.

Hydrophobic molecules, less than approximately 500 Da, are absorbed into PDMS ([Gomez-Sjoberg et al., 2010](#)), which complicates the interpretation of some studies. [Liu et al. \(2010\)](#) compared mouse embryonic fibroblast growth in microchannels on different surfaces and reported that attachment and cell spreading were substantially impaired on PDMS, compared to polystyrene. Embryonic stem cell differentiation was also impaired when the

cells were cultured on PDMS rather than on polystyrene or glass. In these experiments, differentiation of embryonic stem cells was induced with retinoic acid, a small (300 Da) hydrophobic molecule widely used to induce differentiation in various cell types.

The differences reported by these studies suggest that the viability of cells cultured within a microfluidic device depends both on the particular cell type being cultured and on the particular protocol for fabricating the material, e.g., PDMS, within the device. This underlines the necessity of evaluating cell growth, morphology and viability of every cell line, relative to macroscopic culture, when it is introduced to a particular microfluidic culture device.

3.1.1. Surface treatment and coating

The majority of cultured mammalian cells grow as monolayers on an artificial substrate. The surface must be correctly charged to allow cellular attachment and spreading. Polystyrene, by far the most common surface for cell culture, is hydrophobic in its native state and must be rendered hydrophilic before it permits cell adhesion. UV treatment or oxidizing with oxygen plasma is commonly used to reduce hydrophobicity of polystyrene, making it more appropriate for cell culture. Flasks and well-plates whose surfaces have been oxidized in this way are referred to as “tissue culture treated”. The surface of native PDMS is also hydrophobic and must therefore be specifically treated prior to cell culture to facilitate attachment and growth. A number of different methods have been developed to reduce hydrophobicity and coat PDMS surfaces for enhanced cell attachment.

Oxygen plasma and UV treatment are commonly used to activate synthetic surfaces ([Wong and Ho, 2009](#)), thus reducing the surface hydrophobicity. Recently, [van Midwoud et al. \(2012\)](#) compared treatments of different cell culture plastics and PDMS (Sylgard 184). They measured how the hydrophobicity of different materials was reduced by an oxidizing treatment and how stable the treatment was. The hydrophobicity of polystyrene was quickly reduced with either UV or oxygen plasma treatment and the polystyrene surface remained relatively hydrophilic for the duration of the experiment (4 weeks). On the other hand, although the hydrophobicity of PDMS could be reduced with these treatments, the PDMS surface returned to its hydrophobic state within 1 week, a process known as hydrophobic recovery ([Eddington et al., 2006](#)). This effect is due to low molecular weight uncrosslinked PDMS polymeric side-chains diffusing from the bulk to the surface, thus returning it to its hydrophobic state ([Chen and Lindner, 2007](#)). Although the hydrophobic recovery of PDMS can be attenuated to some extent ([Eddington et al., 2006](#)), this limits the feasibility of oxidizing the PDMS surface for cell culture, particularly for long-term culture experiments or when treated devices are stored before use.

Another approach to reduce hydrophobicity is to coat the PDMS culture surface to promote cellular attachment and proliferation, either by coating with charged molecules or extracellular matrix proteins such as fibronectin, collagen or laminin. The strong hydrophobic nature of PDMS causes it to interact with polar substrates either through hydrogen bonding or through polar-polar interactions. Substrates containing methyl or alkyl groups can also interact with PDMS due to van der Waals forces ([Kuncova-Kallio and Kallio, 2006](#)). This means that proteins will adhere to an untreated PDMS surface depending on their structure and surface charge. [Wang et al. \(2010\)](#) studied the effects of different coatings on attachment and proliferation of Caco-2 cells. Incubating the PDMS (Sylgard 184) culture surface with cellular growth medium containing fetal bovine serum was sufficient to promote attachment and proliferation on oxidized PDMS. Adsorbing extracellular matrix (ECM) proteins such as fibronectin or collagen onto native PDMS also facilitated attachment and proliferation.

Zhang et al. (2013) studied the growth and differentiation of SUM159 and MDA-MB-468 breast cancer cell lines on PDMS (RTV 165) with varying stiffness, coated with ECM proteins and bovine serum albumin (BSA). Native or oxidized PDMS proved to be unfavorable for cellular attachment while collagen and fibronectin coating greatly improved cellular attachment, regardless of PDMS stiffness. Although BSA coating also improved attachment, cellular morphology was altered from spindle-shaped to round, indicating weaker adherence. Interestingly, these researchers observed an enrichment of cells expressing cancer stem-cell markers after 2 weeks of culture on BSA coated PDMS. They conclude that coating PDMS with ECM proteins or BSA to facilitate attachment can provide a suitable substrate for culturing breast cancer cells but the phenotypic equilibrium of these cells may be altered in response to cell-to-surface interactions.

Another possible way to treat PDMS surfaces is to coat them with charged molecules such as poly-D-lysine. The amino group on the end of each lysine develops a net positive charge which makes it hydrophilic and may enhance electrostatic interaction between negatively charged ions in the cell membrane (Wang et al., 2010). To screen for cellular attachments and proliferation on different surface coatings and media compositions, Hattori et al. (2011) devised a microenvironment array chip containing an 8×8 array of perfusion chambers that can be independently surface treated with up to four different extracellular matrix proteins (columns) and perfused with four different media compositions (rows). The 96 chamber microfluidic cell culture device designed by Gomez-Sjoberg et al. (2007) offers the possibility of coating each of the chambers independently through 16 inputs and feeding with up to 16 different media compositions. Devices such as these can aid researchers in devising the optimal surface treatment and feeding for any cell type that is to be cultured in a PDMS based microfluidic environment.

Many microfluidic devices contain elaborate medium flow circuits for creating gradients and mixing before the medium enters the culture area. Through these flow circuits, the medium flows over a large surface of PDMS where proteins and hydrophobic analytes in the medium may attach to the free hydrophobic sites of the PDMS channel (Zhou et al., 2012). This can lead to unwanted cellular attachment in the flow channels or non-specific protein adsorption from the culture medium. To overcome this, various surface treatments have been developed to modify the surface and prevent its interaction with proteins (Wong and Ho, 2009). Among the most commonly used surfactants are poly(ethylene oxide)-terminated triblock polymers, such as Pluronic[®] which are able to form a stable adsorbed layer on the hydrophobic PDMS surface, thus preventing non-specific protein adsorption (Wong and Ho, 2009). Yang et al. (2010) developed a method where native PDMS was coated with polysaccharides using a photocatalyzed surface modification method. They found that carboxymethyl cellulose coating repelled both positively and negatively charged proteins while retaining the ability to support cell attachment, proliferation and migration.

3.2. Absorption of hydrophobic molecules

The hydrophobic and porous nature of PDMS enables small hydrophobic molecules to diffuse from the culture medium into the bulk polymer. It has been demonstrated that Nile Red, a small hydrophobic fluorescent dye, is absorbed into PDMS surrounding a microfluidic channel in a matter of seconds, and the fluorescent signal was retained in the PDMS despite multiple washes (Toepke and Beebe, 2006). Regehr et al. (2009) demonstrated that when cell culture medium containing 1 nM estrogen was incubated in microfluidic channels, over 50% of the estrogen diffuses from the

medium into the PDMS during the first hour of incubation. After 24 h, 90% of the estrogen had diffused into the PDMS. Recently, Wang et al. (2012) measured the extent of absorption of five different markers routinely used for in vitro cellular assays. They discovered a relationship between the logarithm of the octanol/water partition coefficient ($\log P$) of the markers and the degree of leaching into the PDMS. A $\log P$ threshold of ≈ 2.5 separated the markers that exhibited low absorption ($\leq 25\%$ after 4.5 h) and those that exhibited high absorption in PDMS ($\geq 75\%$ after 0.5 h). Although $\log P$ values for many chemical compounds are known, the exact values will depend on experimental conditions (Haraldsdóttir et al., 2012). Most media components such as amino acids, glucose and pyruvate have low $\log P$ values (-4 to -1) and should therefore exhibit low absorption into PDMS. However, fat-soluble vitamins such as retinoic acid and calciferol, and lipid derived hormones, have high $\log P$ values (≥ 6) and will therefore be quickly absorbed into the bulk PDMS of the culture chip. Cell culture experiments that rely on these vitamins or hormones for differentiation or stimulation will need to be specifically optimized for microfluidic culture in PDMS devices. Some treatments have been reported in the literature for reducing the absorption of small molecules into PDMS, such as sol-gel and borosilicate glass coating (Gomez-Sjoberg et al., 2010; Abate et al., 2008; Orhan et al., 2008). Although these treatments greatly reduce the absorption of small hydrophobic molecules into PDMS, they also change the surface properties of PDMS and therefore should first be thoroughly tested for their influence on cells in macroscopic culture.

3.3. Oxygen, osmolarity and pH

A valuable property of PDMS for microfluidic cell culture devices is its permeability to gases. This allows for permeation of ambient CO_2 and O_2 through the material to buffer the culture medium and supply the necessary oxygen. However, PDMS is also permeable to water vapor, which can cause drying problems in devices where the media volume is small compared to the surface area. Evaporation of even small amounts of water from the medium can cause a significant shift in medium osmolarity. Most cell lines can tolerate osmolarity ranges from 260 to 320 mOsm/kg but shifts in osmolarity during culture should be avoided (Freshney, 2010). Heo et al. (2007) showed that evaporation in culture chambers containing 500 nL of medium through a thin (120–400 μm) PDMS membrane caused a rapid shift in osmolarity that was sufficient to kill human endothelial cells in only 25 min. Blau et al. (2009) fabricated culture vessel lids, of 1 mm thick PDMS, and reported reduced evaporation and stabilized medium osmolarity in long-term cell cultures.

The optimal pH for most cultured cells lies in a narrow range around 7.4 (Freshney, 2010). This may deviate slightly from one cell line to another, but most commercially available media are designed to keep pH close to 7.4. Cellular respiration produces carbon dioxide that dissolves in the medium to produce carbonic acid, which combined with acidic metabolic byproducts such as lactate, tend to acidify the medium. Therefore cell culture media needs to be buffered to keep pH within a physiological range. Cell culture in flasks or dishes is generally performed in incubators that allow control of ambient CO_2 . Lids or caps are kept un-tight or contain filters that allow diffusion of gas between the culture and the surrounding air and the necessary CO_2 exchange between the medium. As PDMS is permeable to gas, control of the partial pressure of CO_2 in ambient air can be used to buffer media pH. The partial pressure of ambient CO_2 required to maintain pH within a certain physiological range is also dependent on whether the fresh culture media also contains a buffer, such as sodium bicarbonate.

HEPES can also be used to buffer cell culture media at 7.4. HEPES can maintain physiological pH without the need for control of ambient carbon dioxide and might be used when culturing cells in microfluidic devices outside of incubators. Some considerations are necessary when using HEPES to buffer culture media instead of bicarbonate. HEPES is light sensitive and is reported to produce hydrogen peroxide when exposed to ambient or fluorescent light, which may have adverse effects on the culture (Bowman et al., 1985). HEPES buffered media should therefore be kept in the dark as much as possible. This point is especially relevant for microfluidic cell culture with transparent material, such as PDMS, outside of a dark incubator. Addition of HEPES to culture medium will also cause a shift in osmolality so careful evaluation and testing should be carried out if it is to supplement complete media.

Oxygen is necessary for cellular respiration *in vivo*. However, the oxygen requirements of cells cultured *in vitro* vary greatly as many cell lines rely primarily on glycolysis rather than aerobic respiration for energy production. Primary cultures, particularly from late stage embryos or adults, typically require more oxygen than transformed cell lines. Other cells, for example mesenchymal stem cells, proliferate faster and longer and maintain their undifferentiated characteristics better under hypoxic conditions (Ma et al., 2009). Rat embryonic neurons and neuronal stem cells have been reported to grow just as well in anoxic medium as normoxic, provided that the glucose in the medium is sufficient (Wohnsland et al., 2010). During normoxic conditions, these cells will fully metabolize glucose via glycolysis and oxidative phosphorylation. If cultured in an anoxic environment, glucose consumption increases about 5-fold and lactate production increased about 10-fold with no marked reduction in viability. Apart from a direct effect on cell viability, oxygen levels can also greatly affect cellular behavior, morphology and differentiation (Mannello et al., 2011; Panchision, 2009).

In macroscopic culture, oxygen and CO₂ diffusion from the air inside the incubator into the culture medium is usually considered sufficient to supply the cells with the necessary oxygen amount for growth and proliferation and adequate medium buffering (Freshney, 2010). Incubation of microfluidic culture devices generally takes place in either a standard laboratory incubator or mounted onto a live-cell imaging station or bioreactor. In any case, ambient CO₂ levels need to be accurately controlled to maintain the correct pH. The ratio of media-to-cell volume tends to be lower with microfluidic cell culture, therefore, especially if no bicarbonate or HEPES are added to the media, one must ensure that permeation of O₂ and CO₂ can occur at rates sufficient for aerobic respiration and buffering of media pH, respectively. PDMS is highly permeable to gases and allows diffusion of CO₂ and oxygen into the medium inside microfluidic channels, supplying the culture with oxygen, removing carbon dioxide produced by cellular respiration and maintaining physiological pH. Passive permeation of oxygen through PDMS is generally assumed to be sufficient for supply at a rate sufficient for aerobic respiration. How the thickness of PDMS or surface coating for cell adhesion will affect gas exchange is not clear. For example, native PDMS is highly oxygen permeable, but the permeability is subject to change when proteins are adsorbed on it or when the surface is modified by plasma oxidation, as is common in the construction of microbioreactors (Mehta et al., 2007). Kim et al. state that thin PDMS membranes (~100 μm thickness) can be used for gas exchange in a microfluidic perfusion culture (Kim et al., 2007).

In thin-walled PDMS culture channels, containing metabolically active rat hepatocytes, Ochs et al. (2014) report that oxygen was readily replenished (to approximately 16%) by the environment but that the same cells cultured in 5 mm thick PDMS devices

resulted in oxygen levels of to approximately 11.5%. The same study provides calculations for medium perfusion rates necessary to maintain stable oxygen levels within microfluidic culture channels, based on device dimensions, diffusion rates and oxygen uptake rates of the cells. Gases have different permeability and solubility in PDMS than in water (Mark, 1999). This leads them to fractionate within the PDMS so the real concentration of CO₂ and oxygen inside the chip may be different from that in the air around it. To better regulate on-chip oxygen levels or create oxygen gradients, Thomas et al. (2011) developed a microfluidic culture device where precisely oxygenated water is continuously perfused through channels adjacent to cell culture chambers, allowing rapid control of on-chip oxygen levels within physiological ranges while at the same time minimizing evaporation from the culture chambers.

Novel methods to accurately measure pH and oxygen levels inside microfluidic cell culture chambers in real time are necessary as standard laboratory pH and oxygen probes are too large to work with such small media volumes. In addition, the culture medium in most microfluidic culture chambers is closed off within PDMS, making direct measurement challenging. Methods utilizing a ratiometric pH sensitive dye and oxygen sensitive fluorescent probes such as BCECF and RTDP to measure perturbations inside microfluidic channels and chambers have been developed, but they rely on modifying the culture device or medium composition with potentially harmful effects to the cells cultured within the device (Han and Burgess, 2010; Lee et al., 2008). Recently, Magnusson et al. (2013) developed a highly accurate and precise method to indirectly measure microfluidic chamber pH by measuring the shift of light absorption by phenol red as a function of pH. Contrary to previously reported methods, this method requires only a standard cell culture medium, standard microfluidic device design and a standard laboratory microscope with a suitable filter. Real time monitoring is necessary for proper quality control of conditions within microfluidic cell culture chambers.

Different methods are used to supply fresh media to microfluidic cell cultures. Syringe pumps are commonly used to flow media directly into culture lines or chambers. Fresh medium can also be supplied by pressurizing media vials connected to culture lines or chambers with air and using on-chip valves to precisely control flow in and out of the device (Gomez-Sjoberg et al., 2007). Attention must be paid to the composition of the air used to pressurize these media vials. For example, if the medium contains bicarbonate buffer and the media vials are pressurized with ambient air (0.039% CO₂ by volume), the pH of the medium will increase in the vials before it enters the device. Therefore, media vials should be pressurized with air containing the correct percentage of CO₂ and O₂ in order to keep pH and dissolved oxygen at physiological levels.

3.4. Nutrient consumption and medium turnover

In contrast to macroscopic cell culture where medium is typically stagnant in culture flasks or wells, relying on excess amounts of nutrients in the culture medium to feed cells over a number of days, microfluidic systems can be designed for perfusion culture, where medium continuously flows through the culture channels and chambers. This method can create a more realistic culture environment by keeping nutrients and other important media factors constant by continuously supplying fresh media and removing waste products (Kim et al., 2007). Continuous perfusion also offers unique opportunities to create chemical gradients within the cell culture, which are difficult to achieve with macroscopic culture methods (Kim et al., 2007; Chung et al., 2005). However, culturing cells under flow has its own challenges.

Living and proliferating cells consume energy sources, such as glucose, glutamine and amino acids, and produce metabolic waste, such as CO₂ and lactate. Most microfluidic culture devices have a high number of mammalian cells to media volume, which better simulates *in vivo* cell density. If this ratio is 50 times higher in a microfluidic cell culture device than in macroscopic culture, one can assume that metabolites will be depleted and wastes will build up 50 times faster. Medium in a macroscopic culture is generally changed every 2–4 days to replenish nutrients and remove waste. With the aforementioned ratio, this corresponds to total media exchange in a microfluidic chamber every 1–2 h.

Paguirigan and Beebe (2009) found that the baseline glucose consumption per cell of mouse mammary fibroblasts was three to four times higher in a microfluidic culture device than in macroscopic culture. Due to the high cell number to media volume ratio in microfluidic devices, careful validation experiments and feeding schedule calibrations are necessary for every device and cell line. Yu et al. (2007) did an extensive study on medium exchange rates, supplementation and seeding density in microfluidic channels versus macroscopic culture. Among their findings was that increasing the medium exchange frequency from 12 h to 1 h resulted in slower proliferation of normal mouse mammary (NMuMG) cells in both macroscopic culture and PDMS micro channels. Decreasing the medium change frequency from 1 to 4 h had a beneficial effect on cell growth in both platforms, although cell proliferation in micro channels was higher. They concluded that build-up of endogenous growth factors plays a critical role in cell growth. These soluble signaling molecules need to reach a certain concentration in the medium before growth is promoted. Due to the high cell number-to-media volume ratio of most microfluidic channels and chambers, the critical level of soluble factors in the cellular microenvironment can be reached more quickly. A balance is necessary between, on one hand, the rate of media replacement required for replenishment of nutrients, and on the other, the need to keep the concentration of endogenous growth at effective levels.

With computational modeling and experimental testing, Giulitti et al. (2013) optimized medium replacement strategies for long-term culture of mouse myoblasts (C2C12), human fibroblasts (HFF) and mouse embryonic stem cells (mESCs) in PDMS (Sylgard 184) microchannels. They examined constant flow rates, as well as periodic replacement of medium, and found that slow, continuous perfusion had deleterious effects on their cultures, especially downstream while fast, periodic replacement of the same media volumes resulted in uniformly healthy growth of all cell types within the channels. This was attributed to heterogeneous distribution of nutrients (higher concentration upstream) versus endogenous factors and waste (higher concentrations downstream) in slowly perfused culture channels. They conclude that medium delivery strategies are extremely relevant for proper maintenance of cell homogeneity, viability and behavior in microfluidic cell culture.

Another important factor to consider when exposing cells to a perfusion flow in microfluidic channels is shear stress. When rat mesenchymal stem cells, adherent to a microfluidic cell culture chamber, were exposed to sufficient flow induced shear stress (1.3 N m⁻²), Zheng et al. (2012) reported “contraction and re-spread” process, whereby cells contracted during the initial 20–30 min and re-spread in a similar period. While the device fabricated by Zheng et al. (2012) was designed to mimic the flow induced shear stress typical of blood vessels, it is important to recognize the morphological response of cells to shear stress and eliminate it when not desired. Different strategies to minimize the effect of shear stress on culture stability have been published. Kolnik et al. (2012) developed a cell trapping system to load cells

into isolated culture chambers using an on-chip vacuum and evacuating air through the PDMS. While medium continuously flowed past the culture chambers, the cells were not in the direct path of flow, while diffusion replenished nutrients into and removed waste from the culture chambers.

Su et al. (2014) characterized the effect of various culture parameters on cell stress in human embryonic kidney cells cultured in PDMS (Sylgard 184) microchannels, by measuring the expression of a key chaperone gene involved in the unfolded protein response. Without replenishing media, cell stress was significantly higher at 48 h than at 24 h post-seeding, while media replenishment at 21 h reduced stress at 24 h. However, Su et al. also report that increased frequency of media replacement (6–12 h intervals) leads to “abnormal cellular morphology”, which might be related to the “contraction and re-spread” process observed by Zheng et al. (2012). Su et al. (2014) also provide evidence suggesting that increasing the concentration of foetal bovine serum in media can alleviate stress in some cell lines.

4. Conclusions

The development of microfluidics over the past decade has been fast. Microscale bioreactors and analysis systems have grown in ever increasing numbers and complexity, offering new insights into complex cellular behavior. Microfluidics has brought the ability to custom tailor microenvironments, automate experimentation and couple cell culture directly to high throughput analysis systems. As enticing as these new advances may be, there are still hurdles that arise when an established culture platform is changed from macroscale cultures on polystyrene to microfluidic devices made of PDMS or other materials. Different cell types respond differently when moved from macroscopic culture on polystyrene to microfluidic culture on PDMS. Device designs are as diverse as the cell lines cultured within them, making generalizations difficult. In addition, it is likely that negative results, such as sub-optimal cell growth in a given device or other complications, may be underrepresented in the literature.

Although PDMS has a number of properties that are beneficial for cell culture, such as transparency and gas permeability, it should be kept in mind that the primary reason for using this material in microfluidic cell culture devices is from the fabrication perspective, namely, ease of prototyping and low cost (Berthier et al., 2012). For decades, glass and polystyrene have been used for macroscopic cell culture and they have been thoroughly studied and characterized in that respect. Indeed, the majority of new published data on *in vitro* cell biology is still based on cells cultured on these materials. When cell culture is moved from these macroscopic platforms to PDMS based microfluidic devices, where volumes are small, surfaces large in comparison to volume, and feeding schedules different, cellular behavior may very well turn out to be different. This makes direct comparison between experiments performed on these platforms difficult. This is not to say that macroscopic cell culture is more reliable, only that researchers should be aware of the fact that the transition may not be straightforward, as most cell culture protocols in existence have been optimized for macroscopic cell culture. As the field of microfluidic cell culture matures, we will undoubtedly see an increase of standardized microfluidic devices. While design and prototyping of novel devices are simpler and more cost efficient in PDMS, new production techniques such as microfluidic hot embossing in polystyrene (Young et al., 2011) are likely to be favored for mass production. Over the past decade, many companies have emerged that offer standard and customized microfluidic cell culture platforms, with chips fabricated from a wide range

Table 4

A selection of companies offering standard and customized microfluidic cell culture equipment.

Company	Website	Cell culture products	Chip material
Aline	www.alineinc.com	Microslide	Acrylic
BellBrook labs	www.bellbrooklabs.com	Microchannel plates	Standard tissue-culture treated plastic
Cellix Ltd.	www.cellixltd.com	Chips, pumps, software, automated platforms.	Acrylic, Topas
CorSolutions	www.mycorsolutions.com	Pumps, probe stations, probes, flow meters.	N/A
Cytoo	www.cytoo.com	Chips, plates, chambers	Glass, Acrylic
Dolomite microfluidics	www.dolomite-microfluidics.com	Chips, interfaces, pumps, sensors, valves.	Glass, fused silica, PDMS
Elveflow	www.elveflow.com	Pumps, flow control software.	N/A
Epigem	www.epigem.co.uk	Chips, gaskets, ferrules	Polymethylmethacrylate (PMMA), Polyaryletherketone (PEKK)
FlowJEM	www.flowjem.com	Chips, interface accessories.	Polycarbonate (PC), Cyclic olefin polymer, Acrylic, PDMS
Fluidigm Corp.	www.fluidigm.com	Chips, automated platforms.	PDMS
Gradientech	www.gradientech.se	Chips (conc. gradient), software.	Polymer
Merck Millipore	www.emdmillipore.com	Chips, pumps, software, automated platforms.	PDMS
Microflexis	www.microflexis.com	Chips, interface accessories.	Polycarbonate (PC), polystyrene & other polymers
Microliquid	www.microliquid.com	Chips, chip mounts.	PDMS, PMMA, PC
Mimetas	www.mimetas.com	Chips, plates.	Ordyl SY330
SIMTech Microfluidics Foundry	www.simtech.a-star.edu.sg/smf	Chips, interface accessories.	Polymers, elastomer, epoxy

of materials (See Table 4), some of which overcome certain disadvantages of PDMS, for instance compatibility with long term culture (Trietsch et al., 2013).

To the researcher interested in entering the field of microfluidic cell culture, we offer the following general guidelines:

(1) Test the compatibility of any cell line intended to use for microfluidic culture with various formulations of PDMS. A simple experiment where a culture surface is coated with various formulations of PDMS and cells seeded onto these surfaces should quickly reveal any complications with cellular attachment, survival or proliferation.

(2) To test for leaching of toxic or other unwanted species from and medium component absorption into PDMS, one may incubate fresh cell medium with PDMS (with sufficient surface area), separate the medium from the bulk PDMS, then add it to a well characterized macroscopic cell culture and monitor for any changes in response.

(3) Be aware that medium composition may have to be adjusted to suit the device and cells. Small hydrophobic molecules may be lost into the bulk PDMS and proteins, possibly important growth factors, may be adsorbed to the PDMS surface or diluted by too frequent media replacement. Medium buffering and oxygenation may also need to be adjusted according to device dimensions.

(4) The relatively high ratio of cell number to medium volume calls for more frequent feeding. Empirically test different schedules so that medium is replenished when needed but not too often. Endogenous soluble paracrine signaling factors may be important for the viability of certain cell lines and may need time to accumulate to sufficient concentrations within the surrounding medium.

(5) Pay attention to surface treatment of the PDMS. Poly-D-lysine is a good, simple starting point for PDMS surface treatment as it renders the surface hydrophilic and facilitates cellular attachment. Different cell types require different surface treatments for prolonged culture.

Microfluidic cell culture is highly interdisciplinary, relying on the co-operation of engineers to design and fabricate new devices, and cellular or molecular biologists to design and carry out biologically relevant experiments. Due to the ever increasing number of device designs, there is as yet little standardization in the field. In addition, many microfluidic cell culture devices rely on complex external control systems that may be foreign to most

established cell culture laboratories. Therefore, biochemical researchers should have strong ties to experienced engineers if they hope to successfully enter the field of microfluidic cell culture. This being said, if cell biologists are aware of the intrinsic factors that differ between macroscopic and microfluidic cell culture, the latter nascent field has the potential to be used to investigate many hitherto under explored aspects of cell biology.

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