A practical guide to microfluidic perfusion culture of adherent mammalian cells†‡

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Culturing cells at microscales allows control over microenvironmental cues, such as cell–cell and cell–matrix interactions; the potential to scale experiments; the use of small culture volumes; and the ability to integrate with microsystem technologies for on-chip experimentation. Microfluidic perfusion culture in particular allows controlled delivery and removal of soluble biochemical molecules in the extracellular microenvironment, and controlled application of mechanical forces exerted *via* fluid flow. There are many challenges to designing and operating a robust microfluidic perfusion culture system for routine culture of adherent mammalian cells. The current literature on microfluidic perfusion culture treats microfluidic design, device fabrication, cell culture, and micro-assays independently. Here we systematically present and discuss important design considerations in the context of the entire microfluidic perfusion culture system. These design considerations include the choice of materials, culture configurations, microfluidic network fabrication and micro-assays. We also present technical issues such as sterilization; seeding cells in both 2D and 3D configurations; and operating the system under optimized mass transport and shear stress conditions, free of air-bubbles. The integrative and systematic treatment of the microfluidic system design and fabrication, cell culture, and micro-assays provides novices with an effective starting point to build and operate a robust microfludic perfusion culture system for various applications.

1. Introduction

Microscale cell cultures support higher-throughput experimentation in drug testing, in the study of complex biological processes (such as stem cell differentiation), and in testing conditions for large-scale bioreactors when compared with macroscale cell cultures.[1,2] They can also be integrated into microsystems that incorporate cell culture, reactions, and microscale assays into portable devices for on-chip experimentation.[1,3] Culturing cells at microscales further allows for more precise control of the extracellular microenvironment by making use of microscale experimental tools to define cells' interaction with other cells, extracellular matrix (ECM), soluble factors and mechanical forces.[2]

Static microscale cell culture has been successful in enabling novel experiments that take advantage of micropatterning technologies to control cell–cell and cell–matrix interactions.[4–7] Likewise, microfluidic perfusion culture allows novel experiments for controlling the microenvironment, which affects cellular phenotypes.[2,8] Laminar flow in microfluidic systems enables the controlled application of shear stress,[9] and the delivery of multiple laminar streams of different soluble molecules at the cellular or subcellular levels.[10] For tissue types that are highly perfused *in vivo*, such as the liver and kidney, microfluidic perfusion culture may more accurately mimic the *in vivo* microenvironment, where cells are in close proximity with the microvascular network. Having a physiologically relevant *in vitro* model that preserves the *in vivo*-like phenotypes of cells from these tissues enables biologically meaningful data to be extracted during cell-based assays.[3,11] Conversely, microfluidic perfusion culture can effect a defined, artificial microenvironment by continuously controlling the supply and removal of soluble factors.[12] Such defined environments are unobtainable in static culture, where the background

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of soluble factors and nutrients changes constantly over time. Fluid handling operations for higher-throughput analysis can be readily implemented by integrating microfluidic valves, mixers and gradient generators.[1] Furthermore, microfluidic perfusion culture allows the possibility of continuous non-recirculating culture that would be impractical at the macro-scale due to reagent volumes.

A number of microfluidic perfusion culture systems have been developed.[12–22] While some have demonstrated proof-of-concept success in biological studies[23] or drug testing,[24] routine application in biomedical fields has not been achieved. We believe that this may be due to the complexity of such systems, which require significant integration of cell culture and microfluidic operations, thus presenting challenges to the average biomedical scientist (who normally works at the macro level) and microfluidic engineer (who may not be familiar with cell culture). There is also a need for effective integration between microfluidic perfusion culture systems and ultra-sensitive analytical micro-assays to enable higher-throughput extraction of the biological data required in many applications. While there have been separate reviews on the fabrication of microfluidic systems,[25,26] on the design of cellular micro-environments *e.g.*, cell–matrix interactions, and transport phenomena,[11,27] as well as on micro-assays for probing cells,[28,29] these issues are interdependent in microfluidic perfusion culture and need to be discussed in a more integrated fashion. An understanding of the constraints imposed at each stage of performing microfluidic perfusion culture (*e.g.* microfluidic system fabrication, cell culture and assay) will enable one to design and operate a microfluidic perfusion culture system more effectively.

Here we aim to provide novices with a practical guide to expedite the realization of robust microfluidic perfusion culture systems. We provide an overview of relevant design and operational issues and outline guiding principles to implement a microfluidic perfusion culture system (Fig. 1). When appropriate, we use poly(dimethylsiloxane) (PDMS) microfluidic perfusion systems as examples to illustrate these principles; PDMS microfluidic perfusion culture systems are one of the most commonly used systems due to their availability, low cost, and compatibility with rapid prototyping (see supplementary protocol in the ESI for the robust operation of a typical microfluidic perfusion culture system). We also highlight recent developments and challenges involved in integrating existing micro-assays to monitor and probe cellular behaviors in these microfluidic perfusion culture systems.

2. Microfluidic perfusion system design and fabrication

Designing a microfluidic perfusion culture system involves many decisions, including the choice of materials, microfluidic layout, fabrication process, packaging, and sterilization technique. Requirements such as sterility and biocompatibility are non-negotiable; other design parameters are dictated by the intended application and assay method. For example, having independent fluidic addressing to a cell culture array is critical to enable parallelized screening of multiple drugs, while the use of transparent biomaterials is mandatory for live-imaging of cellular dynamics. The incorporation of cells into the system contributes another important factor that affects the system design and fabrication. For example, surface modification procedures to facilitate cell attachment and cell culture configuration, *i.e.* whether cells are cultured as 2D monolayers or in 3D scaffolds or gels, may subsequently place restrictions on the design, fabrication and assembly of the system. Since each requirement narrows the design options, a broad awareness of currently available design solutions is helpful for generating a workable design.

2.1 Biomaterials

The biomaterials for a microfluidic perfusion culture system fall into two main categories: the materials comprising the microfluidic channels and the materials onto which the cells attach. All materials must be, at a minimum, non-cytotoxic. The choice of materials may be further restricted by requirements such as optical transparency, cell patterning, electrical stimulation/recording, chemical sensing, *etc*.

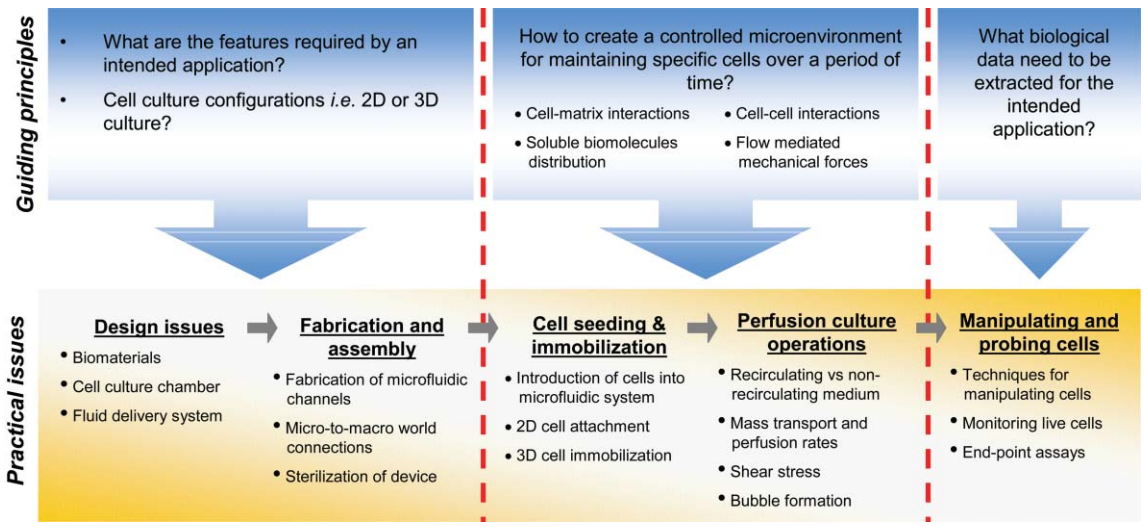

Fig. 1 Guiding principles and practical issues for the robust operation of a microfluidic perfusion culture system for biomedical applications.

Materials for microfluidic channels. Materials for the microfluidic channels must conduct the perfused liquids to the cells. This material may be the same as the cell substrate (*e.g.*, glass microfluidic channels on a glass substrate) or different (*e.g.*, PDMS microfluidic channels on a glass substrate). Microfluidic perfusion culture systems have been fabricated out of many materials (Table 1). The elastomer PDMS is by far the most commonly used material for construction of microfluidic perfusion culture systems.[12,13,16,18–23] PDMS has many desirable qualities as a cell-culture material. It is non-cytotoxic,[30] autoclavable, and since PDMS is gas permeable, thin PDMS membranes (thickness $\sim$ 100 μm) can be used as gas exchange surfaces in microfluidic perfusion culture systems to support cell culture.[16] From a fabrication standpoint, the flexible nature of PDMS allows the creation of integrated mechanical valves and pumps;[31] PDMS also enables patterning structures on the order of microns using soft lithography.[32,33] Finally, PDMS has appealing properties for microscopy; it has low autofluorescence when compared with many plastics used in microfabrication,[34] making it suitable for fluorescence imaging applications. Other materials may have advantages in terms of biological compatibility, fabrication, assay, or specific features required for an application *e.g.*, electrical recording. However, PDMS presents an appealing set of properties that are suitable for constructing general microfluidic perfusion culture systems.

Despite the advantages to using PDMS, the material also highlights challenges faced by such materials for microfluidic perfusion systems. Water vapor and organic solvents can readily permeate PDMS, potentially causing unwanted evaporation and changes in osmolality,[35,36] which is detrimental to cell culture. Heo *et al.* observed evaporation-mediated changes in osmolality when culturing cells in PDMS devices.[35] We have observed that water or ethanol entrained in PDMS can decrease its clarity; the PDMS can appear cloudy, as though micron-scaled particulates are included throughout the bulk material. This cloudiness disappears upon drying. Water transport into PDMS can also cause unwanted permeation-driven flows, which can be eliminated by pre-soaking the PDMS to supersaturate it prior to an experiment or by coating the PDMS with parylene to prevent water permeation.[35,37] For example, a simple solution is to saturate PDMS microfluidics by perfusing the system with culture media for 24 hours prior to cell seeding. Transparent thermoplastics such as poly(methylmethacrylate) (PMMA) typically have lower permeability than PDMS,[35,38] circumventing some of the permeation problems associated with PDMS.

Materials for cell substrates/scaffolds. The choice of cell substrate material is critical for achieving cell attachment in 2D microfluidic perfusion culture systems, especially for fastidious cell types such as primary cells in serum-free media. The cell substrate material allows cell attachment *via* adhesive proteins or peptides adsorbed from the cell culture media, or pre-immobilized cell-attachment proteins such as gelatin, collagen,[17] laminin,[12,55] fibronectin,[18] or poly-L-lysine.[12] In conventional cell culture, adherent cells are typically grown on tissue-culture polystyrene substrates. If standard tissue-culture substrates can be incorporated into the microfluidic perfusion

Table 1 Common materials used for the fabrication of microfluidic networks in microfluidic perfusion culture systems

	Properties relevant to microfluidic perfusion culture systems						
Materials	Fabrication techniques	Visible light transmittance[a]	Autoclavable[b]	Water diffusion coefficient[c] $\times 10^9$/m^2 s^{-1}	Gas permeability[c] $\times 10^{10}$/cm^3 (STP) cm (cm^2 s cm Hg)$^{-1}$	Young's Modulus[d]/GPa	Examples of microfluidic perfusion systems
Poly(dimethylsiloxane) (PDMS)	Soft lithography	Clear	Yes	3–6 (Heo *et al.*[35])	N_2: 280 CO_2: 340 O_2: 600 (Mark[39])	3.6×10^{-4}–8.7×10^{-4} (Armani *et al.*[40])	12, 13, 18, 19, 21, 22, 41–43
Silicon	Micro-electronics fabrication	Opaque	Yes	N/A	N/A	165 (Dolbow *et al.*[44])	45, 46
Glass	Micro-electronics fabrication	Clear	Yes	N/A	N/A	63–73 (Smith[47])	15, 48
Poly(methyl-methacrylate) (PMMA)	Hot embossing, injection molding, laser photoablation	Clear	Yes	0.002 (Rodriguez *et al.*[38])	N_2: 0.039 CO_2: 0.78 O_2: 0.23 (Nakai *et al.*[49])	3.3 (Brandrup *et al.*[50])	14, 51
Polysulfone	Hot embossing, injection molding, laser photoablation	Clear	Yes	0.009 (Schult *et al.*[52])	N_2: 0.2 CO_2: 8 O_2: 1.5 (Hu *et al.*[53])	2.47 (Brandrup *et al.*[50])	54

[a] Affects compatibility with live-cell imaging during microfluidic perfusion culture. [b] Sterilization of microfluidic perfusion culture system at 121 °C, 20 min. [c] Affects culture conditions within the microfluidic perfusion culture system. [d] Elasticity affects chip-to-world interface and integration of other microfluidic components *e.g.* valves.

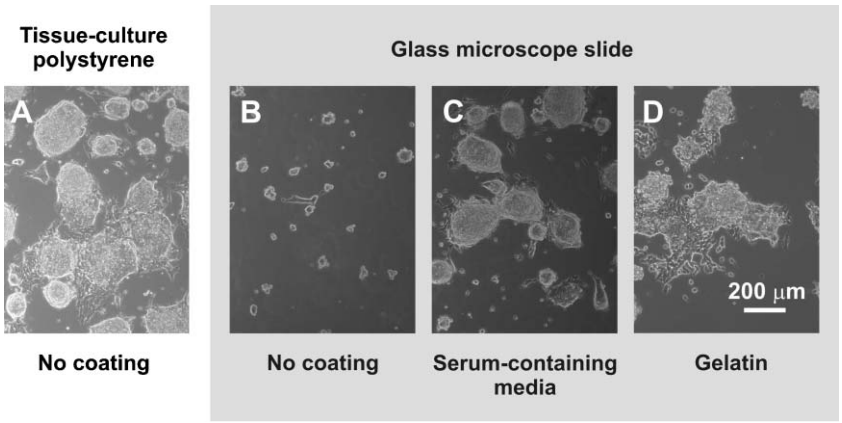

Fig. 2 Surface modification of cell substrates with cell attachment molecules can be used to improve their biocompatibility. Murine embryonic stem cells exhibit different adhesion and morphology on different substrates. (A)–(C) show ABJ1 murine ES colonies on day 3 of static culture. (A) ES colonies on uncoated tissue culture polystyrene exhibited good attachment with typical round, compact colonies. (B) Although ES cells will initially attach to untreated glass microscope slides, by day 3 of static culture, there are few cells remaining. (C) ES growth on glass slide coated with serum-containing media for 1 hour. (D) A separate plating of ABJ1 murine ES showing growth on a glass slide coated with 0.1% gelatin.

culture system, this is a preferred route because it avoids any question of biological effects due to cell substrate. However, tissue-culture polystyrene can be challenging to use because it cannot bond to PDMS; is autofluorescent at the UV/Blue range (360/460 nm); cannot be autoclaved; and is poorly compatible with microfabrication of the substrate (due to its limited solvent resistance). Although it is possible to microfabricate polystyrene, it must be treated to render it partially hydrophilic to be ''cell-compatible'', and even then may not exactly reproduce the surface of standardized tissue culture dishes. Therefore, other materials including glass,[21,54] PDMS,[30] PMMA,[14] silicon,[55] and silicon nitride[56] are commonly used as the cell substrate in 2D microfluidic perfusion culture systems.

As mentioned above, cells do not directly attach to cell substrates but rather to intermediate molecules, typically peptides or proteins. Cell substrates commonly used in microfluidic perfusion culture systems may need to undergo surface modification to improve their compatibility *via* adsorption or chemical conjugation of cell-attachment molecules. The issues governing surface modification with cell attachment molecules in microfluidic perfusion culture systems are substantially similar to those in conventional and microscale static systems, and have been previously reviewed.[57]

In the example with PDMS microfluidic perfusion culture systems, glass is often used as the cell culture substrate because glass can be permanently bonded to the PDMS microfluidic network. While glass is an appealing cell substrate material from a microfabrication and imaging point of view, it can present challenges to cell attachment. As an example, uncoated glass normally does not support the culture of murine embryonic stem cells (mESCs) in serum-containing media, although mESCs grow well on uncoated tissue-culture polystyrene (Fig. 2A and B). By pre-incubating glass with serum-containing media or gelatin for 1 hour, mESCs showed enhanced attachment, permitting them to be cultured in microfluidic perfusion systems. Thus, surface modification of cell substrates to facilitate cell attachment can be critical in microfluidic perfusion culture. In fact, surface modification of cell substrate is more critical in microfluidic perfusion culture systems than in static systems because one typically has a limited time to allow for cell attachment under static conditions before media perfusion must be initiated to replenish depleted nutrients.

In macroscale 3D perfusion culture systems, cells are typically grown on biodegradable 3D scaffolds through which cell culture medium is perfused to create *in vitro* 3D tissues that can later be implanted *in vivo*. Microfluidic perfusion culture systems can serve as miniaturized models for these perfused 3D scaffold-cell constructs. 3D biodegradable scaffolds incorporated into microfluidic perfusion systems include poly(DL-lactide-*co*-glycolide) (PLGA),[58] and poly(glycerol-sebacate) (PGS).[17] Alternatively, cells can also be encapsulated and immobilized in hydrogels to form perfused 3D cultures.[45] Formation of these 3D hydrogels must be localized in the microfluidic system so as not to obstruct fluid flow. Hydrogels that have been adapted into microfluidic perfusion culture systems include complex coacervated polyelectrolytes *e.g.* cationic collagen[59] and anionic 2-hydroxyethyl methacrylate-methacrylic acid-methyl methacrylate (HEMA-MMA-MAA) terpolymer as well as photo-polymerizable poly(ethylene glycol) (PEG).[5]

2.2 Packaging

Robust packaging is crucial for the success of microfluidic perfusion culture systems. The primary packaging challenges are in the sealing of the microfluidic network and the chip-to-world interface. The chip-to-world interface refers to the connections between the microfluidic network and macroscale components such as valves and pumps.

One critical requirement for microfluidic culture systems is the creation of robust sealed microfluidic channels to form flow conduits. The fundamental tradeoff here is between cell accessibility for downstream assays, which requires reversibly sealed or puncturable channels, and channel robustness, which implies a permanently sealed channel.[12] For example, Gray *et al.* have designed two incarnations of the same device, one

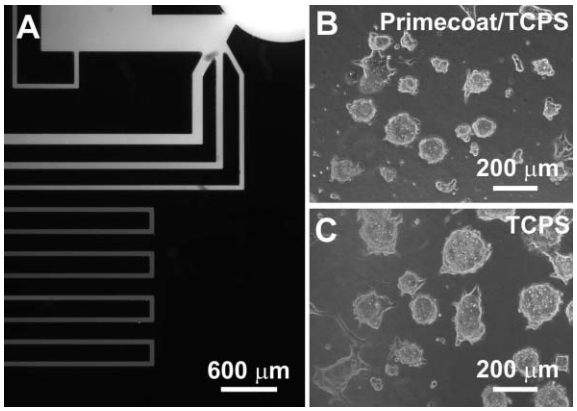

Fig. 3 Although PDMS is usually bonded to glass or silicon, adhesives such as Dow Corning Sylgard PrimeCoat can enable bonding of PDMS to other surfaces, including tissue culture polystyrene and gold. Tissue culture polystyrene was treated with enough full-strength Sylgard PrimeCoat to cover the surface before removing excess PrimeCoat. The coated polystyrene was incubated at room temperature for one hour, and irreversibly plasma bonded to a PDMS microfluidic network. (A) PDMS-bonded-to-polystyrene microfluidic network filled with fluorescein dye. (B) Day 3 perfusion culture of murine embryonic stem cells (mESCs) on a PrimeCoat bonded polystyrene substrate. (C) Day 3 perfusion culture of mESCs on an uncoated, reversibly clamped polystyrene substrate. The stem cells in both (B) and (C) exhibit healthy, round, compact colony morphology, and thus demonstrate that culture on PrimeCoat-modified surfaces does not significantly alter mESC culture.

using a puncturable silicone elastomer for easy access to cells, and one with a glass cover when access to cells during the experiment is not necessary.[46] PDMS can be permanently plasma-bonded to glass, silicon, or PDMS surfaces. PDMS can be reversibly sealed *via* clamping to most smooth, flat materials. When a permanent bond is required but material choices prevent plasma bonding, PDMS can be attached with a combination of adhesive and plasma bonds. The use of the adhesive Sylgard PrimeCoat (Dow Corning #327G) enables plasma bonding between PDMS and other non-glass, non-silicon surfaces, including polystyrene and gold (Fig. 3). We have found that concentrations of 10%–100% of PrimeCoat by volume, diluted in heptane, all created irreversible bonds. It is known that heating devices after bonding can improve the bond strength;[33] we also found this true when devices bonded with PrimeCoat were heated overnight at 60 °C.

In terms of chip-to-world interface, common material choices for tubing and macrofluidics that connect the microfluidic perfusion culture system to the macro world include PEEK, Teflon®, Tygon®, and silicone. Using macrofluidic components such as valves and tubing connectors from High Pressure Liquid Chromatography (HPLC) systems (Upchurch Scientific) are preferred as these parts have low dead volumes and are chemically resistant and autoclavable. Teflon® or Tygon® tubing can be especially useful to route connections because these materials are flexible. While silicone tubing is also flexible, its elasticity and gas-permeability can introduce the risk of unwanted air bubbles during operation if the tubing is inadvertently stretched and a vacuum is created.

2.3 Design of cell culture chamber and fluid delivery system

Cell culture chamber. The cell culture chamber in microfluidic perfusion culture systems defines the effective culture volume (ECV), which is a measure of the cells' ability to control their microenvironment during perfusion culture.[27] The ECV of microfluidic perfusion culture systems is generally characterized by large surface-area-to-volume ratio,[27] although there exist variations among the cell culture chamber designs that affect their mass transport characteristics and general functionality. The simplest culture chamber design has cells adhering and growing as a 2D monolayer at the bottom surface of a microfluidic channel. Cells in this culture chamber experience direct laminar flow in one direction; therefore the ECV in this design is characterized by convective mass transport in one direction *i.e.* the fluid flow axis (Fig. 4A).[27] This design has been adopted by a number of microfluidic perfusion systems,[10,18,19] in particular, to capitalize on the laminar flow profiles for selective delivery of molecules *e.g.*, cytokines and drugs to different regions of the live cell culture.[10,19] This design can also be used to deliver laminar streams of different cell types to achieve cell patterning.[8]

Other microfluidic perfusion culture systems have incorporated an array of simple chambers of different geometrical shapes connected by microfluidic channels to achieve parallelization.[13,16,60] Cells residing in these culture chamber arrays still experience direct convective flow primarily limited to the fluid flow direction *i.e.* x-direction. However, the change in geometrical shape when fluid flows from the microfluidic channel to the cell culture chamber may result in an increase of mass transport distance in the y-direction (Fig. 4B). Significant effects of this change in fluid profiles on cellular responses have not been reported; however, the lack of straight laminar flow profiles in these cell culture chamber arrays limit their application in selective delivery of cells and molecules.

Cell culture chambers have also been designed to simulate the mass transfer characteristics of the *in vivo* tissue environment, which can be described by convective transport of nutrients in capillary vessels that are in close proximity with cells (usually within 100 μm), and diffusive transport across the interstitial space to individual cells. Some microfluidic perfusion culture systems use cell culture chambers that are isolated from the bulk fluid flow so that cells are not subjected to direct convective flow. In such designs, mass transport distances are similar in all directions, resulting in more uniform transport throughout the ECV (Fig. 4C). For example, Lee *et al.* designed a high-aspect-ratio C-shaped cell culture chamber[20] while Powers *et al.*, fabricated micro-wells[61] to shield cells from direct convective flow. Toh *et al.* designed cell culture chambers consisting of a 3D matrix with[22] and without micropillar arrays[59] to support cells in 3D while isolating them from convective flow. The use of the aforementioned cell culture chambers that are isolated from bulk fluid flow can protect cells from detrimental effects of hydrodynamic shear and may be ideal for culturing shear-sensitive cell types such as primary hepatocytes.[62]

Fluid delivery system. Fluid delivery in microfluidic perfusion culture systems can be divided temporally into 3 phases:

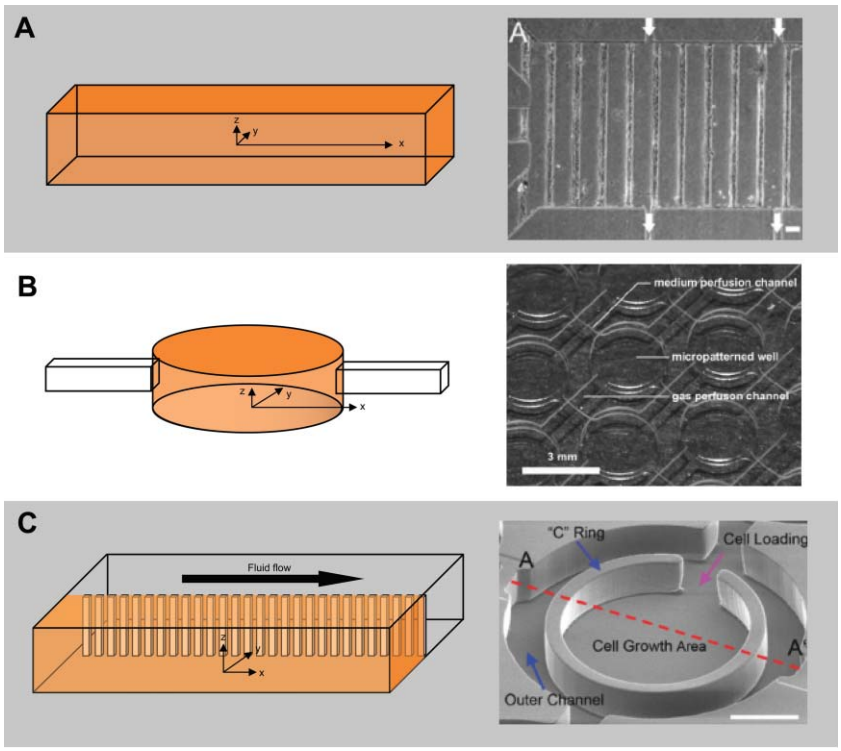

Fig. 4 Cell culture chamber designs for microfluidic perfusion culture systems. Left panels are illustrations of different culture chamber designs; shaded volume indicates the effective culture volume (ECV) where cells exert control over their microenvironment. Fluid flow is directed in the *x*-direction. Right panels are examples of microfluidic perfusion culture systems that adopted the corresponding culture chamber design. (A) Cells are cultured within a microfluidic channel. Laminar flow present in microfluidic channels is used to control the spatial delivery of soluble factors to patterned C2C12 myoblasts.[19] (B) Cells are cultured in simple geometrical chambers connected by microfluidic channels. A hepatocyte cell culture array for parallelized drug testing.[16] Reproduced by permission of American Chemistry Society. (C) Cells are cultured in a volume shielded from direct convective flow by means or microfabricated structures or membranes. A high-aspect-ratio C-shaped cell culture chamber designed to achieve greater uniformity in mass transport properties.[20] Reproduced by permission of Wiley InterScience.

(1) cell seeding, (2) perfusion cell culture and (3) cell testing. In phase 1, microfluidic channels are required to deliver a cell suspension from an external source to the cell culture chamber where the cells are immobilized. During cell culture (phase 2), culture medium is continuously perfused through the cell culture system over a prolonged period of time. Unless high shear stress is desired, fluid velocity during this phase is relatively low and is dictated by the balance between adequate mass transport and limited hydrodynamic effects (see section 3.2). During the 3rd phase, reagents for probing cells such as fluorescent dyes or metabolic substrates are delivered to the cells within a short period of time (usually within a few hours), after which the cells are assayed for cellular activity. The fluid flow during this testing phase is higher ($\sim$3 to 5 times) so that reagents can penetrate the cell culture quickly and uniformly. The fluid delivery network for many microfluidic perfusion culture systems is common for all 3 phases,[16,18,60,63] rendering the microfluidic network design relatively simple. The fluidic control during the 3 phases in such systems is often imparted by external pumps and valves. Other microfluidic perfusion culture systems have independent microfluidic networks to cater to the different flow operations during the 3 phases of fluid delivery.[19,20] For example, separate fluidic inputs for cell suspension and culture media ensure that when phase 2 begins, fresh media (not cell-suspension) is delivered to the cell culture chamber. Independent microfluidic networks can also incorporate built-in designs to alter the fluidic resistance (*e.g.*, smaller microfluidic channel dimensions to increase fluidic resistance) for tuning the mass transport properties independently of external pumping conditions.[20] In addition, the issue of independent fluidic addressing needs to be considered when designing multiplexed systems for higher-throughput cellular assays.[16,20] Individual cell culture chambers must have dedicated fluidic addressing so that discrete microenvironments can be maintained simultaneously for probing cellular responses.

2.4 Sterilization techniques

Effective sterilization of microfluidic perfusion culture systems is critical to maintain the culture over prolonged periods of time. Autoclaving of the microfluidic device and its peripheral components is one of the simplest and most effective sterilization techniques, although it is not always compatible with the biomaterials used[64] or surface modification procedures.[16] For instance, autoclaving systems with micro-patterned collagen for cell attachment will result in the denaturing of the protein. From our experience, polymeric fluidic components, especially some external valves (*e.g.*, Cat. P-782 Upchurch Scientific) can also deform at 121 °C; it is possible to autoclave these

polymeric parts at 105 °C for 30 minutes, which has been previously used to sterilize polycarbonate bioreactors.[65] If autoclaving is not possible, other reported techniques for sterilizing microfluidic perfusion culture systems include flushing the device with ethanol,[13,20,21] incorporating sterilizing chemicals such as chloroform during device fabrication[64] and exposing the device to UV light or oxygen plasma.[63]

3. Microfluidic perfusion culture system operation

The microfluidic perfusion system is operated to optimize the microenvironment for cell functions. Cell seeding and immobilization strategies need to ensure that cells can interact with the extracellular matrix (ECM) and other cells in either 3D or 2D configurations in a controllable and uniform manner. During perfusion culture, nutrients and oxygen must be efficiently transported to the cells while metabolic wastes are removed. Fluid flow needs to be regulated since it can elicit biological responses *via* mechnotransduction. These requirements govern the operating techniques and parameters of the system. Technical issues such as maintenance of the culture media temperature and pH as well air bubble formation must be addressed. In the following sections, we discuss the techniques used to seed and perfusion culture cells in microfluidic perfusion culture systems to achieve robust performance.

3.1 Cell seeding

Cell seeding in microfluidic perfusion culture systems is a dynamic process, where a cell suspension is infused or withdrawn from an external reservoir into the cell culture chamber *via* fluidic connections. This imposes different operational constraints as compared to static seeding that is commonly used in macro-perfusion systems.[66,67] A major challenge lies in controlling the cell density seeded into the cell culture chamber, which affects the extent of cell-cell interactions and consequently influences cellular behavior. The seeding flow rate employed must be relatively low so as not to compromise cell viability. However, low flow rates may cause cells to settle in the reservoir and fluidic connections, causing non-uniformity in the final cell density. The uniformity of the cell density can be improved by: (1) minimizing the distance between the cell reservoir and the cell culture chamber, (2) using a viscous carrier *e.g.*, collagen solution for suspending cells so that settling occurs more slowly, and (3) rotating the cell reservoir. Other issues pertaining to cell seeding are specific to the cell culture configuration *i.e.* 3D or 2D culture and shall be discussed in more detail in the following sections.

Cell seeding for 3D culture. 3D cell culture is implemented by immobilizing and supporting the cells three-dimensionally in the cell culture chamber (Fig. 5A). 3D cell seeding has been accomplished by either cell immobilization in micro-patterned matrices[5,68,69] or confinement of cells into pre-fabricated 3D micro-chambers or scaffolds, coated with a layer of ECM for cell attachment.[61,70,71] In the former configuration, the cell-embedding matrix confers support for both the cells and the cell-matrix structure. The matrix must be sufficiently strong to withstand mechanical stresses during perfusion culture, which

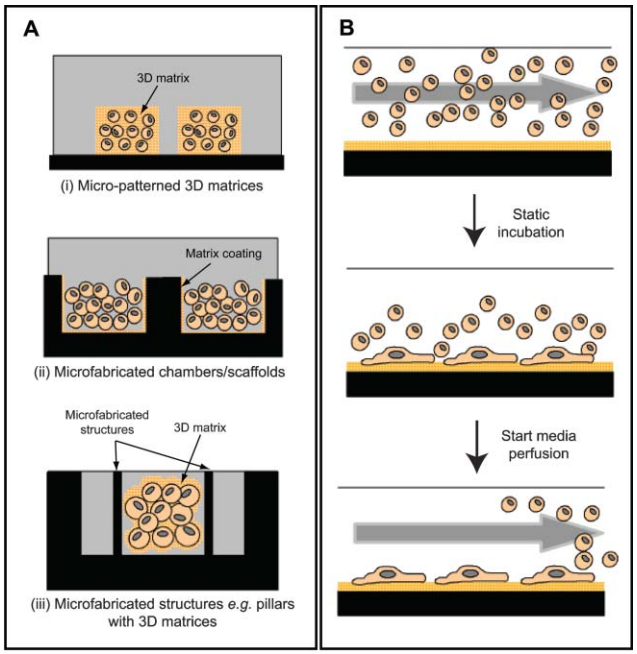

Fig. 5 Methods of cell seeding in microfluidic perfusion culture systems for 2D or 3D cell culture. (A) Cells can be immobilized in 3D within microfluidic systems by (i) micro-patterned matrices, (ii) microfabricated chambers or scaffolds, and (iii) a combination of micro-structures *e.g.*, micropillars and 3D matrices. (B) Cell seeding for 2D culture involves introduction of a cell suspension into the cell culture chamber, followed by a static incubation period to allow for cell attachment, and initiation of media perfusion for perfusion culture.

tends to impose mass transfer limitation of oxygen and nutrients to the cells.[72] This problem is usually compounded by the fact that the choice of biomaterials is limited by the employed matrix-patterning technology (*e.g.*, photo-patterning requires the use of photo-polymerizable matrices) and may not be optimal for supporting cellular functions.[73] In the latter configuration of confining cells in 3D micro-chambers or ECM-coated scaffolds, the cell-matrix interaction is still 2D even though the cells might aggregate to form 3D clusters over extended culture periods.[61] It is possible to use a combination of microfabricated structures, *e.g*, micropillars and a thin layer of 3D matrix, to immobilize and support cells so that they can experience 3D cell-matrix interactions without mass transfer limitations.[22]

Cell seeding for 2D culture. A critical issue in 2D microfluidic perfusion culture is to ensure good cell attachment during cell seeding so that cells are not washed away upon media perfusion. Cell substrate materials and surface modification strategies to facilitate cell attachment have been discussed in section 2.1. Cells are often allowed to attach to the cell substrate in static conditions after seeding (Fig. 5B). There is a time window to allow for sufficient cell attachment without nutrient and oxygen depletion. The duration of this static attachment time may vary from 2 hours[17,74] to overnight,[13] depending on cell type, cell density, cell substrate properties, ECV, and choice of culture medium used during seeding. For example, with mESCs we typically allow 4 hours for static

attachment for 2×10^6 cells mL^{-1} in serum-containing media on a tissue-culture polystyrene substrate.

Some groups have also demonstrated patterned seeding of cells in microfluidic perfusion culture systems by combining established cell patterning techniques with perfusion culture.[16,19,63] These methods involve restricting cell growth along the surface using patterned ECM. For example, Kane *et al.* have demonstrated patterned co-culture of hepatocytes and fibroblasts within a microfluidic perfusion culture system.[16]

3.2 Microfluidic perfusion culture

Once the cells are immobilized and perfusion has started, there may be a range of flow rates at which cell functions, such as proliferation or differentiation, are optimized.[19,21] Perfusion modes can be categorized as recirculating[13,18] or non-recirculating.[12,17,21] In recirculating culture, a given volume of culture media (usually large) is recirculated throughout the microfluidic perfusion culture system, whereas in non-recirculating perfusion, the culture media is perfused through the system and sent to directly to waste. Secreted factors and waste products in recirculating cultures are diluted into the total culture media volume and recirculated back to the cells, whereas in non-recirculating culture, the secreted factors and waste products are permanently removed. Culture media perfusion can be driven by gravity,[19] external syringe pumps[12,54,61] and on-chip peristaltic pumps.[18] Using these methods, long-term microfluidic perfusion culture has been achieved by several groups for longer than 1 week.[12,17,45,74]

Operating environment of microfluidic perfusion culture systems. Appropriate temperatures and pH must be established to ensure maintenance of the cell culture. The entire system can be placed in a conventional 37 °C, 5% CO_2 incubator to maintain the culture medium at the appropriate temperature and pH;[19,21,70] however, this may hamper live-imaging or monitoring of cells during perfusion culture. Temperature can be maintained at 37 °C outside the incubators using hotplates[22] or transparent indium-tin-oxide (ITO) heaters.[14,74] Most culture media are formulated to achieve a constant pH of 7.2–7.4 under 5% CO_2 and will shift towards a higher pH when equilibrated under atmospheric conditions.[65] Culture media pH can be maintained outside the CO_2 incubators by the addition of HEPES buffer[22,65] or by pre-equilibrating the cell culture media in a 5% CO_2 environment before perfusing it through a microfluidic perfusion culture system with low gas permeability. CO_2-independent medium for cell culture at atmospheric conditions has also been used to operate microfluidic perfusion culture systems outside the CO_2 incubators.[74] The microfluidic perfusion culture system should also have minimal light exposure when operated outside an incubator since light can cause reactive oxygen species (ROS) generation, especially in culture medium containing HEPES buffer and lacking phenol red.[75]

Mass transport of soluble factors. Various factors affect the steady-state distribution of nutrients and oxygen as well as secreted growth factors and metabolic wastes in the system. There may be a temporal dependence of this distribution as different cells' uptake and secretion rates may change over time. Some of the factors affecting transport include culture media flow rates, cell culture chamber geometries, cell type, cell densities, and circulating/non-recirculating perfusion modes. In addition, the desired soluble microenvironment depends on cell type. For instance, some cells (*e.g.*, hepatocytes) require high oxygen concentrations,[54,62] whereas other cells, such as embryonic stem cells, proliferate better at lower oxygen levels.[76] In general, perfusion culture provides the opportunity to increase nutrient delivery when compared with static culture. Perfusion culture may also affect the local concentration of secreted factors by diluting them into a larger volume (in a recirculating system) or by permanently sweeping away secreted factors (in a non-recirculating system). In addition to the perfusion mode, it is possible to adjust the local accumulation of secreted factors by tailoring the culture chamber geometry and culture media flow rate.

Changing the culture media flow rate and/or the cell culture chamber geometries, most commonly chamber height, are the most straightforward means of optimizing mass transport of nutrients and oxygen to the cells as well as controlling the local concentration of secreted factors and metabolites. In general, cell types (such as hepatocytes or embryonic stem cells) which require frequent feeding in static culture also need higher culture media flow rates in perfusion culture than less demanding cell types (such as fibroblasts) when grown in the same culture volume. Adequate nutrient delivery as well as removal of waste products such as lactate,[77] which can be toxic at high concentrations, is facilitated by increased culture media flow rates and higher cell culture chamber heights. By adjusting these parameters it is possible to achieve adequate nutrient delivery while maintaining low shear stress (see next section). It is useful to understand the relationship between culture media flow rate and chamber height, which collectively determine the average fluid velocity within the cell culture chamber. For example in a rectangular cell culture chamber, the average fluid velocity is given by: $v = \frac{Q}{wh}$, where v = average fluid velocity (ms^{-1}), Q = culture media flow rate ($m^3\ s^{-1}$), w = chamber width (m), and h = chamber height (m). Increasing the flow rate to augment mass transport of oxygen and nutrients at a given chamber height increases the fluid velocity, exposing cells to increased shear stress (see next section). If constant fluid velocity is desired, increasing the chamber height causes increased media consumption (increased Q) in non-recirculating systems. Typical culture media flow rates vary greatly depending on cell type and system design (*e.g.*, culture chamber height and perfusion mode). We list some example culture media residence times in Table 2 as a rough guide for microfluidic perfusion culture of various cell types. Culture media residence time is defined as the time needed for one complete change of culture media in the cell culture chamber, calculated from the chamber dimensions and assuming a uniform average fluid velocity. While the parabolic flow profile obtained in these Poiseuille-flow (also known as laminar-flow)[78] based systems will increase the true culture media residence time, our metric provides a useful starting point for comparison.

To minimize empirical optimization, computational modeling has been used to predict the optimal design and operating conditions for maintaining a specific soluble cellular

Table 2 Reported microfluidic perfusion conditions for various cell types

Cell type	Culture chamber height/μm	Media residence time/min	Estimated shear stress, if provided/dyn cm^{-2}	Recirculating flow?	Reference
Primary rat hepatocytes	100	0.12–0.4	—	Yes	22
	100	2	—	No	15
	100	0.0026	0.7	Yes	81
	100	1.24	—	No	16
	150	0.039–0.77	—	Yes	54
	85–500	0.042–15.63	0.01–21	Yes	62
H4IIE rat hepatocytes	20	0.63	—	Yes	60
HepG2 human hepatocytes	100	0.4	1.4–16	No	82
	100	0.12	—	Yes	22
	50	0.033–4.17	0.001–4	No	20
	20	0.63	—	Yes	60
C3A human hepatocytes	20	0.63	—	Yes	60
3T3-L1 adipocytes	100	2	—	Yes	60
L2 rat lung epithelial cells	20	0.033	—	Yes	60
Primary bovine endothelial cells	50	0.033–4.17	0.001–4	No	20
Bovine aortic endothelial cells	100	0.009	20	No	46
HMEC-1 endothelial cells	35	2.6	—	No	83
HeLa cells	40	24	—	Yes	74
	50	0.033–4.17	0.001–4	No	20
	1250/7500	74/443	—	Yes	14
MCF7 human breast cancer cells	100	0.12	—	Yes	22
Human neural stem cells	100	24	0.0005	No	12
Human SY5Y neuroblastoma	50	0.033–4.17	0.001–4	No	20
3T3 fibroblasts	50	0.033–4.17	0.001–4	No	20
	83	<115	—	No	21
Bone marrow stem cells	100	0.12	—	Yes	22
MC3T3-El osteoblasts	100–200	8	0.05–0.7	Yes	70
C2C12 myoblasts	30	0.3 1.5 7.7		Yes	18
	250	<7.2		No	19
ABJ1, D3 murine embryonic stem cells	83	<3.1		No	21

microenvironment. Several groups have used analytical methods to model nutrient uptake, oxygen transport, and accumulation of secreted growth factors.[54,79] Others have performed finite element modeling simulations to assess filling of microfluidic chambers and nutrient delivery.[74,80] These methods can be helpful in explaining and predicting cell behavior under microfluidic perfusion culture; however, these predicted conditions eventually need to be validated with experimental data using biological read-outs.

Hydrodynamic shear stress. Shear stress is an inherent part of microfluidic perfusion culture systems and is often perceived as a limiting factor in microfluidic perfusion culture due to its detrimental effects on cells at high levels. However, it is possible to design and operate microfluidic perfusion culture systems such that applied shear stresses are often orders of magnitude below those at which adverse effects are observed.[12,20,21] Methods for mitigating shear stress include lowering fluid velocities,[21] designing high aspect ratio cell culture chambers,[20] and including micropillars or microwells[84] to shield cell cultures. On the other hand, some microfluidic perfusion systems use high levels of shear stress to investigate biological phenomena, such as endothelial cell function[46,85] or cell adhesion.[9] In addition, acceptable levels of shear stress can vary widely depending on cell type (Table 3).[81,86–88] For microfluidic perfusion culture in 2D Poiseuille flow systems, the resulting parabolic flow profile yields a simple estimate of shear stress at the wall:[21] $\tau = \frac{6\mu Q}{h^2 w}$, where μ = viscosity (kg $m^{-1}s^{-1}$), Q = flow rate (m^3 s^{-1}), h = chamber height (m), and w = chamber width (m). For a given flow rate Q, the shear stress may be reduced to acceptable levels by increasing the channel height (lowering the fluid velocity). Since these changes (increasing the height and lowering the fluid velocity) affect not only the shear stress, but the content of the soluble microenvironment, the effects on nutrient delivery and secreted factors must also be considered. The parallel-plate shear stress estimate is useful when dealing with simple rectangular cell culture chambers; however, finite-element simulations may be used to estimate shear stress in devices with more complicated geometries.[20] Another method for assessing the effect of shear stress on cells in a microfluidic perfusion culture system is to assay for stress-induced markers.[88]

Air bubble formation during microfluidic perfusion culture. Microfluidic perfusion culture systems are susceptible to failure by air bubble disruption of the cell culture due to their enclosed nature, small dimensions and the constant introduction of new culture media. At the macroscale, air bubbles introduced by gas spargers to oxygenate culture media in bioreactors have been shown to be detrimental to cell viability.[89,90] Similarly, the presence of air bubbles in a microfluidic perfusion culture system is undesirable as they obstruct fluid flow and kill cells at the gas–liquid interface (Fig. 6A). Air bubbles can arise from residual air due to incomplete priming of the system or spontaneous formation at defect sites. Air bubble kinetics can be divided into 2 phases *i.e.* nucleation and growth. Bubble nucleation is generally not

Table 3 Biological effects of shear stress

Cell type	Shear stress/ dyn cm^{-2}	Experimental conditions	Biological effect	Reference
Vascular endothelial cells	10–100	*in vivo*	Normal *in vivo* range	86
Hepatocytes	<2	*in vivo*	Normal *in vivo* range	81
Mouse embryonic stem cells	6.5	*in vitro*	No significant negative effects on proliferation or self-renewal.	87
Human umbilical vein endothelial cells (HUVEC)	4	*in vitro*	Same c-fos levels as in static controls	88
	25	*in vitro*	Elevated c-fos levels compared with static controls	88
Bovine aortic endothelial cells (BAEC)	4	*in vitro*	Same c-fos levels as in static controls	88
	25	*in vitro*	Elevated c-fos levels compared with static controls	88
HeLa cells	4	*in vitro*	Reduced c-fos levels compared with static controls	88
	25	*in vitro*	Elevated c-fos levels compared with static controls	88
Chinese hamster ovary (CHO)	4	*in vitro*	Same c-fos levels as in static controls	88
	25	*in vitro*	Elevated c-fos levels compared with static controls	88

well understood although it has been suggested that deformation sites such as crevices may be a source for air bubbles.[91] For example, in PDMS systems, tears in fluidic interconnects resembling crevices can result in spontaneous nucleation of microbubbles (Fig. 6B; ESI video). It is important to ensure that PDMS interconnects are free from defects by using sharp coring devices that are commercially available (Technical Innovations, Inc.) or modified from syringe needles, as well as co-molding the interconnects with the microfluidic channel (Fig. 6C).[92] The growth of existing air bubbles within the microfluidic system is governed by the Laplace equation: $P_i = P_{atm} + \frac{2\sigma}{R}$, where P_i is the internal bubble pressure (Pa), P_{atm} is the ambient pressure outside the air bubble (Pa), σ is the surface tension (Nm^{-1}) and R is the air bubble radius (m).[91] The condition for stability *i.e.* $P_i > P_{atm}$ tends to drive gas diffusion out of the air bubble, which helps to collapse it. Therefore, priming of the microfluidic perfusion culture system under high pressure >5 psi, known as "blind filling"[31] helps to collapse residual air bubbles that are not flushed out of the system. Pre-priming the system with low surface tension liquids such as ethanol before filling with culture media also facilitates the removal of air bubbles. Alternatively, the perfusion culture system may be operated under high pressure, akin to a hyperbaric chamber used to treat decompression sickness,[93] to limit air bubble growth. We have investigated various strategies of creating and maintaining a pressurized closed-loop perfusion system and found that elevation of the culture medium reservoir relative to the cell culture chamber to be the most robust method (Fig. 6D).

In the event that air bubbles occur, bubble traps located directly upstream of the cell culture can be useful in preventing air bubbles from disrupting the cell culture.[21,94] This is especially important if the perfusion system must be interrupted, disconnected, and reconnected during the course of an experiment. If large-volume bubble traps are incorporated upstream of the cell culture chamber, cells should be seeded *via* a separate fluidic input to avoid cell settling within the bubble trap. Other techniques for avoiding bubble formation during loading of the device include droplet merging[95] and choosing syringe orientation such that air bubbles are not perfused through the device.

4. Assessment of cellular phenotype and function

Various microfluidic-based analytical systems for manipulating, testing and analyzing cells have been extensively reviewed.[28,29] Here, we highlight microfluidic perfusion culture-specific methods that are useful for extracting biological data from live or processed cells and their microenvironment. In general, the enclosed nature and small dimensions of microfluidic perfusion culture systems pose challenges to cell assays.

4.1 Manipulation during perfusion culture

One motivation for performing microfluidic perfusion culture is to enable the application of microscale cell-manipulation techniques not available at the macroscale.[3] However, microfluidic perfusion culture can also make tasks that are easy at the macroscale more challenging such as the passaging of adherent cells for long-term culture. Hung *et al.* have developed one approach to this problem by perfusing trypsin through the system for a limited time to remove some cells from culture, while retaining others.[43] While this method does remove cells to avoid confluency, it does not fully replicate macroscale cell passaging because many cells remain adherent throughout the process. Lifting cells from the culture substrate and separating the cells from one another to create a single-cell distribution upon replating is a requirement for some cell types, such as mESCs. One could envision a microsystem designed to passage cells in exactly the same way that it is performed at the macroscale. However, this function would have to be specifically designed into the device, as is the case with many types of on-chip cell-culture manipulations.

4.2 Monitoring live-cells and their microenvironment

Microfluidic cell culture systems are ideal for applying small perturbations to the cellular microenvironment and monitoring the kinetics of the resulting cellular response. For example, a micro-patterned microfluidic culture system has been used to monitor axonal transport of central nervous system (CNS) neurons using live-imaging.[23] Cellular dynamics can be monitored *via* imaging or fluidic integration with microanalytical devices to detect metabolites or proteins produced by the cells. Imaging using exogenous fluorophores or transfected fluorescent reporters[96] is ideal for collecting data from microdevices because it enables dynamic measurements; is non-invasive to the microfluidic perfusion culture system; and takes advantage of existing biological imaging tools, since cellular constructs in transparent microfluidic systems are usually less than 100 μm thick (see Table 2). Transfected

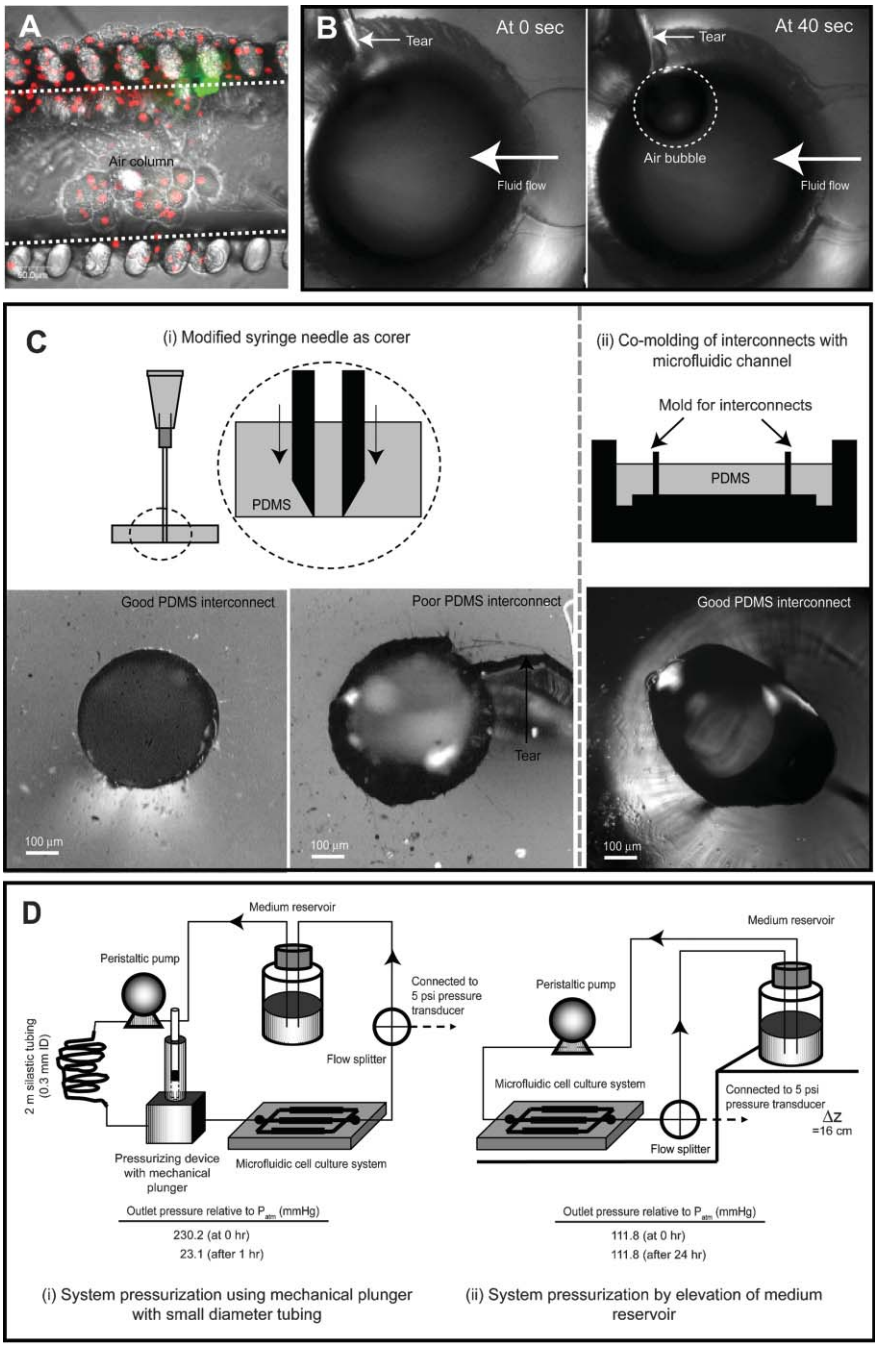

Fig. 6 Strategies to eliminate air bubbles and prevent their formation in microfluidic perfusion culture systems. (A) Presence of air columns (demarcated by dotted lines) in microfluidic perfusion culture system is detrimental to cell viability. Cells are stained with Calcein AM (green, live cells) and Propidium iodide (red, dead cells) (Molecular Probes, USA). (B) Presence of defects in PDMS interconnects causes spontaneous nucleation of microbubbles (circled in white). Images are snap-shots of real-time video acquisition under a light microscope at 20 s interval. (C) Methods of fabricating high quality PDMS interconnects include using a sharp corer to punch holes or co-molding with the microfluidic channels. (D) Strategies for creating and maintaining a pressurized closed-loop microfluidic perfusion system to limit bubble growth by (i) using a mechanical plunger device with small-diameter silastic tubing (0.3 mm ID) and (ii) elevation of medium reservoir from the cell culture system. Both (i) and (ii) can create a pressurized system; only (ii) can maintain the pressure over 24 hours.

reporters are simple to use in microfluidic systems because they require no washing or incubation steps; however development of a stably transfected cell line is time-consuming and may not be possible for some cell types (*e.g.*, primary mammalian cells) or proteins of interest (*e.g.*, large fluorescent proteins that disrupts the cell physiology). Exogenous fluorophores are readily obtained, but their use in microfluidic systems is slightly more complicated as they must be perfused through the system. When perfused at a flow rate such that the residence time of the exogenous dye in the entire perfusion system is short (*i.e.* <1 minute), the staining durations used in static cultures can generally be applied in microfluidic

perfusion cultures. Microfluidic systems mounted onto microscopes with transparent heating systems such as an ITO-resistor plate can serve as live-imaging chambers without suffering from evaporation loss seen in conventional imaging chambers.[97] We have observed that the reduced liquid height of culture media in microfluidic perfusion systems (usually >5 times thinner than in conventional petri dish culture) can significantly reduce autofluorescence due to media containing serum or phenol red (data not shown). Microfluidic systems that allow for optical-based measurement of intra-cellular Ca^{2+} fluxes in response to ATP or glucose stimulation have been reported by using the Ca^{2+} indicators, Fluo3 and Fluo4.[98,99] Non-invasive imaging modalities such as second harmonic generation (SHG) and back-scattering confocal or multi-photon microscopy that do not require exogenous labeling may be useful in characterizing ECM remodeling by cells subjected to various environmental perturbations in a microfluidic system.[100] Fluid flow properties can be monitored in real time using scanning fluorescence correlation spectroscopy (sFCS) for correlation to biological functions.[101]

The majority of the micro-analytical systems are developed for the extraction and analysis of intracellular contents for metabolites and genetic material.[28] Relatively few systems have been reported for the detection of secreted biochemical molecules by live cells although efforts have been made using amperometric detection with a carbon fiber microelectrode,[102] micro-electrophoresis based immunoassay[103] and on-chip ELISA systems.[104,105] Current microfluidic perfusion culture systems routinely measure metabolite and protein production as indicators of the cells' condition in the system, *e.g.* albumin production to measure synthetic function of hepatocytes[16,24] and p-nitrophenol to measure alkaline phosphatase activity in osteoblasts;[70] however measurements are typically performed using macroscale analytical techniques that may require a larger number of cells. More research efforts are needed to develop ultra-sensitive, miniaturized analytical techniques for detecting the plethora of biochemical molecules secreted by a very small number of cells present in these microfluidic perfusion culture systems.

4.3 Endpoint assays

Although live-cell assays are ideal, many useful assays disrupt the cell culture itself and thus can be performed only at the endpoint of an experiment. As with biochemical analytical methods, the small scale of microfluidic perfusion systems creates challenges for endpoint assays because of low cell numbers. There has been much work on micro-versions of the traditional macroscale assays, such as RT-PCR and cytometry.[28,29] However, these technologies are still under development and to date have not been widely integrated with microfluidic perfusion culture. Endpoint assays for microfluidic perfusion culture currently involve mostly on-chip fixing/staining or removing cells from the device for use in macroscale biological assays *e.g.*, RT-PCR. Fixing and staining cells on chip is easily compatible with microfluidic perfusion systems since the reagents can be perfused through the system. Cells may be fixed on chip by injection of paraformaldehyde, particularly in the case of 3D culture where tissue sections are desired.[17] Microfluidic devices may be also disassembled to allow further access to cells. For example, Chung *et al.* detached the PDMS microfluidic network from the glass substrate to perform immunocytochemistry.[12] Gray *et al.* performed an on-chip patch clamp using a microfluidic device with a removable lid to access cells.[46]

Whole or lysed cells can also be extracted from devices using traditional enzymatic methods (such as trypsin) or lysis buffers. However, even if there are adequate numbers of cells in the device, some percentage is often lost during extraction. Once the cells are extracted, they can be analyzed using traditional techniques, such as RT-PCR or fluorescence activated cell sorting (FACS) if there are enough cells. They can also be analyzed using hybrid techniques that fall between macro- and microscale assays. For example, cells can be collected from a microfluidic perfusion culture system, triturated to a single-cell suspension, and then pipetted into a standard well for image-based analysis of cell number and fluorescence. Such a method could be used if there are too few cells to perform conventional flow cytometry.

5. Conclusion

Microfluidic perfusion culture systems are of great interest because they extend the capabilities of macroscale perfusion and microscale static systems. Most reports on microfluidic perfusion systems have focused on elucidating the features of the final system;[3,28] understanding the rationale behind the design, fabrication and operation of such systems is important so that they can be readily adopted. Technical issues, such as device assembly, cell seeding and perfusion regimes, are crucial for the successful implementation of a bubble- and leak-free, integrated, sterile microfluidic perfusion culture system capable of sustaining cell functions. Here, we have highlighted some of the general issues pertaining to the robust operation of microfluidic perfusion systems and proposed strategies to address them in a systematic and integrated fashion. For a microfluidic perfusion culture system to be eventually integrated into microsystems with integrated micro-assays or used as stand-alone biomedical devices for diagnostic or research purposes, operational problems specific to the microfluidic perfusion culture system must be identified, and suitable techniques addressing these issues must be developed, miniaturized and eventually automated. The routine application of microfluidic perfusion culture in biomedical research may potentially shape the approach to solve many biological and medical problems.

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