

# Macro- and microscale fluid flow systems for endothelial cell biology

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Recent advances in microfluidics have brought forth new tools for studying flow-induced effects on mammalian cells, with important applications in cardiovascular, bone and cancer biology. The plethora of microscale systems developed to date demonstrate the flexibility of microfluidic designs, and showcase advantages of the microscale that are simply not available at the macroscale. However, the majority of these systems will likely not achieve widespread use in the biological laboratory due to their complexity and lack of user-friendliness. To gain widespread acceptance in the biological research community, microfluidics engineers must understand the needs of cell biologists, while biologists must be made aware of available technology. This review provides a critical evaluation of cell culture flow (CCF) systems used to study the effects of mechanical forces on endothelial cells (ECs) *in vitro*. To help understand the need for various designs of CCF systems, we first briefly summarize main properties of ECs and their native environments. Basic principles of various macro- and microscale systems are described and evaluated. New opportunities are uncovered for developing technologies that have potential to both improve efficiency of experimentation as well as answer important biological questions that otherwise cannot be tackled with existing systems. Finally, we discuss some of the unresolved issues related to microfluidic cell culture, suggest possible avenues of investigation that could resolve these issues, and provide an outlook for the future of microfluidics in biological research.

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## 1 Introduction

Endothelial cells (ECs) compose a biologically active cellular monolayer (*endothelium*) that lines all blood-contacting surfaces of the cardiovascular system. Because of their anatomic location, ECs experience a multitude of mechanical forces from their local microenvironment.<sup>1</sup> Hemodynamic forces from the luminal blood and adhesive forces between cell-surface anchoring proteins (*integrins*) and the basement membrane (*basal lamina*) contribute to a complex set of mechanical signals that are



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Specific focuses include the pathobiology of heart valve disease, strategies to regenerate tissues using mesenchymal stem cells, and the development of microfluidic tools to study cell mechanobiology.

known to regulate vascular function through a myriad of mechanotransduction-related signaling pathways.<sup>1–3</sup> Mechanical cues from the local EC environment are thus responsible for biological responses at the molecular level, and ultimately, changes in cellular structure and function. The nature and magnitudes of the mechanical stimuli together play a major role in maintaining vascular homeostasis, and regulating the development of cardiovascular pathologies.

Studying endothelial mechanobiology requires engineered tools that can maintain ECs in a controlled *in vitro* culture environment while exposing them to mechanical stimuli. Over the last quarter century, many *in vitro* systems have been developed to study ECs under physical force. The majority of these systems incorporate fluid flow over adhered, confluent EC monolayers to mimic hemodynamic shear stress experienced by ECs in their native environments. These cell culture flow (CCF) systems form a broad class of tools with varied designs tailored to answer specific questions about EC behavior under stress. Much of our current understanding of endothelial mechanotransduction can be attributed to the research work performed using a handful of well-characterized shear flow devices that have become mainstays in typical endothelial cell biology laboratories.

Despite their widespread use, current CCF systems have significant drawbacks: (1) CCF systems are typically custom-designed and cannot be easily modified once the system is fabricated; (2) CCF systems often require auxiliary pieces of equipment that occupy valuable laboratory space, or require dedicated rooms with controlled ambient conditions; and (3) CCF systems probe large EC populations that require sizable volumes of chemicals and reagents both during and after experimentation. Altogether, these issues make efficient large-scale experimentation formidable because of cost and space limitations.

In the past decade, microfluidics has emerged as an advanced technology with wide-ranging capabilities for chemical, biological, and biomedical applications. Microfluidics is dominated by viscous, interfacial, and diffusional forces that have minor roles at larger length scales, but have led to new strategies for manipulating, handling, detecting, and analyzing small discrete fluid samples at the microscale. Many of the notable advantages of microfluidics can be exploited to study effects of mechanical forces on ECs in an efficient and economical manner. For example, laminar flow in microchannels is suitable for generating predictable, flow-induced shear stress on EC monolayers. The small length scales and minute sample volumes within microfluidic systems translate to reduced consumption of reagents. Also, fabrication of microfluidic devices by soft lithography permits short design-to-device turnaround times, making rapid prototyping an affordable option. Therefore, microfluidic systems eliminate many of the aforementioned drawbacks associated with macroscale setups, and thus have the potential to bring forth new scientific discoveries in less time than previously achievable.

Reports on the application of microfluidics for EC studies have emerged from the vast body of “lab-on-a-chip” literature in the last few years. With these new affordable alternatives available to researchers, choosing a suitable system among macro- and microscale options becomes a critical first step in experimental design. The main purpose of this review is to provide a comparison between different CCF systems used to

study effects of mechanical forces on ECs. The focus is to discuss basic principles of macro- and microscale systems, compare their advantages and disadvantages, and uncover new opportunities for developing novel technologies that have potential to both improve efficiency of experimentation as well as answer important biological questions that otherwise cannot be tackled with existing systems. To justify the need for various designs of CCF systems, we first outline main properties of ECs and their native environments. We then review and compare macro- and microscale systems, categorizing each apparatus by the endothelial characteristic for which it was designed to study. In the end, we discuss the outlook for technological advancements and propose studies that could have immediate impact on how biologists in the research community perceive the usefulness of microfluidic systems. Our intention is for this review to serve not only vascular biologists interested in latest technological developments, but also engineers hoping to design creative microfluidic devices that can genuinely benefit the biological research community.

## 2 Endothelial cell properties

For CCF systems to be truly useful for EC studies, engineers designing the systems need to be knowledgeable about endothelial properties, and aware of the relevant questions in basic EC biology. This section broadly summarizes EC properties, and is intended specifically for those interested in a survey of the current problems facing biologists. The hope is to bridge the gap between cell biology and engineering, and facilitate the development of novel, accessible tools from the microfluidics community. We cover several main properties of ECs that are affected by flow-induced mechanical forces, including adhesion strength, migration, adaptive response to shear stress, and permeability. We also briefly discuss the importance of phenotypic heterogeneity within the endothelial population, and how it pervades other important EC characteristics. The scope of the current overview is limited, but other more detailed discussions on these topics by Davies<sup>1</sup> and Aird<sup>4,5</sup> are available, and interested readers are directed to these landmark reviews as reference.

### 2.1 Endothelial heterogeneity

ECs line all blood vessels, including arteries, arterioles, veins, venules and capillaries. ECs also cover outer surfaces of other cardiovascular tissues such as heart valves. Because of the diversity of vascular locations, and the distinct physical properties of the local microenvironments in which ECs reside, ECs are heterogeneous both in structure and in function.<sup>4,5</sup> In arteries, ECs align parallel to the direction of blood flow, elongate to different aspect ratios depending on region, and are known to be less permeable to blood-borne factors than in capillaries and veins.<sup>6</sup> In contrast, ECs in veins are polygonal instead of elongated in shape, lack specific orientation, and are generally more permeable than those in the arterial system. These distinguishable traits are closely correlated to differences in both the hemodynamic environments in which ECs reside and the functional purpose of the vessels to which they belong.

Endothelial heterogeneity has a large impact on *in vitro* experimentation with ECs. Because of the diversity of the

endothelial population, specialized isolation techniques and characterization studies are necessary for each EC type, and a large body of work on such methods has been accumulated.<sup>7–12</sup> Perhaps the clearest example of endothelial heterogeneity *in vitro* was a comparative study of various ECs isolated from different organs of fetal pigs, which showed that typical markers of ECs, including von Willebrand factor, Weibel–Palade bodies, uptake of acetylated low density lipoprotein (diI-ac-LDL), and cobblestone morphology were all organ- and tissue-specific.<sup>13</sup> Indeed, some eminent discoveries on the effects of mechanical stimuli on the endothelium can be generalized to all ECs,<sup>14</sup> but the above example of marker specificity between cell types is clear evidence of the diversity of the endothelium, even in *in vitro* systems.

## 2.2 Adhesion and integrins

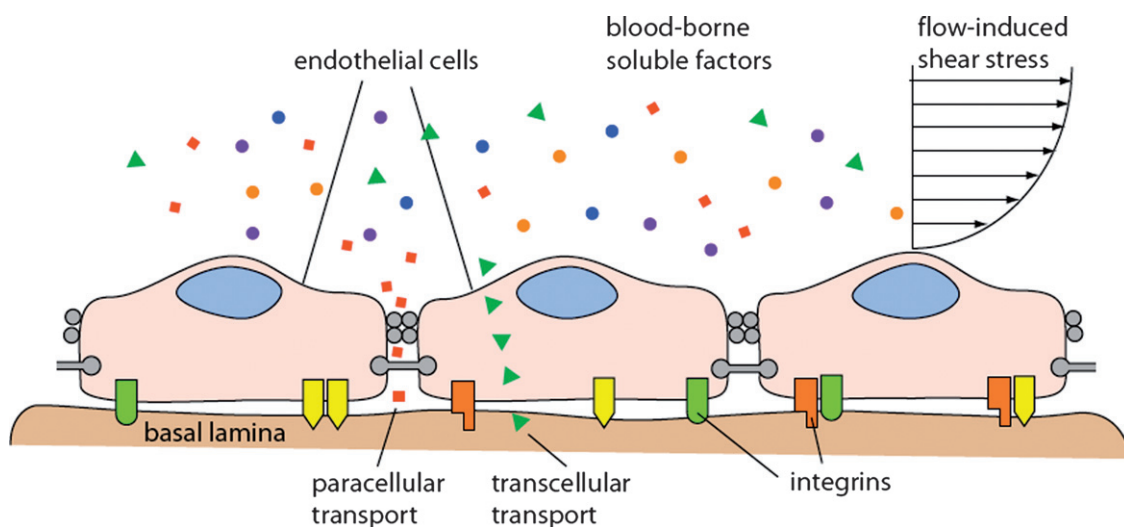
ECs are anchorage-dependent cells that only function when adhered to a substrate. *In vivo*, ECs adhere to a basement membrane, or basal lamina, composed of various extracellular matrix (ECM) proteins that include varying amounts of fibronectin, vitronectin, type IV collagen, and laminin.<sup>15</sup> Adhesion is mediated at the cell surface by integrins, a family of transmembrane heterodimeric glycoproteins that physically link the ECM, the cell surface, and the intracellular cytoskeleton (Fig. 1).<sup>16</sup> Integrins are not only important for establishing focal adhesions as physical anchors to the ECM, but are also responsible in part for transducing mechanical signals generated by blood flow-induced shear stress.<sup>17</sup> Integrin expression levels and their distributions on the cell surface are cell-type specific. Immunohistochemical staining of different regions of the heart showed that ECs from distinct anatomic locations expressed integrins to varying degrees.<sup>18</sup> Because of this specificity, focal adhesions likely have varying strengths, and mechanical signals are likely transduced distinctly for different EC types. Exploration of integrin–ECM interactions for the diverse endothelial population is therefore of fundamental importance for

understanding mechanisms of mechanotransduction. Furthermore, adhesion strength of ECs to matrix proteins is particularly important for tissue engineering applications that require vascular endothelialization of scaffold materials or other biocompatible surfaces for potential implantation. Without proper EC adhesion on scaffold surfaces, tissue-engineered constructs may lack endothelial monolayer integrity, leading to loss of function and ultimately poor long-term performance.<sup>19</sup> Adhesion assays using CCF systems to measure binding affinity and strength of cells on a specific surface have been devised to measure adhesion properties so that we can better understand cell–matrix interactions between cell types and matrix protein components. Thus, development of new and improved methods for testing adhesion have the potential to further the basic understanding of EC biology as well as advance the ability to generate tissue-engineered products for eventual use in the clinical setting.

## 2.3 Migration

Migration of ECs is an important aspect of vasculogenic and angiogenic processes that are critical for both embryonic development as well as the progression of many diseases.<sup>20</sup> Understanding the motility of ECs is therefore critical to fundamental biology and pathology, and to tissue engineering studies that rely on the appropriate spatial organization of cells for proper tissue synthesis and regeneration.

Most often, EC migration is induced by the presence of local gradients of environmental cues, including gradients of chemical factors (*chemotaxis*), substrate rigidity (*durotaxis* or *mechanotaxis*),<sup>21</sup> surface adhesion sites (*haptotaxis*),<sup>22</sup> or some combination of these cues.<sup>20,23</sup> In addition, ECs are extremely motile under exposure to shear stress because of their tendency to elongate and realign with the direction of flow, and the subsequent need for individual cells to reposition themselves within the monolayer to maintain confluence. In the event that confluence is



**Fig. 1** Endothelial cells (ECs) interact with their physical and biochemical microenvironments. ECs are adhered to ECM proteins of the basal lamina *via* signal-transducing and mechanosensing integrins, and rely on this anchorage to resist blood flow-induced shear stress on their apical surfaces. Some blood-borne soluble factors send chemical cues to ECs while others are shuttled through the permeable endothelial barrier *via* paracellular (red squares) or transcellular (green triangles) transport.

lost due to endothelial injury, ECs at the wound edge quickly migrate to seal the gap in the monolayer. Indeed, this property of ECs is exploited in wound healing studies where normal confluent endothelial monolayers are deliberately compromised by scraping the culture cells to generate a wound.<sup>24,25</sup>

Aside from the motile tendencies of ECs themselves, the endothelium – in its relative quiescence – plays a crucial role in the migration of other cell types known to physically interact with the cellular monolayer. Two cell types of particular interest in this regard are leukocytes and circulating cancer cells, both known to interact with the endothelium through recruitment cascades that ultimately lead to transmigration, or extravasation, of these cells through the endothelium. Regardless of whether leukocytes are reacting to an inflammatory response or whether neoplastic cells are homing into a new metastatic location, such complex extravasation events are yet to be fully understood and continue to require intense examination. Elucidating the underlying mechanisms will no doubt require the proper design and implementation of appropriate CCF systems.

## 2.4 Shear stress response

All ECs *in vivo* are exposed to blood flow-induced shear stress (Fig. 1). These forces are imposed directly on the apical surfaces of the ECs, and are transmitted to the cell cytoskeleton *via* transmembrane mechanosensors at the surface.<sup>1,14</sup> As mentioned, the cytoskeleton is also linked to integrins found on the basal surface of ECs.<sup>1</sup> The cytoskeleton therefore forms a bridge between mechanosensors on the luminal side and the integrins on the abluminal side, which have been described as both mechanotransducers<sup>1</sup> and mechanosensors.<sup>26</sup> Shear stress has been shown to mediate endothelial morphological adaptations,<sup>27–29</sup> endothelial permeability,<sup>30</sup> vasoregulation,<sup>31</sup> arterial remodeling,<sup>32,33</sup> and pathophysiological processes leading to atherosclerosis and other cardiovascular diseases.<sup>34</sup> Due to its importance, shear stress effects on endothelial function have been studied extensively in the last forty years, both *in vivo* and *in vitro*.

*In vivo*, morphological adaptations of the endothelium were first observed when Flaherty and co-workers demonstrated that endothelial nuclei from a cell patch that had been rotated 90 degrees from its original orientation (*i.e.*, long axis aligned perpendicular to flow) had the ability to realign their long axis in the direction parallel to flow.<sup>29</sup> Since then, others have found evidence that link distinct *in vivo* flow patterns (laminar *versus* disturbed) to endothelial cell shapes,<sup>1,28</sup> as well as to focal genesis of atherosclerotic lesions.<sup>35</sup>

Although *in vivo* experimentation provides unique insight into shear stress responses of ECs, fundamental studies of shear stress effects have mostly been facilitated by controlled *in vitro* experimentation using CCF systems that mimic physiological conditions while reproducing trends observed *in vivo*. Alignment and elongation of ECs in the direction of flow have been thoroughly demonstrated *in vitro*,<sup>36–40</sup> and these morphological responses have since become standard validation measures for ensuring that CCF systems such as parallel plate flow chambers can reproduce *in vivo* observations with fidelity. Biochemical responses of ECs related to protein synthesis,<sup>41</sup> and production and secretion of vasodilators,<sup>42,43</sup> vasoconstrictors,<sup>44</sup>

thrombogenic factors,<sup>45</sup> and other cytokines<sup>46</sup> have also been extensively studied with controlled fluid flow. In most of these studies, shear stress magnitudes and waveforms are carefully chosen to mimic physiological conditions. Laminar steady flow is the most common mode because it is simple and provides the most direct assessment of the effect of shear in comparison to static culture conditions. Because blood flow during the cardiac cycle is pulsatile, other experiments have incorporated various types of unsteady flow (*e.g.*, oscillatory, non-reversing pulsatile) to evaluate the dynamic effects of pulsatility, such as frequency, amplitude, and degree of flow reversal.<sup>47,48</sup> Of these studies, the most physiologically relevant model was that of “atheroprone” and “atheroprotective” waveforms, which were obtained from a combination of *in vivo* imaging and computational fluid dynamics simulations.<sup>49</sup> Because shear stress waveforms are so complex and varied between vascular locations as well as across species, choice of which shear stress pattern to use *in vitro* becomes an important consideration.

## 2.5 Permeability

The endothelium acts as a semi-permeable barrier between flowing blood and the underlying tissue matrix. As one of its primary functions, the endothelium selectively traffics fluid, solutes, and macromolecules from lumen to interstitium in an effort to maintain specific protein gradients or molecular balances between the two sides. Selective permeability depends on both molecular size and the location within the vasculature. Smaller molecules less than 3 nm in radii pass between intercellular endothelial junctions *via* a paracellular mechanism, while larger macromolecules require vesicular carriers within endothelial cells to carry them through the cell in a transcellular fashion (Fig. 1).<sup>50</sup> Within the vasculature, capillary beds and post-capillary venules are more permeable than large arteries,<sup>4</sup> and this re-emphasizes the heterogeneity of the endothelial population. Permeability controls the passage of molecules through highly regulated transport pathways.<sup>50</sup> Because overall permeability is dependent on junctional integrity as well as density of surface-bound proteins, it is not surprising that permeability is also tightly regulated by cytoskeletal mechanisms<sup>51,52</sup> and by the glycocalyx.<sup>53</sup> Furthermore, loss of junctional integrity and barrier function results in increased permeability, and usually signifies a breach in the endothelial monolayer accompanied by subsequent inflammatory responses. Thus, understanding permeability of ECs is imperative to elucidating pathogenic processes of the vasculature.

Permeability studies *in vitro* are paramount to the pharmaceutical industry where pharmacokinetic properties of drug compounds need to be screened for their ability to absorb through an endothelial layer such as the blood–brain barrier.<sup>54</sup> In these studies, ECs are typically grown on membrane inserts seated in well plates, and albumin often serves as a model protein whose movement is tracked across the endothelium. Albumin plays a central role in permeability because of its abundance in plasma and its interactions with ECM proteins and the glycocalyx.<sup>50</sup> In shear response studies, it was demonstrated that albumin permeability increases at the onset of shear stress, and decreases to baseline values at the cessation of shear.<sup>55</sup> This important finding suggests that flow-induced shear regulates

permeability, and that CCF systems that incorporate flow with permeability measurement are valuable to our experimental repertoire.

## 2.6 Neighboring cells of ECs

All cardiovascular tissues contain cells within the interstitium below the basal lamina on which ECs reside. These neighboring cells of ECs generally help maintain cardiovascular homeostasis. For example, smooth muscle cells (SMCs) reside in the tunica media of blood vessels, and are responsible for contraction and dilation of the vessel walls. In the aortic valve, valve interstitial cells (VICs) reside in the leaflet interstitium and are responsible for regular maintenance and repair of the surrounding valve matrix.<sup>56,57</sup> However, the role of SMCs and VICs as mediators of vascular health also implicates them as sources of disease initiation. Lesions form in the artery wall due to proliferation of and excessive matrix protein synthesis by SMCs, usually initiated by endothelial dysfunction.<sup>58</sup> Calcification and fibrosis in valve leaflets also involves matrix remodeling by VICs, leading to thickened leaflets with stiffened matrix properties.<sup>56</sup>

ECs are naturally involved in the regulation of SMC/VIC function due to their location as a barrier between flowing blood and the interstitium. Perhaps the best example of EC–SMC communication regulating SMC function is the role of nitric oxide (NO) in smooth muscle contraction.<sup>15</sup> Activated nitric oxide synthase (NOS) within ECs produces NO gas that readily diffuses across EC membranes and into SMCs where it produces cyclic GMP and ultimately relaxes the SMCs. Because NO, and many other cytokines and secreted factors that are important to tissue function, have short half-lives, their ability to induce an SMC response *via* paracrine signaling is dependent on the proximity between communicating cells. Thus, recreating spatial relationships in *in vitro* models is imperative to study transfer of molecular signals from one cell to another. Coculture models provide a method to bring both ECs and SMCs/VICs into the same culture where they can share certain biological products within the same media while still maintaining sufficient separation from each other (so as not to have a non-physiological mixed aggregate of cells).

## 3 Fluid flow principles

CCF systems are designed to provide an enclosed and controlled culture environment where ECs can be exposed to *in vivo*-like hemodynamic conditions. Because fluid flow must be predictable to properly control shear stress on the endothelial monolayer, the geometries of the systems are usually simple, and have analytical solutions for velocity and pressure fields that are based on the theory of viscous flow in ducts. For completeness, we briefly summarize the basic principles of fluid flow relevant to our discussion. Interested readers are referred to a more in-depth review by Beebe on the physics of microscale fluid flow, including discussions on diffusion, surface tension, capillary forces, and surface area-to-volume ratios.<sup>59</sup>

The Reynolds number,  $Re$ , is an important dimensionless parameter in viscous flows, particularly when different length scales are involved and inertial and viscous forces dominate.  $Re$  is defined as

$$Re = \rho UL/\mu \quad (1)$$

where  $\rho$  is the fluid density,  $U$  is a representative velocity of the flow,  $L$  is the characteristic length, and  $\mu$  is the fluid viscosity.  $Re$  is derived from non-dimensionalization of the Navier–Stokes equations, and represents a ratio between inertial and viscous forces. Low Reynolds number ( $Re < 10^3$ ) signifies viscous laminar flow where fluid streamlines are steady and predictable; high Reynolds number ( $Re > 10^4$ ) signifies chaotic turbulent flow where fluid motion is random and unpredictable, and where analyses are only possible by time-averaged approaches. Between these two regimes lies a third ( $10^3 < Re < 10^4$ ) where flow transitions from laminar to turbulent.<sup>60</sup> For the purpose of designing well-characterized CCF systems, flows are usually restricted to  $Re < 10^3$  (and often times  $Re < 100$ ) to ensure laminar streams are maintained, and predictable shear stresses can be applied at the walls of flow sections. Indeed, almost all microfluidic geometries guarantee low  $Re$  and laminar flow patterns, a key characteristic that makes them ideal for CCF applications.

Most flows in CCF systems are steady pressure-driven flows, and because of the dominance of viscous terms, the Navier–Stokes equation can be simplified to

$$0 = -\nabla p + \mu \nabla^2 \vec{u} \quad (2)$$

where  $p$  is the pressure field and  $\vec{u}$  is the velocity field. This basic equation, along with appropriate boundary conditions, can be manipulated to provide velocity profiles, pressure fields, and other important flow parameters for a given problem. For CCF systems used to study ECs, an important measure is the shear stress on the endothelium. For systems that employ parallel flat plates separated by a narrow gap of height  $h$ , the wall shear stress  $\tau_w$  can be calculated by

$$\tau_w = 6\mu Q/wh^2 \quad (3)$$

where  $Q$  is the flow rate, and  $w$  is the width of the flat plates. This two-dimensional approximation assumes an ideal case of infinite flat plates, but because actual flows in CCF systems are confined by side walls, the near-wall regions experience shear stress that deviates from the above model. The extent of this deviation depends on the cross-sectional aspect ratio,  $\alpha = h/w$ , where  $0 \leq \alpha \leq 1$ . Low  $\alpha$  signifies wide plates and a slit-like geometry that closely mimics the two-dimensional model. In contrast, high  $\alpha$  (*e.g.*,  $\alpha = 1$ ) signifies a square-like geometry where wall effects substantially alter flow profiles. An exact solution to the three-dimensional velocity profile in rectangular ducts can be derived *via* Fourier series expansions.<sup>61</sup> To avoid the computational rigour inherent in solving Fourier series expansions, a simple approximation has also been derived,<sup>61</sup> resulting in a modified version of eqn (3) in algebraic form:

$$\tau_w = \frac{2\mu Q}{wh^2} \left( \frac{m+1}{m} \right) (n+1) \quad (4)$$

In eqn (4),  $m$  and  $n$  are empirical constants, with  $m = 1.7 + 0.5\alpha^{-1.4}$  and  $n = 2$  for aspect ratios  $\alpha < 1/3$ . Although wall effects are typically negligible for the majority of macroscale systems, these effects have become more important in microfluidic geometries because of the desire to increase parallelization, and hence reduce microchannel widths. In the process, aspect ratios are higher, and wall effects are more pronounced.

Eqn (3) and (4) apply to the channel regions where flow is fully developed. Near the channel inlet, a region of developing flow is

characterized by non-parabolic velocity profiles where eqn (3) and (4) do not apply. For flow between plates, this entrance length,  $L_e$ , can be calculated based on channel height  $h$  and Re:

$$L_e = ah \cdot \text{Re} \quad (5)$$

where  $a$  is an empirical proportionality constant (e.g.,  $a = 0.06$  for circular tubes;  $a = 0.08$  for rectangular channels). It is important to determine  $L_e$  prior to experimentation to allow proper delineation of the developing and fully developed regions. This ensures that the uniform section of the sheared surface can be appropriately analyzed.

Because of the convenience of soft lithography, it is straightforward to design and fabricate multichannel networks of various configurations. This opens the door for highly organized and integrated fluidic circuits for multilevel on-chip processing. Although flow analyses for networks are inherently more complex, calculations can be simplified by treating individual channel sections as resistances within the flow circuit (i.e., analogous to an electrical circuit). This was previously demonstrated in an analysis of fluid flow within multichannel droplet generators.<sup>62</sup>

## 4 Macro- versus microscale systems

We now focus on introducing existing CCF systems designed to study endothelial properties in the form of adhesion, migration, flow-induced mechanotransduction, and permeability. Excellent comprehensive reviews on these topics from a biological perspective are available in the literature, and interested readers are directed to them.<sup>1,2,50,52,53</sup> The purpose of the overview here is to bring together the various macro- and microscale designs in an effort to facilitate comparisons focused on experimental procedures, protocols, measurement methods, and analyses as opposed to the interpretation of biological outcomes (we leave that for the aforementioned topical reviews).

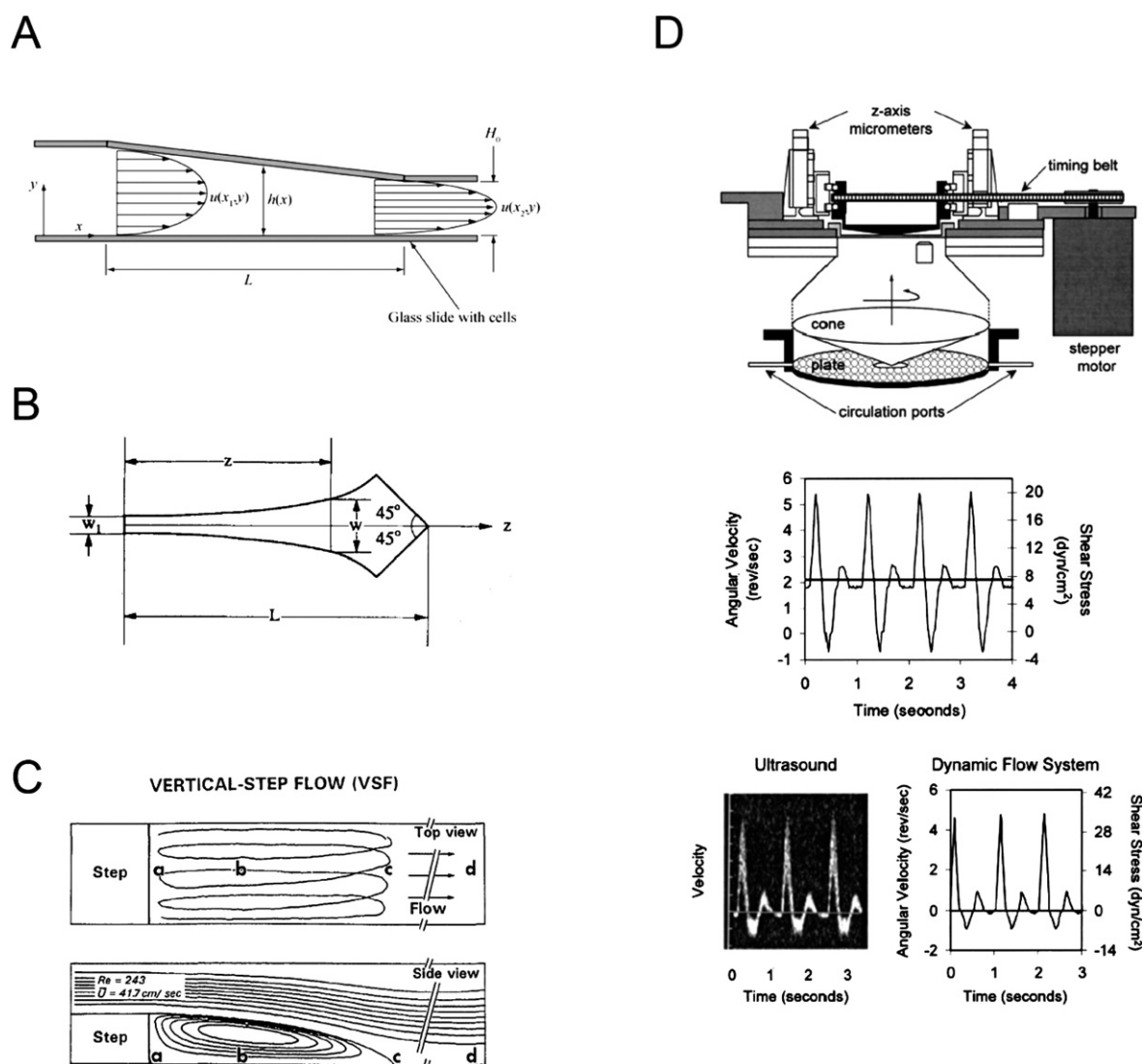
### 4.1 Adhesion

Adhesion assays are designed to measure the strength of attachment between anchorage-dependent cells and their underlying substrates. There are two main ways to quantify adhesion strength: (1) count the fraction of cells that detaches when exposed to a specific shear stress, or (2) determine an estimate of the total force per bond between cell and substrate. The first method is a global measure of adhesion that assesses overall preference of a cell type for a particular surface; the second is a fundamental measure of bond strength that requires determining number of bonds present in each cell, cell contact area, and models for bond and force distributions. The global measure is often sufficient and ideal for testing overall suitability of a biomaterial for cell attachment, spreading, growth, proliferation, and migration, as well as for determining the limits of forces which the cell population can resist. CCF systems designed to study adhesion often easily allow cell counting under different shear stresses and at different timepoints, but may require substantial modifications to allow proper visualization *via* microscopy to permit accurate predictions of individual cell-to-surface bond strengths.

Traditional CCF systems for studying adhesion include the parallel plate flow chamber (PPFC) and a number of variations on its basic design.<sup>63–65</sup> PPFCs are also the most commonly used apparatus for EC shear stress response studies, as discussed in Section 4.3. In PPFC adhesion assays, cells are loaded into the flow chamber, allowed to adhere, and then applied shear stress is incrementally increased to produce a ramped shearing protocol. Because flow chamber geometry is uniform throughout the device and samples of adhered cells are treated with the same conditions for a given experiment, the PPFC is limited to studying a single experimental condition at once. Furthermore, to apply different shear rates, the flow rate must be adjusted manually during the ramping procedure. To avoid this tedium, PPFCs have been modified to allow the same flow rate to simultaneously generate different shears at different locations. Variable-height flow chambers<sup>64</sup> rely on a sloped top plate such that shear stress varies as a function of height,  $h(x)$ , along the  $x$ -direction of the bottom plate (Fig. 2A). The variable-width flow chamber designed by Usami *et al.* employs a tapered channel where side walls follow an inverse function, thus producing a linear shear stress gradient from inlet to outlet (Fig. 2B).<sup>65</sup> Both systems permit simultaneous application of a range of shear stresses, but the tapered channel is easier to fabricate because of the uniform height, and thus lends itself well to microfabrication techniques (see below). Alternatively, one can take advantage of radial and circumferential flows to create ranges of shear stress from the non-uniform velocity fields. Radial flow can be generated by either divergence from a single point source, or convergence to a sink. If flow is confined by parallel plates, velocity and shear stress are both dependent on radial position alone. Circumferential flows may also be developed to generate similar shear stress gradients in the radial position. These fluid flow principles have been implemented for cell adhesion studies in a myriad of designs, including radial flow chambers,<sup>66,67</sup> spinning disc apparatuses,<sup>68</sup> and cone-and-plate viscometers.<sup>69</sup>

Although variable shear stress devices can produce a range of shear rates using a single volumetric flow rate, all these systems suffer from the major drawback that only one combination of cell type and specific surface can be tested at one time. Many bioengineering studies involve investigating multiple cell types on different surface functionalizations to screen for appropriate biomaterials and surface coatings that provide optimal adhesion of a given cell type. Yet none of these macroscale devices can determine adhesion strength metrics (e.g., critical wall shear stress<sup>64</sup>) for multiple cell and surface conditions at once. Recent developments in microfluidics have demonstrated that such capabilities are indeed possible, particularly when the benefits of the microscale are fully exploited.

Examples of microfluidic devices for adhesion studies are plentiful. Katak and co-workers were one of the first to take advantage of parallelization in microfluidics and the convenience of soft lithography by investigating platelet adhesion to different fibrinogen-coated substrates on four individual straight microchannels on the same chip.<sup>70</sup> By applying different surface coatings in each, Katak and co-workers demonstrated one of the most basic advantages of microfluidics, i.e., increased efficiency of experimentation by parallelizing multiple conditions. Though simple in channel design and fabrication, it was rather impractical because it required separate injection into each



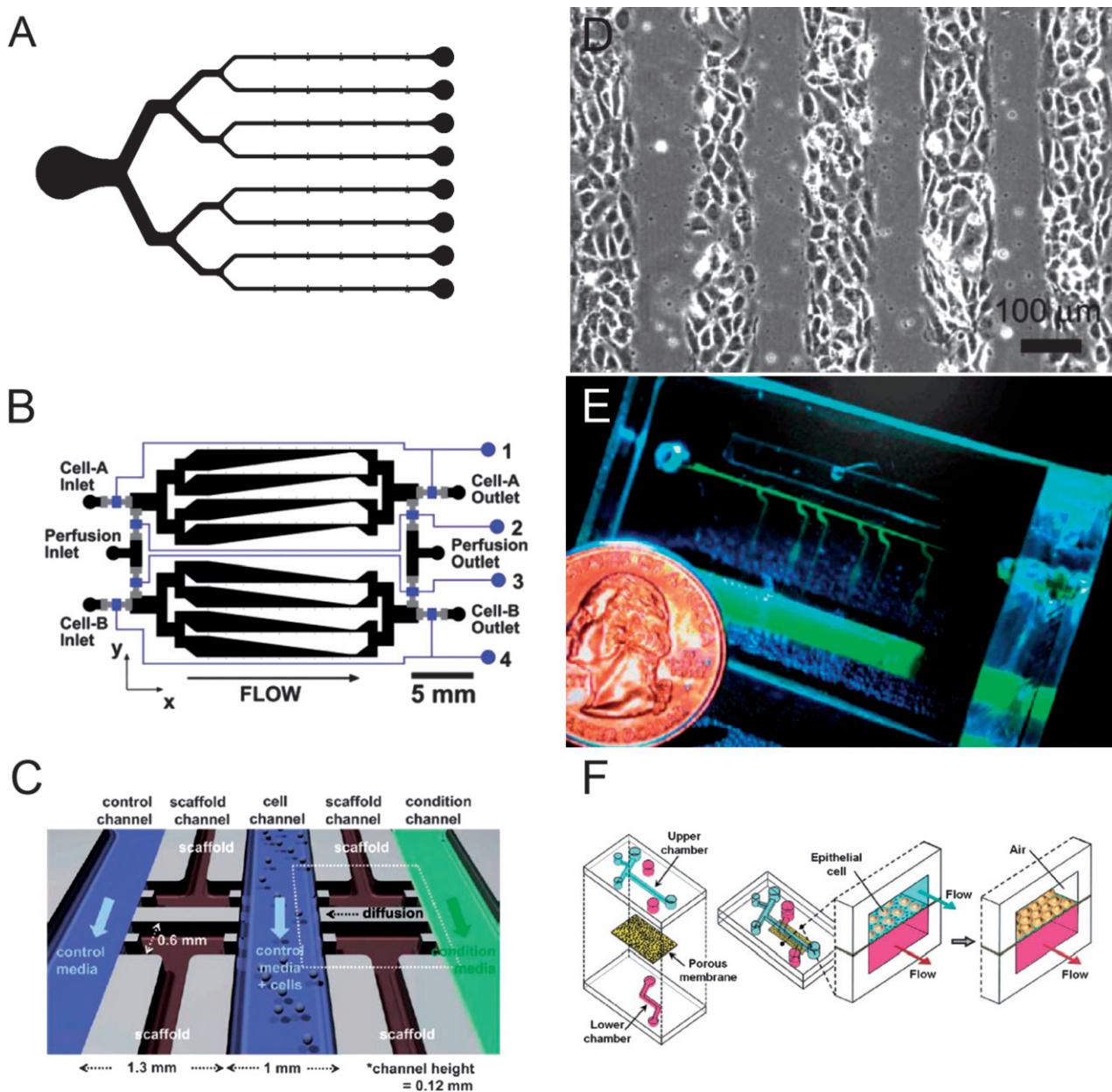
**Fig. 2** Various macroscale CCF systems. (A) Variable-height flow chamber used by Xiao and Truskey (ref. 64). (B) Variable-width linear shear stress flow chamber (source: Usami *et al.* (ref. 65), with kind permission from Springer Science and Business Media). (C) Parallel plate flow chamber with inlet step to produce recirculation zone (source: Chiu *et al.* (ref. 114)), and (D) cone and plate viscometer with microstepper motor for mimicking arterial waveforms (source: Blackman *et al.* (ref. 105)), both reprinted with kind permission from the American Society of Mechanical Engineers (ASME), Journal of Biomechanical Engineering, copyright © by ASME.

microchannel using individual syringes mounted on a multi-syringe pump. However, keeping the microchannels separate ensured no cross-contamination between samples.

To further streamline experimentation, Lu and co-workers designed parallel multi-microchannel networks to study adhesion of fibroblasts.<sup>71</sup> In each of two separate designs, Lu employed a single inlet port that required only one syringe to feed four interconnected microchannels, obviating the need for multiple syringe hook-ups. Young *et al.* adopted a similar configuration with eight parallel microchannels, and used it to reveal subtle yet important differences in adhesion properties of vascular and valvular ECs (Fig. 3A).<sup>72</sup> Flow analyses in these types of networks relied on principles of hydraulic resistance (see Section 3). While these designs reduced the number of connections, they introduced the possibility of cross contamination between connecting branches of the network. When care is taken

to ensure that hydraulic resistances are equal between parallel branches, cross contamination can be largely avoided.

While these designs have benefited from parallelization, they still suffered from the same drawback as mentioned for PPFCs, *i.e.*, that shear stress must be manually adjusted by changing flow rate in the system, in this case *via* the syringe pump. Gutierrez and Groisman designed a microfluidic network that incorporated variable shear stress flow chambers in a parallel configuration, combining the efficiency of multiple shear rates within one chamber with the flexibility of multiple experimental conditions across separate chambers of the same network (Fig. 3B).<sup>73</sup> In addition, the design integrated pressure-actuated membrane valves that added an extra level of sophistication and permitted the simultaneous loading and experimentation of two separate cell populations. To date, this is perhaps the best demonstration of the benefits of microfluidics for testing



**Fig. 3** Various microscale CCF systems. (A) Eight-channel parallel microfluidic network for EC adhesion assays. (source: Young *et al.* (ref. 72), reproduced by permission of The Royal Society of Chemistry). (B) Parallel microfluidic networks with variable shear flow chambers (source: Reprinted with permission from Gutierrez *et al.* (ref. 73) Copyright 2008, American Chemical Society). (C) Microfluidic coculture migration system (source: Chung *et al.* (ref. 100), reproduced by permission of The Royal Society of Chemistry). (D) Micropatterned substrates for HUVEC culture in microfluidic channels (source: Rhee *et al.* (ref. 120), reproduced by permission of The Royal Society of Chemistry). (E) PDMS-based EC shear device with flow actuated by Braille pins (source: Reprinted with permission from Song *et al.* (ref. 126) Copyright 2005, American Chemical Society). (F) Application of membrane device for modeling the lung airway system (source: Huh *et al.* (ref. 134) Copyright 2007, National Academy of Sciences, USA).

adhesion: increased functionality coupled with simultaneous application of a range of shears over several different experimental conditions.

Several reports by Murthy and co-workers have employed linear shear stress flow chambers for studying adhesion strength as well as for selective enrichment of cell populations.<sup>74–76</sup> The design is identical to the tapered chamber of Usami, and while the devices implemented by Murthy are in fact physically larger than the original Usami design, they were fabricated using soft

lithography and this provides the benefit of allowing future parallelization of multiple linear shear stress chambers.

## 4.2 Migration

Cell motility is quantified by measuring the speed of the migrating cells in terms of distance traveled over time. Migration assays come in many formats, and the appropriateness of each depends on the application. Migration can be tested across

barriers or membranes, on two-dimensional surfaces, or within three-dimensional gels or scaffolds. Either average migration rates of cell populations can be detected, or single cells can be tracked and random walk patterns analyzed to determine individual cell migration speeds. Important considerations include how well the *in vitro* apparatus recapitulates the *in vivo* environment of the cell type of interest, and whether population-based measures are sufficient as an endpoint. Some migration assays are specifically designed for ECs, while others are more applicable to non-endothelial cell types that do not require cell–cell contacts in a monolayer to elicit physiologically relevant cell function. For this review, we focus mainly on migration assays and CCF systems designed for ECs, but also mention the other assays for completeness, as well as to motivate discussion.

The most widely used conventional assay for migration studies is the Boyden chamber assay (also known as the filter membrane, Transwell migration, or chemotaxis assay).<sup>77,78</sup> Briefly, cells are cultured on one side of a porous filter membrane and allowed to migrate through the membrane pores to the opposite side where they are detected, usually by fluorescence. Migration is usually induced by addition of a chemo-attractant to the culture medium on the opposite side. For such chemotaxis studies, a population-based assay is sufficient to determine whether the chemo-attractant of interest has an overall migratory effect on the cells. This assay is popular because of the applicability to some of the most important biological problems studied, including the extravasation of both leukocytes in inflammatory responses<sup>79</sup> and neoplastic cells in cancer metastases.<sup>80</sup> ECs are generally involved in these types of studies as a static monolayer cultured on the membrane surface through which the migrating cells must traverse, but can also be the migrating cell type under study.

Other migration assays used to study ECs are typically conducted in two dimensions. This is an ideal format because ECs exist in sheet-like monolayers *in vivo*, and migrate mostly to maintain confluence within this two-dimensional cellular layer. Commonly used surface assays include the Teflon fence<sup>81</sup> and free migration assays<sup>82,83</sup> where ECs are grown to confluence in an area restricted by an obstacle or “fence”. The fence is removed when desired, allowing the ECs to migrate into the open area. Prior to culturing the ECs, surfaces can be modified to incorporate spatial gradients of surface-bound factors (haptotaxis)<sup>82,83</sup> or substrate stiffness (durotaxis). In contrast, the scratch assay<sup>84,85</sup> is regarded as the gold standard for wound healing studies, and typically starts with a fully confluent monolayer, upon which a deliberate scratch in the monolayer is generated. Regardless of which surface assay is used, cell motility is quantified by tracking and measuring movement of the endothelial fronts.

An important enhancement to these assays is to investigate EC migration under shear flow conditions. This has been previously demonstrated by incorporating a trip bar in a PPFC to generate shear stress gradients that induced EC migration.<sup>86</sup> Furthermore, to study wound healing with shear flow, endothelial monolayers can be first scratched intentionally before introducing into a PPFC.<sup>87–89</sup>

Finally, novel techniques exist for tracking and measuring motility of individual migrating cells. Phagokinetic track assays<sup>90,91</sup> rely on migrating cells to ingest colloidal gold particles that initially coat the entire culture surface. As cells migrate, they

leave behind a particle-free trail that traces the cells’ paths from which persistent lengths can be measured and speeds of individual cells calculated. Time-lapse video microscopy can be employed to achieve similar traces.<sup>92</sup> Perhaps the most novel recent development for single cell motility analysis was the quantitative morphodynamic study by Dieterich *et al.*,<sup>93</sup> who demonstrated tracking of ECs that were not only dynamically imaged at confluence, but were maintained under shear flow conditions with a cone-and-plate device.

While these conventional migration assays offered a number of viable options to researchers in the past, the recent advances in microfluidics have further expanded our repertoire to include functionalities that were previously unachievable. Migration studies stand to benefit most from the ability of microfluidics to generate various gradients that are instrumental to chemo-, hapto- and durotactic experiments. Early demonstrations of gradient generation and micropatterning<sup>94–96</sup> using laminar flow and the versatility of soft lithography fabrication have paved the way for numerous functionalities at the microscale, and have since become landmark examples of how microfluidics can impact modern biology. These ideas have since been exploited for various migration studies, including those involving ECs.

Hsu and co-workers<sup>97</sup> used elastomeric stamps made by soft lithography to micropattern collagen-coated strips on a glass slide to study haptotaxis of ECs under shear. Although the glass slide was loaded into a standard macroscale PPFC for shear stress application, the use of microchannels to prepare the substrate represents an important demonstration of how microfluidics could be used to improve experimental design.

For chemotaxis, Barkefors *et al.*<sup>98</sup> used microfluidics to generate Gaussian-like gradients of VEGFA and FGF2 across the 800- $\mu\text{m}$  wide channel, and studied migration of both artery and vein ECs from human umbilical cords. In a slightly different configuration, Shamloo *et al.*<sup>99</sup> generated linear chemical gradients of VEGF across the 1-mm wide channel to also study migration of human umbilical vein ECs (HUVECs). A major difference between the two studies is that the former generated the gradient by flowing laminar streams lengthwise and in the process created low shear stress over the cells ( $0.1 \text{ dyn cm}^{-2}$ ), while the latter study avoided the shear effect by forming the gradient with perpendicular perfusion channels. The former design is advantageous if synergistic effects between flow and chemical gradients are desired.

The most recent demonstration of the versatility of microfluidics comes from the Kamm group, who were able to culture human dermal microvascular ECs in the vertical plane of microchannels, and monitor capillary morphogenesis within the lateral plane as ECs migrated into collagen gels (Fig. 3C).<sup>100,101</sup> Controlled chemical gradients were generated within the gel to induce migration *via* chemotaxis. Although the three-dimensionality of the experiment was a novel approach to visualize capillary morphogenesis, it was unclear whether shallow microchannel depths adversely affected the formation of an endothelial monolayer with proper morphology and confluence.

In the examples thus far, shear stress effects on migration have been largely avoided to isolate influences from chemical factors. Recently, however, the authors discovered that shear stress gradients in microchannels also induce strong migratory responses, similar to that demonstrated by Tardy *et al.* at the

macroscale.<sup>102</sup> Using time-lapse video microscopy, ECs from porcine aortas were exposed to shear (20 dyn cm<sup>-2</sup>) for 48 h in a 1.5-mm wide microchannel, and were found to continuously migrate toward the channel center away from the shear gradient close to the walls. Because of this migration, the confluent ECs crowded the middle of the channel, and elongated in the transverse (migratory) direction.

Finally, we mention a non-EC example to illustrate how microfluidics can be employed to study durotaxis. Sundararaghavan *et al.*<sup>103</sup> recently employed a simple H-channel to generate a diffusion gradient of a crosslinker (genapin) into a collagen solution. After gelation, the collagen displayed a gradient of mechanical stiffness, which influenced neurite growth. Presumably, similar gradients in mechanical stiffness of gels can also be generated by gradient photopolymerization. Given the recent discoveries on the influence of matrix stiffness on cell behavior,<sup>104</sup> such techniques for investigating durotaxis in microfluidic systems could play an important role as a method of experimentation.

### 4.3 Shear stress response

The objective in shear stress response studies is to expose endothelial monolayers to uniform shear forces while maintaining sterile cell culture conditions. This is achieved by applying well characterized flow fields on sufficiently large endothelial surfaces. Monolayer size dictates the amount of biological material, *e.g.*, secreted factors, proteins, or mRNA, that can be extracted for analysis at the end of experiments. Knowing endpoint measures and having an estimate of the amount of material needed for analysis aids in choice of flow chamber size.

Because the goal is to expose ECs to uniform shear stress, many of the variable shear flow chambers designed for adhesion assays are not suitable for shear response studies. The applicable systems are the PFCs and the cone-and-plate viscometers.<sup>105–107</sup> The others generate shear stress gradients undesirably, leading to varied levels of mechanical stimuli across the endothelial sample, and ultimately local variations in cellular response that cannot be easily isolated. To ensure uniform shear stress across the monolayer, some designs employ baffles<sup>39,108</sup> or perforated plates<sup>109</sup> to produce laminar streams upstream of the test region. Most of the time, however, laminar shear flow is produced without difficulty, as long as the inlet geometry is properly designed.

Once a uniform shear stress is established, the next step is to choose appropriate experimental parameters such as flow rates and duration of experiment, with due consideration for the output measure of interest. A typical first metric of endothelial response to shear is cell morphology and structure. Morphological responses in the form of cell elongation and preferential orientation to flow have been shown to require 24 to 48 h before the response can be detected.<sup>37,38,40</sup> In some cases, such as the full restoration of linear adherens junctions at borders of sheared ECs, the response may need up to 96 h.<sup>110</sup> For other applications, such as studying the regulation of specific shear stress response elements (SSREs), responses may occur in less than 6 h.<sup>111</sup> Thus, while the duration of adhesion assays appears to only depend on the time needed to ramp through a range of shears, duration in

shear stress response assays depends invariably on the output measure of interest, and therefore requires careful consideration.

The majority of studies use steady shear stress in the range of 10 to 40 dyn cm<sup>-2</sup> to mimic physiological conditions.<sup>37,40,110,112,113</sup> Steady continuous shear is a natural starting point for understanding EC responses to shear *in vitro*. However, to model more closely the *in vivo* hemodynamic environment, particularly in the arterial system, consideration should be given to unsteady pulsatile flow, where past studies have demonstrated unique time-dependent adaptations to cell morphology depending on whether flow is steady, purely oscillatory, or pulsatile (with and without reversing patterns).<sup>47</sup> A further advance was replacing simple sinusoidal patterns with accurate arterial waveforms that were generated by precision micro-stepper motor technology on a cone-and-plate device, resulting in accelerations and decelerations of fluid with high temporal resolution.<sup>49,105</sup> For the majority of studies, steady shear remains the simple, reliable choice for inducing shear-related responses unique from static conditions, but the subtle differences afforded by more accurate waveforms are particularly important for correlating with *in vivo* experiments.

A number of studies have attempted to recreate flow patterns *in vitro* that mimic vascular regions of disturbed flow where atherosclerotic lesions are known to originate. The simplest way is to introduce a lateral protruding step or “trip bar” across the flow chamber to perturb the laminar fluid stream, and in the process create shear stress gradients in the direction of flow.<sup>86,114</sup> This was discussed previously for studying migration. The design is far from an accurate depiction of vascular geometry, but it provides a simple solution to a complex fluid problem, *i.e.*, the controlled and predictable generation of recirculation zones and reattachment points that model aspects of the turbulent regime.

Many of the systems reviewed thus far fully enclose the sample in large contraptions to provide a leak-proof seal and contamination-free environment. As a consequence, access to the sample during experimentation is limited to before and after the application of flow. Real-time monitoring of live cells *via* video microscopy can provide unparalleled insight into the dynamics of EC response to shear,<sup>115</sup> and studies have demonstrated the power of this technique through novel quantitative morphodynamic analyses of cultured and sheared ECs.<sup>93</sup> Live cell imaging setups have become increasingly sophisticated over the years, incorporating features such as direct heating, and perfusion ports within systems designed for optimal optical performance (Bioptechs).

In choosing an appropriate CCF system, or designing a new one for a specific application, the first priority is to determine the endpoint measure and the amount of biological material needed to carry out the desired analyses. Some morphological studies rely on post-shear immunostaining to determine localization of specific intracellular proteins associated with cytoskeletal remodeling.<sup>38,40,110,113</sup> In these cases, it may be a simple matter to divide the tested glass slide into separate compartments to allow staining of multiple markers. For measurement of secreted factors and proteins such as NO,<sup>116</sup> prostacyclin (PGI<sub>2</sub>),<sup>43</sup> tissue plasminogen activator (tPA),<sup>45</sup> or fibronectin,<sup>41</sup> it is pertinent to estimate the amount of substance needed to detect differences between experimental samples. This is important when choosing assays with the appropriate level of sensitivity. More

importantly, however, it impacts experimental design because it necessitates proper selection of media volumes and endothelial surface area such that suitable levels of protein are produced and secreted for analyses.

Though the variety of CCF systems for shear stress response studies is mostly limited to PPFCs and cone-and-plate viscometers, there is no shortage of variations on these two themes. Simple modifications to existing systems and more sophisticated implementations are available to the end user using either platform. Fig. 2 summarizes some of the noteworthy macroscale designs discussed. As previously stated, a clear shortcoming is that these systems test only one condition at a time. As an example, immunostaining of sheared ECs under different chemical or mechanical stimuli and for multiple timepoints would require a single PPFC for each condition. Clearly, more high-throughput means are needed to expedite the experimental process, and this is an area where microfluidics can make an immediate and significant impact.

A multitude of microfluidic cell culture devices have been reported, yet only a select few have focused on shear stress-induced responses of ECs. Many more studies have directed their attention to investigating other cell types including neurons, hepatocytes, osteoblasts, fibroblasts, and others, and this is understandable given the suitability of microdevices for all cellular scale studies.<sup>117–121</sup> The major challenge in these studies is the need for long-term culture of cells and the assurance of their viability, which was not necessary for the short duration of experiments for most microfluidic adhesion assays. The challenge of culturing ECs is amplified by the need to achieve a confluent monolayer with proper cell–cell contacts, the absence of which has been shown to impact normal cell response. In terms of fluid mechanics, microfluidics is suited for shear stress studies because Reynolds numbers are low and guaranteed to be laminar. Consideration, however, must be given to channel aspect ratios to ensure wall effects on velocity profiles are minimized.

To our knowledge, the first shear response study of ECs in microfabricated channels was conducted by Gray *et al.*<sup>122</sup> who fabricated a device from silicon by deep reactive ion etching (DRIE), and cultured ECs to confluence to assess cell shape in varying channel widths. Bovine aortic ECs aligned with the direction of flow in a 200- $\mu\text{m}$  wide microchannel after 16 h of shear at 20  $\text{dyn cm}^{-2}$ , demonstrating the feasibility of converting macroscale experimentation to the microscale. Perhaps a more interesting result from the study was the natural tendency for ECs to elongate and align with channel lengths even in the absence of flow for widths less than 200  $\mu\text{m}$ . Thus, to maintain proper EC morphology under static conditions in microenvironments, channel width was shown to be an important design parameter.

One of the often cited advantages of microfluidics and soft lithography is the ability to produce micropatterned surfaces. As one example of micropatterning for EC culture, Rhee *et al.* created adhesive patches on glass with a PDMS patterning piece, and bonded a second microfluidic PDMS piece over the patches to form enclosed channels where ECs were seeded (Fig. 3C).<sup>120</sup> They demonstrated adhesion and spreading of ECs on the patterns over a 5 h culturing period, but did not apply shear stress to the cultured cells. Despite the relatively short culturing time and the absence of applied shear, the study was a novel

demonstration of how in-channel micropatterns could be generated to spatially control growth of ECs.

An important advance in microfluidics technology with biological relevance was the work of Spence and co-workers who fabricated an in-line carbon ink microelectrode for amperometric detection of NO production from pulmonary artery ECs.<sup>123,124</sup> A general concern of microfluidics is the low volume, and thus, low numbers of detectable analytes. NO measurements at the macroscale are typically performed by sampling milliliters of media over specified time periods on the order of tens of minutes, but volume limitations make this impractical at the microscale. In-line amperometric detection allows real-time monitoring of NO production and reduces the amount of NO lost due to autooxidation to  $\text{NO}_2^-$ . Oblak *et al.* have since used fluorescent microscopy for NO detection as an alternative technique with improvements over amperometry.<sup>125</sup> Further investigation is needed to ensure EC behavior is directly scalable from macro- to microscale so that no loss in biological activity is introduced. If this is the case, on-chip measurements of secreted factors would be a large step toward advancing microfluidics for EC studies.

Finally, we discuss one of the most innovative techniques for generating flow-induced shear on ECs in microfluidic channels. Microchannels cast in PDMS were used to culture ECs to confluence before recirculating flow was introduced *via* Braille pin actuation (Fig. 3E).<sup>126</sup> On-chip valves were incorporated by fabricating thin film PDMS membranes that were deflectable by computer-programmed actuating pins to pump fluid in a pulsatile fashion through the microchannels. Using this device, Song and co-workers measured EC morphological response and obtained data on elongation and orientation consistent with the literature, *i.e.*, low shear of 1  $\text{dyn cm}^{-2}$  did not align cells while high shear of 9  $\text{dyn cm}^{-2}$  aligned cells parallel to flow. One caveat of this system is that only peristaltic pressures can be generated, and although this is sometimes desirable for mimicking physiological environments more closely, data cannot be easily compared to the large body of literature on steady shear EC responses.

#### 4.4 Permeability

The goal in permeability assays is to assess the structural integrity of a given endothelial monolayer by determining the flux of molecules from luminal to abluminal sides. Two main methods are usually employed: (1) tracking the movement of molecules from one side to the other by labeling and detecting the permeate using fluorescence or other means;<sup>55,127,128</sup> and (2) measuring transendothelial electrical resistance (TEER) by passing a current, measuring a voltage across the monolayer, and using Ohm's law.<sup>129–131</sup> The first method is more direct in that actual flux of particles are tracked and counted; the second method relies on the concept that the endothelial monolayer can be treated as an impedance layer or barrier. In either case, endothelial permeability can be tested against various chemical and mechanical stimuli to induce a differential response that sheds light on underlying mechanisms related to endothelial barrier function.

Regardless of the measurement technique, the first priority in permeability experiments is to culture ECs on a supporting porous membrane. This is most conveniently done using

Transwell cell culture inserts that can be seated in well plates while bathed in culture media. Inserts, or the membrane alone, can then be transferred to a setup that holds the membrane in media and contains both upper and lower compartments where abluminal and luminal sides are exposed for interrogation. For electrical resistance measurements, current passing electrodes are placed on either side; for fluorescence detection of labeled molecules, a specified concentration of solutes is added to the luminal side, and the concentration on the abluminal side is measured to determine flux across the monolayer.

Most permeability measurements are done while ECs are in static conditions. However, knowing the effects of shear stress on ECs, and the role of shear-regulated EC components in endothelial permeability (see section 2.5), it is not surprising that endothelial permeability is shear-dependent. Only a few reports have incorporated shear stress on ECs with their permeability measurements. Since our focus here is on different CCF system designs, we review two apparatuses that incorporate flow with measurement of endothelial permeability for comparison.

The first, developed by Jo and co-workers,<sup>55</sup> consisted of a shear chamber that housed the membrane containing the endothelial monolayer, and two separate loops that included an abluminal non-circulatory loop and a luminal circulatory loop. The purpose of the luminal flow loop was to provide shear stress over the endothelial monolayer and supply a constant stream of fluorescently-labeled albumin molecules. The purpose of the abluminal loop was to maintain a prescribed transmembrane pressure across the membrane while providing access to the sampling region in the lower compartment under the monolayer. The second, from Sill and co-workers,<sup>128</sup> employed a rotating cylindrical disk on the luminal side to produce circumferential flow patterns and corresponding shear stress on the endothelial monolayer. In this case, volume flux of fluid (rather than solute flux) was measured in real-time by tracking the motion of a bubble in a glass tube through spectrophotometric measurements.

These two examples provide us with a glimpse of the complexities involved in shear-dependent permeability experiments. Shear stress can be produced using similar concepts as before, with either linear flow through parallel plates, or circumferential flow *via* cone-and-plate setups. In both cases, control of pressure on luminal and abluminal sides is critical to proper detection of permeability. The size and complexity of these apparatuses limits them to laboratories dedicated to understanding specific problems of the endothelium, where targeted molecules can be analyzed in detail and high-throughput is not necessary.

To date, no studies have measured permeability of ECs in microfluidic channels. Despite the absence of specific studies pertaining to permeability, advances in microfluidics have helped introduce practical fabrication methods suited for endothelial permeability experiments. The most important feature of a permeability measurement system, based on macroscale setups, is a porous membrane that supports endothelial cell growth while also allowing passage of molecules from luminal to abluminal sides of the endothelium. In microfluidic systems, the simplest way to incorporate membranes is to sandwich a commercially available membrane between two pre-cured PDMS slabs each containing their own microfluidic channel patterns, as reported

by Ismagilov *et al.*<sup>132</sup> This approach introduced new opportunities for multilayer fabrication in which microchannels could be separated by a permeable layer to communicate in a three-dimensional microfluidic network. Others have since adopted this technique for their own applications.<sup>133–135</sup> Indeed, there are other fabrication options available for introducing membrane layers within microfluidic devices, and these are critically reviewed by de Jong *et al.*<sup>136</sup> The incorporation of commercially-available membranes, however, is the most direct and economical of the alternatives.

It is worth mentioning here the novel work of the Takayama group, who used a membrane-based microfluidic device to culture small airway epithelial cells and study the effects of rupturing liquid plugs in a liquid-air two-phase flow system (Fig. 3F).<sup>134</sup> The study demonstrated the feasibility of culturing ECs on membranes for long-term within microfluidic environments. More importantly, however, the work was a reminder that once cells are properly cultured on the membrane, microfluidics is a natural platform for imposing shear stress on the endothelial monolayer.

#### 4.5 Coculture

Coculture of ECs with either SMCs or VICs is important for understanding cell–cell communication and how it affects homeostasis and pathogenesis within the cardiovascular system. Allowing interaction of two cell types within the same environment adds a level of experimental sophistication that has potential to capture paracrine-dependent effects that otherwise would be lost in the absence of either cell type. Coculture models can be configured in a number of ways depending on the desired amount of contact between cells. Using membrane cell culture inserts, ECs can be grown on the top of the membrane while SMCs or VICs are grown inside the wells in a fully separated configuration suitable for conditioned media testing.<sup>137</sup> Alternatively, SMCs or VICs can be grown on the bottom of the membrane opposite the ECs, permitting extension of cell processes through membrane pores and allowing controlled physical contact *via* myoendothelial bridges.<sup>138,139</sup> Finally, SMCs or VICs can be seeded directly on EC monolayers in a model that allows full contact between the two cell types without segregation.<sup>140</sup> Each option offers its own advantages, and answers different questions (*e.g.*, separated coculture examines paracrine signaling and effects of conditioned media, whereas controlled contact coculture examines cell–cell communication *via* physical bridging); the choice is dependent on application.

Many of the early coculture studies simply focused on ensuring viable coexistence of both cell types in the same vicinity.<sup>138,139</sup> Proliferation rates and cell counts were determined to ensure cell viability, and scanning electron micrographs were taken to assess cell shapes and possible extension of cell processes through membrane pores. Others investigated secretion rates of certain molecules in the presence of the second cell type. One specific example was the report of C-type natriuretic peptide (CNP) production by ECs in the presence of SMCs.<sup>140</sup> Interestingly, Komatsu and co-workers discovered marked increases in CNP production when high densities of SMCs were grown directly on ECs, but no significant increases when SMCs were seeded at low densities or in separated coculture.

To more closely mimic *in vivo* conditions, shear stress of ECs in any coculture system is a desirable feature. Although the majority of coculture studies are conducted with ECs in static condition, a number of coculture systems that incorporate shear have also been reported in the last decade. Three major types of systems have emerged. A series of papers published by Redmond and co-workers reported cocultured semipermeable capillary tubes featuring ECs grown inside the tubes and SMCs grown on the outer walls of the tubes.<sup>141–143</sup> ECs were sheared and perfused with media flown through the tube “lumen”. In this setup, cells were separated by the tube wall such that there was no physical contact between cell types. One shortcoming of this system involves the many challenges related to post-experimental analyses of cells grown on curved tubes.

A coculture technique that has gained widespread popularity involved growing ECs and SMCs on opposite sides of a membrane insert (similar to that discussed above), and mounting the insert to a parallel plate flow chamber such that the ECs were exposed to flow.<sup>144–147</sup> This has several advantages over the capillary systems of Redmond, including simple assembly and disassembly to access either cell layer, and easy conventional coculture maintenance prior to inducing flow.

A third class of coculture systems that employed shear flow was an adaptation of the coculture using PPFCs above. Instead of growing both cell types on the same membrane, Ziegler and co-workers embedded SMCs in a matrix of collagen gel before seeding the gel surface with ECs.<sup>148</sup> The cocultured gel layer was then inserted into a PPFC where the ECs were sheared. Interestingly, Butcher and co-workers have recently used a similar coculture model to investigate aortic valve ECs with VICs,<sup>149</sup> and found perpendicular alignment of valve ECs to flow. This result agreed with previous results from the same group, who saw perpendicular alignment of sheared valve ECs in monoculture.<sup>112</sup>

Microfabrication technology has recently aided advances in coculture studies. However, none of the reports to date have discussed a microfluidic system for coculture of ECs and SMCs/VICs while the ECs are exposed to shear stress. Published reports of coculture have mainly focused on controlling spatial relationships of cells and chemical gradients of reagents in a microscale platform consisting of culture arrays and perfusion networks,<sup>121,150,151</sup> except for the recent series of migration/coculture studies by Kamm and co-workers,<sup>100,152</sup> none of these involve ECs. Others who have studied ECs in coculture have utilized the benefits of laminar flow in microfluidics to assemble a model of a blood vessel consisting of three separate layers of cells in distinct matrices.<sup>153</sup> One coculture example of note was from Genes and co-workers who cultured ECs on one side of the membrane in a microchannel and streamed red blood cells on the opposite side of the membrane to stimulate NO production from the ECs.<sup>154</sup> But perhaps the best demonstration of ECs in coculture was the most recent work of Song and co-workers who used a membrane microfluidic device to study adhesion of breast cancer cells on a microfluidic endothelium.<sup>155</sup> Although demonstrative of the coculture capabilities of microscale systems, these reports have yet to take advantage of the inherent benefits of microfluidic geometry to apply shear stress over EC monolayers to induce a proximal SMC/VIC response. With the membrane-bound devices described here and in the previous section, such a coculture system appears to be within reach in the near future.

## 5 Critical analysis: microfluidic cell culture

The current review of CCF systems has summarized the last quarter century of engineering developments aimed at providing necessary tools to EC biologists. The systems developed during this period can be delineated into two generations: the first fifteen years were devoted to early CCF systems with macroscale dimensions, and the last five to ten years have shifted toward miniaturization and the advancement of microscale systems. Not surprisingly, the delineation coincides with the birth of novel microfabrication techniques, such as the advent of soft lithography, which opened up new methodologies for approaching scientific problems.

Microfluidic systems to date have exhibited extreme versatility and wide applicability to numerous studies while often showcasing the newest engineering breakthroughs in miniaturization. There is little doubt a major shift from macro- to microscale systems is upon us, and the potential impact of microfluidics on biological and biomedical research will be profound. Yet, amidst all the hype surrounding this potential, only a small portion of devices ever designed will see widespread use in biological laboratories, whether through commercialization as often promised, or through collaborative research efforts with engineering institutions. For the foreseeable future, vascular biologists will likely resort to the tried and true macroscale systems as their tools of choice. For those continuing to pursue research in microfluidics for cell culture studies, two important questions should be considered as the field collectively moves forward: (1) What do biologists truly need from microfluidic systems? and (2) How can microfluidics technology be harnessed to provide biologists with those needs? To shed light on these questions, we reflect on design considerations of the past and present, discuss and analyze outstanding concerns regarding microfluidics as a valid methodology, and propose potential avenues of pursuit for the future.

In the past, macroscale CCF systems were designed and built for long-term reliable use, and materials chosen for their construction were usually autoclavable, chemically resistant, and optically transparent to aid visualization. Designs were usually based on well-characterized two-dimensional steady fluid flow patterns so that known theoretical solutions were available to aid analyses (*e.g.*, PPFCs, and cone-and-plate viscometers). Because of the size of the platform, large populations of cells could be studied at once, and this was advantageous for global population-based studies where it was desirable to smooth out heterogeneities and also make available enough biological products for gene and protein expression analyses. Many of these claims are still regarded as advantages of conventional CCF systems. And because much of the current literature on ECs was established using conventional systems, it has been convenient to resist major change and continue with traditional methods so that results between studies can be objectively compared.

In comparison, the majority of microscale CCF systems are currently fabricated by soft lithography and replica molding of elastomeric materials such as PDMS. This fabrication technique is convenient, versatile, and relatively economical, meaning new designs of complex channel networks can be implemented quickly over short concept-to-device turnaround times. Elastomeric materials are not ideal for all applications, but serve as

good materials for rapid prototyping of different designs for proof-of-principle experiments. Other polymeric materials such as polyimide (PI), poly(methyl methacrylate) (PMMA), polystyrene (PS), and cyclic olefin copolymer (COC) are also popular in the microfluidics community, but require fabrication and bonding methods that are more sophisticated and less convenient than those required for PDMS.<sup>156</sup> Regardless of the material used, current microfabrication techniques easily allow feature sizes as small as five microns, offering high spatial resolution for micropatterning, and high density of microchannels for parallelization and integration with other on-chip processes. Microchannel sizes for cell-based studies often do not need to be smaller than 100 microns, but the flexibility to pattern the internal surfaces of larger microchannels is an added bonus. Naturally, small microchannel cross-sections mean high surface area-to-volume ratios and a significant reduction in reagent consumption. Because length scales are small, low Reynolds number laminar flows dominate, and this can be exploited to generate controllable concentration gradients of soluble factors. Thus, the cellular microenvironment in terms of chemical, mechanical, and topographic cues can be tailored to researchers' needs. These advantages are simply not available at the macroscale.

The aforementioned advantages of macroscale setups, including their durability and capacity for large population-based studies, are in fact becoming less favorable when one considers the new options available to us through microfluidics. Macroscale CCF systems may be durable, but often require regular maintenance and repair. Microfluidics offers an affordable option that can be made disposable and at the same time amenable to existing infrastructure, so that microfluidic "cartridges" akin to Petri dishes and well plates can be employed to streamline experimentation. The argument that macroscale CCF systems can study large populations of cells and smooth out heterogeneities is also weakened with advancements in small population-based studies and single cell analyses.<sup>157</sup> For example, the Quake group at Stanford has demonstrated single cell mRNA isolation and analysis,<sup>158</sup> while Ginsberg has reported methods to amplify RNA from a single cell.<sup>159</sup> Microfluidic systems give us the option of obtaining information from single cells when needed, while population averages can be reassembled from the single cell data if so desired. Therefore, previous claims to macroscale benefits are perhaps simply misconceptions stemming from the time when these technological advances had yet to mature.

These arguments seem to give microfluidic systems a clear advantage over their macroscale counterparts. In reality, however, microfluidic systems are not without their own flaws. A number of outstanding concerns remain regarding their appropriateness for cell culture studies. And it is conceivable that these concerns have lessened the supposed impact on biological research that microfluidics had promised a decade ago.

First, the validity of biological outcomes obtained during microfluidic experimentation is still being questioned because of the uncertainty surrounding both the effects of culturing in low media volume and those of using device materials that have yet to be fully characterized (*i.e.*, PDMS). Secondly, the tools and devices designed by microscale engineers are often too cumbersome and complex for translation into another laboratory, and

this limits the potential marketability and accessibility of many designs. Thirdly, even when multiple researchers agree on the suitability of a microfluidic design, different versions of the same approach inevitably emerge from the literature, confounding the selection process of an appropriate system for any given application. Based on these concerns, what biologists truly need from the microfluidics community is (1) a concerted effort toward the *validation* of microscale results to macroscale data, (2) a strong focus on designing devices for true *end-user accessibility*, and (3) an emphasis on proper *standardization* of methods and procedures for common applications. Efforts in these three areas will likely have a major influence on the eventual mainstream utility of microfluidic systems for cell biology.

The first priority should be proper validation of biological results at the microscale. All the microscale advantages mentioned above are of little consequence if the results obtained do not corroborate those from macroscale data with either equal or better sensitivity. Because of the complexity of cellular behavior, validating results of cell studies will require more effort than demonstrating the fidelity of results for biochemical work. Validation should start with the goal of determining effects of low media volumes during microfluidic cell culture. Surface area-to-volume ratios in microenvironments are much higher than in conventional Petri dishes and wells, and this leads to higher concentration of cytokines, faster nutrient depletion and waste accumulation, and potential increase in cellular sensitivity to minor fluctuations in culture conditions. The importance of the soluble factor microenvironment have been discussed at length in several previous reports.<sup>160,161</sup> In addition, several recent reports have also emerged detailing the effects on cellular behavior due to shifts in pH,<sup>162</sup> osmolarity,<sup>163</sup> and oxygen tension<sup>164</sup> at the microscale, all important parameters for measuring cell culture viability.

Another area that needs further characterization is the inertness of PDMS during cell culture studies. It has been previously shown that hydrophobic molecules can absorb into the bulk of the PDMS.<sup>165</sup> Depending on curing temperature and time, PDMS also contains uncrosslinked oligomers that have a tendency to migrate from the bulk to the PDMS surface, allowing the surface to recover its hydrophobicity even after oxygen plasma treatment.<sup>166</sup> Most recently, the uncrosslinked oligomers have also been found to leach out from the bulk, into the media, and onto the surface of cultured cells in microchannels, affecting cellular behavior.<sup>167</sup> Based on this evidence, it is crucial to our general understanding of PDMS-based cell culture studies that we carefully characterize the effects of using this material for biological applications. In the absence of such characterization, it would be prudent to consider alternative materials and techniques for fabricating microchannel devices, including the use more mainstream tissue culture substrate materials like polystyrene.<sup>168</sup> While PI, PMMA and COC should also be given consideration as alternative materials for cell-based microfluidic applications, it is uncertain whether biologists will be as receptive to them as they would be to PS given that PS is the most commonly used material in laboratory cultureware. Like PDMS, these materials would need to be characterized to ensure their inertness to biological outcomes.

A specific validation metric for ECs under shear is the well-accepted property that ECs elongate and align in the direction of flow. Yet, as mentioned above, alignment can sometimes be

detected even under no flow conditions when microchannels or micropatterned protein strips are narrow enough.<sup>169,170</sup> Thus, it is imperative to design experiments that properly validate the chosen dimensions of microchannels such that biological artifacts are not generated. Recently, a pilot study was carried out by the authors to test the alignment of porcine aortic ECs in both macroscale PFFCs and microfluidic channels.<sup>102</sup> This was the first documented attempt at a side-by-side comparison between macroscale and microscale flow systems. Also worth mentioning is the recently published work by Paguirigan and Beebe, who likewise performed a parallel study between macroscale and microscale measurements, comparing Western blots to microfluidic protein detection (In-Cell Westerns) using an infrared laser scanner (Licor Odyssey).<sup>171</sup> Altogether, the emergence of such validation studies in the literature is an encouraging sign for both the engineer and the cell biologist, but continuing efforts must be made to improve our understanding of potential artifacts and establish a set of reliable design principles for microfluidic systems.

In terms of designing microscale systems for end-user accessibility, it is imperative for the microfluidics design engineer to understand the typical procedures executed on laboratory benches, and the common equipment available for biochemical analyses. Specifically for flow studies on ECs, some of the typical operations performed on endothelial monolayers during analysis

include fixation and permeabilization prior to immunostaining, mRNA and protein extraction, culture media collection for molecular secretion measurements, and live-cell imaging using video time-lapse microscopy. Fixation, permeabilization and immunostaining procedures can be easily performed by sample injection or passive pumping of solutions directly into channel devices. Although single-cell mRNA isolation was demonstrated using microfluidics (mentioned above), the complexity of integrated fluidic circuitry displayed in that specific example will likely prohibit its widespread use. The ongoing challenge will be to provide a simple and effective method to lyse cells and collect mRNA and protein products for online or offline analysis. Depending on microchannel dimensions, however, fluid volumes may be too low to handle manually, and online analysis may be the only option. In other cases, larger microchannels may still be employed to take advantage of offline techniques while offering significant improvements in sensitivity and efficiency over macroscale systems.

Another common experimental measurement is the protein secretion rate, as determined by media collection and subsequent analysis using either radioimmunoassays or ELISA. A typical question regarding microfluidics is whether the small channel dimensions will hold enough ECs to yield sufficient quantities of secreted products. Table 1 lists a panel of molecules with varying functions (vasodilation: CNP, NO, PGI<sub>2</sub>; vasoconstriction: Et-1;

**Table 1** Secreted molecules from endothelial cells under shear stress, macroscale vs. microscale quantities.<sup>a,b</sup>

Molecule (MW)	Cell type	Shear stress/dyn cm <sup>-2</sup>	Method (sensitivity)	Secretion rate/ pg cm <sup>-2</sup> h <sup>-1</sup>	Macroscale, 60 × 25 mm (total pg/24 h)	Microscale, 10 × 1.5 mm (total pg/24 h)	Reference
CNP (2198 Da)	BAEC	0 25	RIA	1.8 5.7	<b>650</b> 2050	6.5 21	Zhang (1999) <sup>46</sup>
Et-1 (2492 Da)	HGMEC	6.5 13	RIA (5 pg ml <sup>-1</sup> )	12.5 21	4500 7560	45 76	Wang (2000) <sup>174</sup>
Et-1 (2492 Da)	HUVEC	0 1.8 6 12 25	RIA	~20 <sup>c</sup> ~30 <sup>c</sup> ~15 <sup>c</sup> ~12 <sup>c</sup> ~10 <sup>c</sup>	7200 10800 5400 4320 3600	72 108 54 43 36	Kuchan (1993) <sup>44</sup>
NO (30.01 Da)	HUVEC	0 25	Griess method	~0.5 <sup>c</sup> ~2.4 <sup>c</sup>	180 860	1.8 8.6	Kuchan (1994) <sup>116</sup>
PAI-1 (~46 kDa)	HUVEC	0 – 40	ELISA	3.2 × 10 <sup>3 d</sup>	1.2 × 10 <sup>6</sup>	1.2 × 10 <sup>4</sup>	Diamond (1989) <sup>45</sup>
TGF-β (25 kDa)	BAEC	0 0.18	ELISA	88 <sup>d</sup> 175 <sup>d</sup>	32000 64000	320 640	Cucina (1998) <sup>175</sup>
PGI <sub>2</sub> (352.5 Da)	HUVEC	0 10	RIA (50 pg ml <sup>-1</sup> )	40 <sup>d</sup> 240 <sup>d</sup>	14400 86400	144 <b>864</b>	Frangos (1985) <sup>43</sup>
tPA (~70 kDa)	HUVEC	0 4 15 25	ELISA (50 pg ml <sup>-1</sup> )	10.0 <sup>d</sup> 8.3 <sup>d</sup> 20.8 <sup>d</sup> 30.4 <sup>d</sup>	3600 3000 7500 11000	36 30 75 110	Diamond (1989) <sup>45</sup>

<sup>a</sup> Abbreviations: CNP = C-type natriuretic peptide; Et-1 = endothelin-1; NO = nitric oxide; PAI-1 = plasminogen activator inhibitor-1; TGF-β = transforming growth factor-β; PGI<sub>2</sub> = prostacyclin; tPA = tissue plasminogen activator; BAEC = bovine aortic EC; HGMEC = human glomerular microvascular EC; HUVEC = human umbilical vein EC; RIA = radioimmunoassay; ELISA = enzyme-linked immunosorbent assay. <sup>b</sup> Notes: Sensitivity of method is shown only if provided in reference; macroscale area of 60 × 25 mm as suggested in references, and represents area of cell monolayer exposed in typical PFFC; microscale area arbitrarily chosen, in accordance with typical microfluidic cell culture channel dimensions. <sup>c</sup> Reported as mol/mg total protein; conversion assumed 200 pg total protein per cell (range of ~100–300 pg per cell typically cited). <sup>d</sup> Reported as pg/10<sup>6</sup> cells h<sup>-1</sup>; conversion assumed 60000 cells cm<sup>-2</sup> at confluence, as suggested in Diamond *et al.* (ref. 45).

blood clotting: tPA, PAI-1; proliferation: TGF- $\beta$ ) secreted by ECs under static and shear flow conditions. It is apparent that for specific cell types and shear conditions, the amount of some secreted proteins at the microscale is on the same order of magnitude as the amount of other detectable proteins secreted at the macroscale (e.g., compare CNP at macroscale with PGI<sub>2</sub> at microscale). This approximation assumes a 100-fold decrease in available endothelial surface area for microscale estimates. Of greater importance are the assumptions that protein secretion rates, as a biological response to shear, will be equal for both macroscale and microscale experiments, and that no amount of protein will be lost due to absorption into PDMS walls. Again, only direct comparison experiments can verify the validity of these assumptions. Finally, microfluidic systems are ideal for time-lapse videomicroscopy because of the optically transparent materials used to fabricate microdevices. An important consideration, however, is the proper spatial and temporal acquisition of images when high throughput studies are monitored, and cellular events occur in short time.

Two notable examples come to mind when discussing the lack of standardization in microfluidic studies. First, fabrication procedures involving cured PDMS vary widely between laboratories. Curing temperatures range from 70 to 120 °C, while curing times range from 2 h to overnight incubations in the oven. Given the known effects of curing parameters on PDMS properties,<sup>166</sup> the lack of standardization raises skepticism about the implications on biological findings at the microscale. Secondly, specific to maintaining long-term cell culture in microfluidic channels, there is a need for constant media replenishment. To achieve this, researchers can choose to perform intermittent feeding (preferably automatically) based on a schedule,<sup>172</sup> or use perfusion to continuously replace the media.<sup>117,134,173</sup> In either case, the practice to date has been mostly a trial-and-error process, and each cell type appears to prefer a different culturing protocol. It may not be possible to completely generalize a perfusion condition for all cell types inasmuch as it is equally difficult to generalize the maintenance of cultures in Petri dishes. However, there is a need to develop a systematic way of predicting, selecting, and testing the most likely conditions to yield a successful culture at the microscale.

## 6 Future outlook

For over twenty years, macroscale CCF systems have been important laboratory instruments for basic research in endothelial cell biology. Yet, within just the last five to ten years alone, developments in microfluidics have revolutionized the way we approach cell-based research, and have called into question the utility and efficiency of existing setups. To realize the promise of microfluidic technology, however, it is imperative for the research community to bridge the gap between engineers, who design novel devices that often do not translate to user-friendly systems, and biologists, who are the end-users of these devices and ultimately want evidence of biological fidelity in microscale results, accessibility in the final design, and standardization of certain routine procedures for common applications. In the next ten years, the true measure of whether microfluidics has made significant impact on biology will be the number of biologists who have accepted microfluidics as an alternative to macroscale

formats, and the number of significant findings originating from microfluidic-based studies, which otherwise would not have been discovered with macroscale experiments. The role of the engineer is to facilitate this revolution by designing with the biologist in mind.

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