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MICROFLUIDIC PLATFORMS FOR STUDIES OF ANGIOGENESIS, CELL  
MIGRATION, AND CELL-CELL INTERACTIONS

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Running title: Microfluidics for angiogenesis, migration and coculture

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**ABSTRACT**

Recent advances in microfluidic technologies have opened the door for creating more realistic *in vitro* cell culture methods that replicate many aspects of the true *in vivo* microenvironment. These new designs (i) provide enormous flexibility in controlling the critical biochemical and biomechanical factors that influence cell behavior, (ii) allow for the introduction of multiple cell types in a single system, (iii) provide for the establishment of biochemical gradients in 2- or 3-dimensional geometries, and (iv) allow for high quality, time-lapse imaging. Here, some of the recent developments are reviewed, with a focus on studies from our own laboratory in three separate areas: angiogenesis, cell migration in the context of tumor cell-endothelial interactions, and liver tissue engineering.

**Cell culture, cancer, tissue engineering, liver, vascular networks**

List of abbreviations

## INTRODUCTION

This paper presents a brief review of microfluidic systems used in the study of several basic biological functions -- cell migration, angiogenesis, and organ formation *in vitro* - along with a more detailed description of some of the platform technologies and applications currently being developed in our own laboratory. We begin with a short overview of some of the technologies used in the past and today that incorporate cell culture in microfluidic systems. While a number of the existing technologies are mentioned here, for a more comprehensive review of the literature, the reader is referred to one of several excellent recent reviews.<sup>61,68</sup>

### *Early in vitro assays*

Many cellular processes, such as cell migration, angiogenesis, and cell–cell interactions, have been studied using a variety of conventional culture models. In the Teflon fence assay, for example, a cell-free region is established on a substrate using a physical fence, and cell migration is measured after the fence is removed.<sup>76</sup> Another method, the wound assay, involves scraping off a narrow band of cells and quantifying the migration of cells from the boundary into the scraped region.<sup>11,74,78,90,97,98</sup> This method has also been integrated with electrical impedance detection (electric cell-substrate impedance sensing (ECIS) migration assays) to automatically monitor and quantify cell migration.<sup>22, 33, 55</sup> While these methods have been widely employed,<sup>65,71</sup> they have limitations: cell migration occurs over a 2-dimensional (2D) substrate, quantification is somewhat arbitrary, and it is difficult to create environments like those found *in vivo* that typically include a chemical gradient, mechanical stimulation or interactions with other cell types.

In studies of chemotaxis, an agarose well is sometimes used to isolate the cells. For these experiments, an electrode can be integrated into the system to monitor and quantify migration.<sup>37</sup> For studies of neutrophil transendothelial migration or for measures of adhesion, an adherence assay has been used.<sup>7,8,80</sup> These studies have revealed that neutrophil transendothelial migration occurs independent of tight junctions and preferentially at tricellular corners. While this method is quite realistic, there remains a need for 3-dimensional (3D) models that better mimic migration through tissue.<sup>24,95</sup> Cells undergoing migration in 3 dimensions differ considerably from those migrating in 2 dimensions in terms of their morphology, their cell-cell and cell-ECM (extracellular matrix ) interactions, and their tendency to induce cell differentiation.<sup>104</sup>

To meet the needs of mimicking a 3D environment, several assays have been developed. In one such assay, the Boyden chamber, cells are cultured on top of a porous membrane and induced to migrate from top to bottom. Cell migration is monitored by counting the cells that migrate through the membrane and appear beneath it.<sup>1,42,58,79</sup> This experiment can mimic some features of the physiological 3D environment, but does not lend itself to quantification of cell migration in real time. In addition, restrictions on membrane material choice make it difficult to analyze cell-cell or cell-ECM interactions or 3D morphogenesis within the ECM. However, it is a well-established platform and is commercially available.<sup>40,41</sup>

Plating cells on beads that can be suspended in a gel provides a means of monitoring cellular morphogenesis within an ECM.<sup>4,32,64,92</sup> It is simple and effective; nevertheless, sprouts initiate from a solid bead in a manner that differs considerably from capillary formation *in vivo*. Additionally, with this technique it is difficult to produce a well controlled microenvironment with similar dimensions to tissue structures *in vivo*, such

as the distance of tumor cells to blood vessels.<sup>10</sup> Assays adapting hydrogel scaffolds were also developed,<sup>20,82,85</sup> but these often do not allow for the inclusion of many physiological factors such as a fluid-matrix interface and fluid shear as experienced by the cells *in vivo*. A need therefore exists for technologies that recognize, quantify and permit controlled perturbation of local cellular morphogenesis in a 3D matrix while also allowing for chemokinetic and chemotactic effects.

### *Microfluidic approaches*

Microfluidics has been applied to make *in vitro* assays more realistic and adaptable to various applications, whereas typical cell migration assays are unable to integrate complex environmental factors, particularly those that facilitate 3D cell migration. Microfabrication technology has the potential to overcome these challenges in studying cell migration by allowing for precise control of multiple environmental factors.

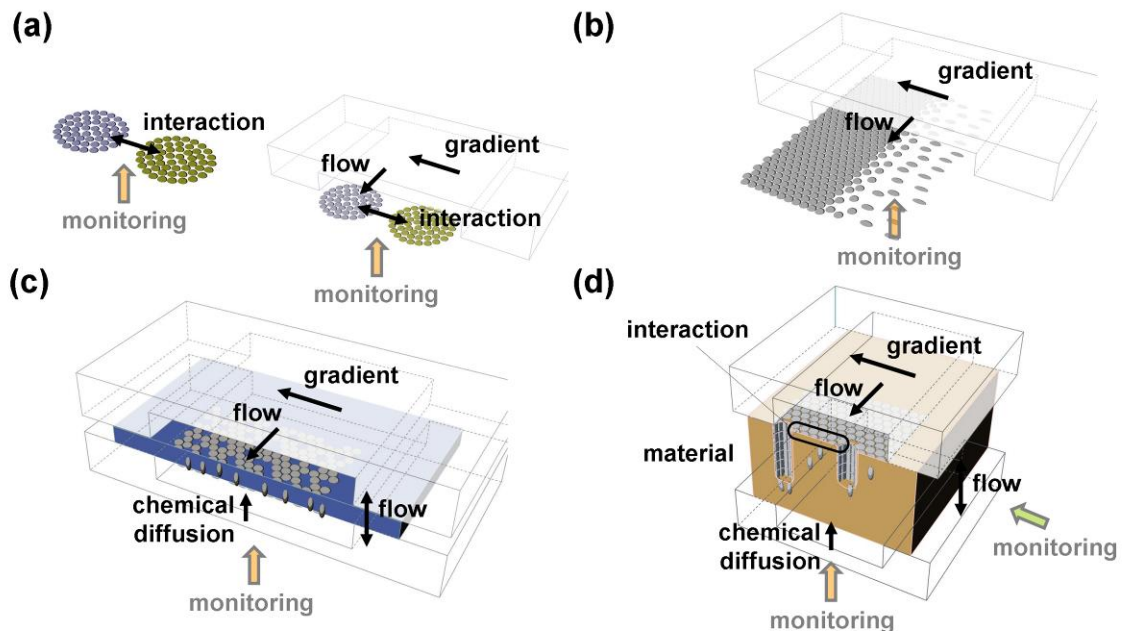


Figure 1. Microfluidics integrated cell migration studies. Orange arrows indicate typical viewing direction. (a) Two cell types interacting on a 2D surface. (b) Interactions in the

presence of a gradient in chemoattractant. (c) Migration through a gel in the presence of flow, either over the cell-seeded surface or through the interstitial space represented by the gel. (d) Similar to (c), but with the gel region of sufficient thickness to allow for sprouting angiogenesis from surface-seeded endothelial cells.

Early studies with micro- and nano-fabricated systems employed topographically modified surfaces with the intention of controlling cell morphologies.<sup>19</sup> Such systems can indeed enhance biocompatibility of artificial organs, bio-interfaced devices, stents, biomedical replacements and implants; induce cell differentiation; and affect cell orientation, gene expression, and protein secretion.<sup>18,63</sup> Previous reviews have summarized many of the advantages of incorporating micro- and nano-fabricated patterns into substrates.<sup>45,49,63</sup> Microfabricated substrates were used to determine whether migrating cells could detect variability in substrate stiffness.<sup>13,77</sup> Microfluidic technology has enabled the precise control of biochemical gradients and quantification of the resulting cell migration (Fig.1(a) and (b)).<sup>47</sup> Some examples include the migration of neutrophils,<sup>27,46</sup> leukemia cells,<sup>88</sup> stem cells,<sup>15</sup> bacteria<sup>75</sup> and cancer cells<sup>89</sup> under varying chemical gradients in microfluidic channels. In these studies a precisely controlled chemical gradient was applied to the cells in a microfluidic channel. However, the spatially- and temporary-controlled chemical gradients were applied to the cells migrating on a 2D surface or suspended in medium.<sup>49</sup>

To explore more realistic conditions, others have employed microfluidics integrated with micro filter structures (Fig.1(c)) to capture increasingly complex 3D tissues.<sup>36</sup> This approach offers the potential for applying continuous mechanical stimuli under controlled chemical environments. In other studies, hydrogel scaffolds have been

cast in microfluidic channels to mimic the ECM. Molecular diffusion across the scaffolds was analyzed under flow condition,<sup>9, 14</sup> and various cell types (i.e. neutrophils,<sup>75</sup> hepatocytes,<sup>51, 84</sup> carcinoma cell lines,<sup>84</sup> astrocytes,<sup>29</sup> and macrophage-like cells,<sup>94</sup>) were cultured within a hydrogel scaffold and exposed to a linear chemical gradient. Kim et al.<sup>51</sup> also reported that hepatocytes formed 3D structures when they were mixed and cultured within a hydrogel scaffold. However, successful 3D cellular morphogenesis within the scaffolds mimicking *in vivo* conditions has not yet been realized in a microfluidic platform.

Microfluidic devices made of hydrogel can be expected to induce 3D responses of the cells in a channel into the surrounding hydrogel.<sup>12,54,67</sup> One reference reported a cell-seeded hydrogel microfluidic device that cultures the cells inside the gel under chemical gradient.<sup>54</sup> Other works used hydrogel microfluidic devices with a linear chemical gradient but without flow.<sup>12,67</sup> However, they reported limited cellular morphogenesis into the hydrogel due to handling difficulties and the need for support structures.

Other approaches have introduced hydrogel into specific regions of the microfluidic device<sup>29,75</sup> to induce physiologically relevant 3D capillary morphogenesis and to culture various types of cells under well controlled microenvironments (Fig. 1(d)).<sup>16,59,81,86</sup> In these studies, cells are seeded and cultured in one channel in direct contact with hydrogel scaffolds and a chemical gradient is applied via a separate channel to induce migration through the scaffolds.<sup>16,59,81,86</sup> Mechanical factors such as shear stress and/or interstitial flow can also be applied with the capability to monitor cellular dynamics in response to changes in their microenvironments (green arrow). Different cell types can be co-cultured in the opposing channels or in the scaffolds to



simulate biological conditions such as tumor angiogenesis and metastasis,<sup>16</sup> smooth muscle cell recruitment,<sup>59</sup> etc.

#### *Applications of microfluidic systems to cellular processes*

Although conventional cell and tissue culture models are useful to investigate certain biological functions, the recent advances in micro-culture techniques described above have opened the way to the next generation of *in vitro* culture models. In particular, microfluidic systems possess the unique capability to spatially and temporally control biophysical and biochemical factors in culture. Hence, these microfluidic culture systems can be applied to a wide variety of *in vitro* studies, such as angiogenesis models, cultures of organ function, and interactions between tumor cells and endothelial cells (ECs).

1) *In vitro angiogenesis models.* The transformation from a quiescent to an active endothelium during angiogenesis is a result of the integration of both pro- and anti-angiogenic signals from the surrounding microenvironment. The *in vivo* microvasculature is subjected to metabolic stress, mechanical stresses (due to fluid shear stress, cyclic stretch and pressure differential), pro- and anti-angiogenic molecules, and other biochemical factors. Several *in vivo* and *in vitro* approaches have been taken to better understand the basic mechanisms involved, with a general consensus to preserve vital physiological conditions. With regard to developing more physiological models, the ability to control one or more relevant environmental features on demand is critical. Recent advances in microfluidic technology have paved the way for the integration of such complexities into microfluidic-based cell culture systems. Additionally, small working distances (between sample and microscope objective) permit continuous monitoring and visualization during morphogenesis.

2) *In vitro cultures of organ function.* Since tissues and organs are typified by complex geometries, 3D microenvironments tend to be more physiologic. Despite this, many studies have been performed using cells grown on flat 2D tissue culture substrates. Consequently, there has been an increasing trend toward reproducing the true complexity of tissues and organs *in vitro* as models of pathophysiological processes. Microfluidic systems can meet these demands by providing both the capability of placing multiple cell types in close proximity and of providing cells a well controlled 3D microenvironment.

The interactions between epithelial cells and ECs are important since most endocrine glands consist of clusters or cords of secretory cells surrounded by capillary networks permeating the tissues. Liver is a vital organ due to its roles in detoxification and producing plasma proteins.<sup>48,100</sup> Its structure is highly organized and composed of several cell types such as hepatocytes, sinusoidal endothelial cells (SECs), hepatic stellate cells (HSCs), Kupffer cells, and biliary epithelial cells. Hepatocytes are the main functional cells in the liver, and organize in interconnected plates. These plates of hepatocytes are surrounded by capillaries, the liver sinusoids that are lined by SECs. HSCs are found in the space of Disse that separates the SECs from the hepatocytes. The surface of each hepatocyte is in contact with the surface of adjacent hepatocytes as well as the wall of the sinusoids, through the space of Disse. These structures suggest that hepatocytes, SECs, and HSCs experience close interactions and collectively account for the structure and essential functions of the liver. Microfluidic systems provide the capability to investigate such interactions needed for organ function.

3) *Tumor-endothelial cell interactions.* Cancer cell migration and signaling interactions with the endothelial cells play a critical role in the invasion of tumor cells

into the vasculature. Once cancer cells have entered into the circulation they can spread to distinct target organs and form lethal metastases.<sup>99</sup> Many studies have emphasized the importance of the tumor microenvironment, consisting of the ECM, biochemical and biophysical forces, and other cell types, in regulating cancer metastasis.<sup>95</sup> Although existing *in vitro* assays have provided significant insight into the cellular and molecular mechanisms of tumor cell motility, they are typically limited to two-dimensions, include only a single cell type, and lack control of the microenvironment cues. Microfluidics allows for the integration of these critical tumor microenvironmental factors with multiple cell types. This allows for novel assays to be designed that better mimic *in vivo* conditions. Assays based on microfluidics therefore provide robust platforms for studying 3D cell migration and heterotypic cell-cell interactions in cancer invasion and for testing the efficacy of potential treatment drugs.

Microfluidic systems need to be designed for specific *in vitro* applications as described above. In particular, the microfluidic systems are designed so as to provide a 3D gel scaffold, controlling pressure and flows, manipulate multiple cell types, and image in real time at high resolution. Detailed design considerations are described below.

## **DESIGN CONSIDERATIONS AND METHODS**

### **A modular approach**

In an attempt to design a system that meets the stated objectives, the approach in our lab has been to reduce the system to a small number of component elements, and to assemble these in various ways with different geometries to meet the demands of each particular experiment, specified in terms of the *controllable factors*, discussed below.

The modular components are (i) the access channels, (ii) gel or matrix regions, and (iii) a means for controlling the flow of fluid, its magnitude and direction (Fig.2).

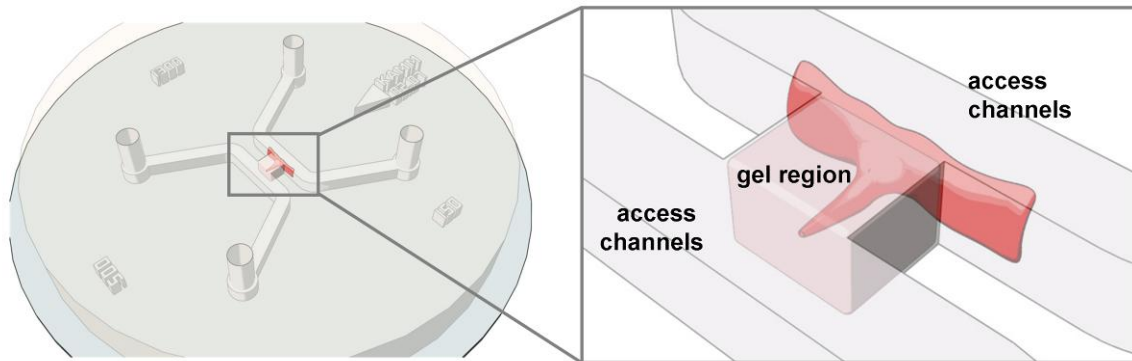


Figure 2. Microfluidic device incorporating ECM scaffolds. Gel or matrix regions located between two access channels enable cell culturing under controlled condition.

*Access channels.* These provide a means for seeding cells, changing media, and regulating flow. We have chosen to use channels that are comparable in size to small venules or arterioles (hundreds of microns) that are (i) large enough to seed single cells or cell clusters without blockage, ii) but not so large as to cause significant dilution of soluble factors released into the media or unduly increase the system volume relative to the region of interest, namely the portion of the channels adjacent to the gel region.

*Gel/matrix regions.* If access channels are the vascular or lymphatic vessels, then the gel/matrix region is the tissue or ECM that these vessels permeate. Volumes are again critical when cells are seeded in 3D, and dimensions are critical in terms of establishing gradients in biochemical factors (distances between capillaries vary widely, but often fall in the range of several hundred  $\mu\text{m}$ ). In addition, high resolution imaging dictates that the cells in both the access channels and the gel/matrix regions be within a few hundred microns from the surface through which imaging is performed.

Several methods of introducing gel that we have used involve different designs of the gel region. In certain cases, the gel needs to be microinjected prior to covering the gel region with a coverslip (Fig.3(a)).<sup>86</sup> This is most often done when cells are seeded in 3D so as to eliminate the stress cells might experience as a result of injection through a long filling duct, and to reduce the time prior to gelation or equilibration with medium. In many experiments, however, it is more convenient to close the system prior to introducing matrix material. In this case, gel-filling channels are added to the microfluidic system, with filling ports similar to those at the entrance to the access channels (Fig.3(b)).<sup>16, 59</sup> Focused laminar flow also can form gel/scaffold region between two channels (Fig.3(c)).<sup>94</sup>

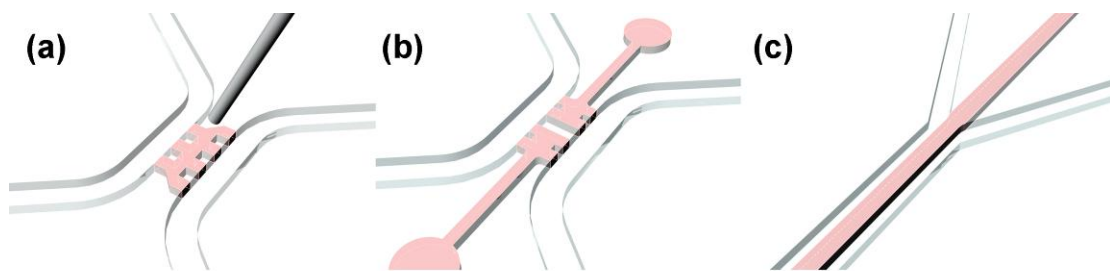


Figure 3. Gel/scaffold filling methods. a) Microinjection into the gel region prior to closing. b) Gel injection via channels incorporated into the fabricated surface. c) Injection of a gel solution into a laminar flow between two non-gel fluids.

*Flow control* . Methods of flow regulation can be further sub-divided into pumps (active) and valves (passive) components, and several designs exist for both. Pumping can sometimes be accomplished with external systems, although this greatly increases the total fluid volume required, adds complexity, and limits the mobility of the system. The simplest method for producing flow is by adjusting the relative size of the droplets

at the entrance and exit to the access channels.<sup>87</sup> Surface tension then provides the pressure gradient needed for pumping, although the “strength” of the pump varies with time, as the sizes of the two droplets change. Other, onboard methods have been employed: for example, one involving the use of multiple fluid channels, separated from the access channels by a compliant membrane, that can be sequentially pressurized to collapse the medium channel in a way that induces unidirectional flow.<sup>30,31,101</sup> Finally, small reservoirs can also be attached to each entrance port, and filled to various levels to both regulate the mean pressure within the channel and to produce pressure gradients.<sup>81,86</sup> Depending on the rate of flow, these reservoirs can be relatively small (e.g. the tip of a micropipette). Valves can obviously be created in a similar manner, using channel collapse as a means of preventing flow in one or more access channels. It is also possible to prevent flow by simply blocking the entrance to access channels.

### **Controllable factors**

In order to faithfully replicate as much of the *in vivo* microenvironment as possible, it is necessary to maintain control over a variety of biophysical and biochemical factors. Some of these are listed and discussed here.

*Pressure and flows.* Fluid or interstitial pressure gradients *in vivo* vary over a range of up to about 15 kPa (e.g. between an artery and a vein) over distances as small as about 1 mm, or even higher in load-bearing connective tissues. More generally, interstitial pressure gradients tend to be much lower, since much of the vascular pressure change occurs across the relatively impermeable vessel wall. Although considerable evidence has been published to demonstrate that cells respond to hydrostatic pressures, much of the mechanotransduction literature focuses on the effects of flow or, more directly, fluid shear stress, either on the vascular endothelium or cells

in the ECM. Interstitial flow velocities have been reported to be in the range of about 10  $\mu\text{m}/\text{min}$ .<sup>38,83</sup> Given the dimensions of the matrix region in our systems ( $<1\text{mm}$ ) and the desire to reproduce physiological flow magnitudes, we found it necessary to generate pressure gradients across the gel region of about 100 Pa. Larger gradients might be useful for some experiments, but they increase the likelihood of problems either with system leaks or with maintaining an intact gel volume. In this connection, design features, such as posts added to the gel region and surface treatments to help the gel adhere to the walls of the device, have both proven useful.

*Matrix materials.* Various matrix materials, both biological and other biocompatible materials, have been employed in various microfluidic systems. Some of the matrices include collagen gels, Matrigel, fibrin gels, self-assembling peptide gels, and polyethylene glycol gels. The desirable mechanical and biochemical properties of gels vary depending on the host tissue of cultured cells, such as liver, pancreas, brain, and heart.<sup>21,23</sup> While the gel modulus can always be varied by changing its concentration, it is often useful to have a concentration-independent means of controlling the modulus. This has been accomplished by the introduction of cross-linking, as in the case of PEG gels, or simply by changing the conditions during polymerization (e.g. by changing the pH during collagen I polymerization).<sup>96</sup> Pore size (affecting diffusion coefficients and hydraulic permeability) is also a critical factor, but more challenging to control in a reliable manner.

*Gradients.* Many biological processes occur in the presence of gradients of various growth factors, chemoattractants and other biological agents. Among those most extensively studied are angiogenesis and cell migration. Numerous investigations have reported the effects of gradients on migration on a 2D surface,<sup>13,53,73</sup> and a few have also

generated 3D migration in a gel.<sup>24,35,36,95</sup> The ability to establish a gradient of known magnitude stable for periods of hours to days, is a useful capability in a microfluidic system. Such gradients can be demonstrated by a variety of methods, but one common approach is to introduce a fluorescent tracer so that the concentrations gradients can be visualized *in vitro*.

*Surface treatments* . Since the surface to volume ratio tends to be greater in microfluidic systems as compared to traditional culture methods, the nature of the surface, its behavior in combination with seeded cells or the gel matrix is of central importance. A variety of surface treatments have been used, both to promote cell adhesion (e.g. collagen, fibronectin, laminin) and adhesion of the gel (e.g. collagen, poly-D-lysine).<sup>39,72</sup> One issue that becomes critical when attempting to maintain stable flows or concentration gradients is the ability to prevent gel shrinkage due to cell contraction, as is facilitated by the use of an appropriate surface treatment or by limiting cell density.

*Time-dependent delivery*. Most biological processes proceed through a series of stages, and each stage is characterized by a different local environment in terms of concentrations or gradients of factors. To recreate this evolving process, one needs the capability to change the bathing medium over time in some predetermined manner. In macro-culture methods, this is typically accomplished by changing the medium on a daily basis. In a microfluidic system, medium can be convected through the access channels, and if desired, varied as a function of time. While we know of no experiments that have yet attempted to do this in a continuous fashion, the capability exists.



### **Additional considerations**

*Imaging capabilities.* One of the major advantages in microfluidic systems for cell culture is that high resolution imaging is possible, given the small overall dimensions. Using thin coverslips and transparent substrates such as polydimethylsiloxane (PDMS), the imaging distance can be maintained in the range of several hundred microns. This allows for real-time imaging using an environmental chamber, including confocal microscopy to observe structures such as vascular sprouts extending in 3D. Using a variety of fluorescent markers, various intracellular processes can be visualized in real time, although these methods are still under development.

*Multiple cell types.* It has become increasingly evident that in order to replicate *in vivo* behavior requires the interaction among several different cell types. One method that has been employed is the use of conditioned medium. Another is to use porous wells, with one cell type seeded on the top and another on the bottom. Still, traditional methods lack flexibility in the use of multiple cell types in the correct biological arrangement. Microfluidic systems, due to the existence of multiple gel regions or access channels, have the capability to introduce two or more cell types in close proximity. Two examples are provided below, but researchers are just now beginning to realize the full potential of this new capability.

*Quantification methods.* Much work is needed to cast the results that can be obtained from microfluidic cell culture in quantitative form. To date, the results have largely been reported in qualitative terms since much of the data are obtained from imaging. Certainly in the case of cell migration, the various well-established 2D methods can be extended to 3D. In the case of angiogenesis, capillary sprouts can be quantified in a number of different ways (number, total length, length of projected

images, branch points), but to date there has been relatively little quantitative analysis of the biochemical signaling that occurs.

This raises one of the current limitations of microfluidic systems: the small number of cells involved and the restrictions that imposes on the use of more traditional biochemical assays. Medium volumes often lie in the range of micro liters, and total cell numbers may be in the hundreds, so the total amount of protein or mRNA may be insufficient for Western or Northern blot studies. This highlights the need for high throughput or multi-culture systems that both provide for larger amounts of protein and mRNA, as well as aiding statistical analysis.

## **RESULTS AND DISCUSSION**

### **Angiogenesis**

Angiogenesis - the growth of new micro vessels from preexisting blood vessels, is orchestrated by a range of pro- and anti-angiogenic factors.<sup>26</sup> This process occurs either naturally during development or wound healing or as a consequence of a pathological process that is associated with various ‘angiogenesis-related diseases’<sup>10</sup> including cancer,<sup>25</sup> rheumatoid arthritis,<sup>17,52,91</sup> and atherosclerosis,<sup>66,93</sup> among several others.

In our system, we localize hydrogels in the gel region of the microfluidic device to achieve physiological relevance, in turn promoting 3D capillary morphogenesis under well-controlled microenvironments.<sup>16,29,59,75,86</sup> In addition, relevant cell types (e.g. pericytes, cancer cells) may be included for co-culturing studies. In these approaches, ECs are cultured in one channel in direct contact with hydrogel scaffolds. Once a confluent endothelial monolayer is formed, induction of an *in vivo*-like angiogenesis is achieved by applying a chemical or physical angiogenic stimulus (e.g. fluid shear stress,

interstitial flow and fixed gradients of growth factors (e.g. vascular endothelial growth factor (VEGF))). In the case of a chemical gradient, a growth factor is applied from the channel opposite to the cell monolayer to induce invasion into the scaffolds, allowing us to evaluate and quantify capillary morphogenesis from an intact cell monolayer. For example, angiogenic factor (VEGF gradient across hydrogel) was applied to the ECs cultured in the microfluidic channel, and during several days of culture, the length and projected area of sprouting structures into the scaffolds were monitored and quantified. We found that ECs in contact with the VEGF gradient were highly active and rapidly migrated into the scaffold, but cells in contact with the control scaffold were more restrained and demonstrated markedly less migration (Fig. 4). Confocal microscope images confirmed 3D migration and also the cross-sectional distribution of the cells in the scaffolds.<sup>16,86</sup> We also evaluated the EC response in co-culture with physiologically relevant cell types, including cancer cells as discussed further below.<sup>16</sup>

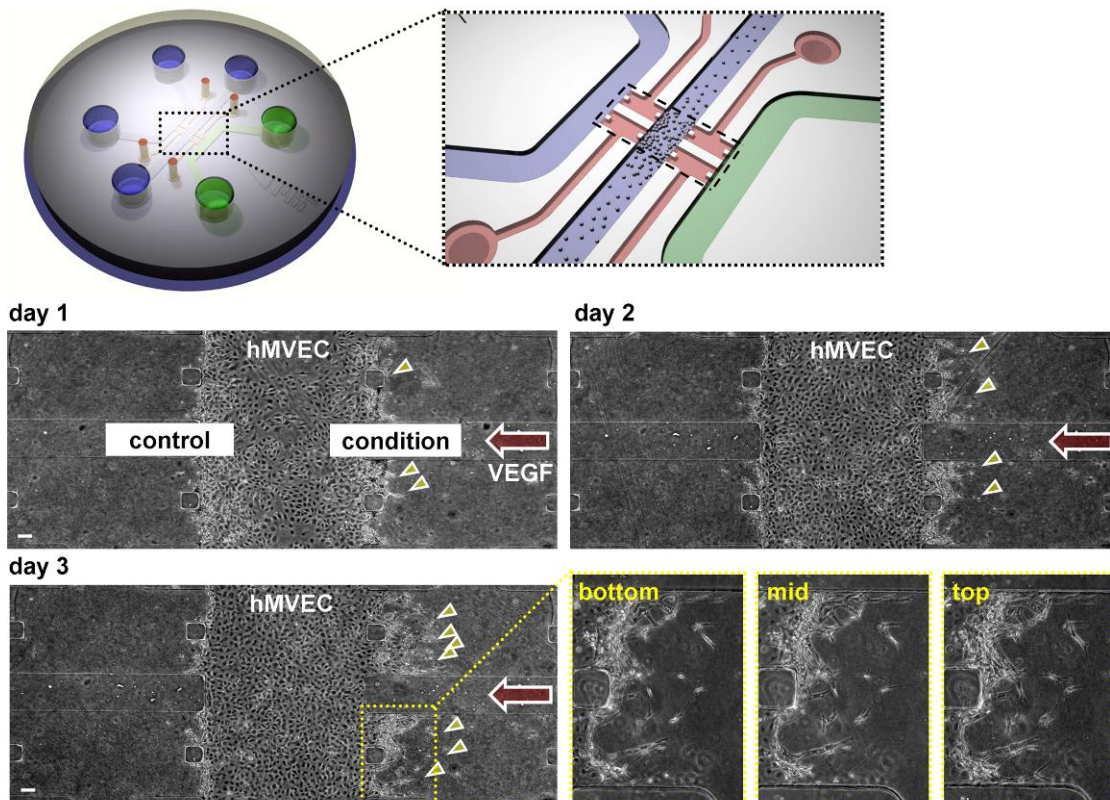


Figure 4. Microfluidic *in vitro* 3D angiogenesis assay. Human microvascular endothelial cells (hMVEC) are cultured in the center channel followed by the addition of growth factor (VEGF) to the condition side (green). Biased angiogenic response on the condition side is evident with the 3D nature confirmed by the pictures taken at different focal planes (from left to right; bottom, middle and top).

### Liver tissue engineering

Liver has a remarkable capacity for regeneration; hepatocytes can actively proliferate and restore the original liver mass in response to partial hepatectomy in animals and humans. It has been difficult, however, to reproduce the regenerative capacity of liver cells *in vitro*. When hepatocytes are isolated from rats and cultured on a tissue culture dish, they lose their cuboidal morphology and their differentiated functions within one week of culture.

To reconstruct liver tissue *in vitro*, microscale culture techniques were first applied in 2D culture systems. Advances in microfabrication technology provided a means for precisely controlling the distribution of the liver cells. In these systems, hepatocytes along with the other cell types are cultured in a restricted area with the aid of micropatterning, and heterotypic cell interactions have been investigated.<sup>5,43,50,102,103</sup>

3D culture of liver cells is essential if we hope to replicate the *in vivo* situation. Recent advances in microfluidic systems have provided the opportunity to culture liver cells in a precisely controlled microenvironment, including growth factor gradients and interstitial flow conditions. A microfabricated microarray bioreactor has been developed for the culture of hepatocytes under physiological flow conditions.<sup>35,44,69,70</sup> Using this reactor and under interstitial flow conditions, hepatocytes formed 3D tissue-like structure and maintained liver-specific functions for over 2 weeks. However, these reactors are difficult to image due to their geometry and size.

Our microfluidic system overcomes some of these limitations in that it enables real-time imaging of cell morphogenesis and migration.<sup>16,86</sup> Applying this microfluidic system to liver cell culture, hepatocytes were cultured under flow and static conditions.<sup>81</sup> When hepatocytes are cultured under interstitial flow, they formed 3D tissue-like structures; the interstitial flow appears to enhance cell–cell cohesion, leading to the formation of 3D tissue-like structures through a different mechanical balance between cell-substratum adhesion and cell-cell cohesion. In addition, this microfluidic system was also applied to the co-culture of hepatocytes and ECs, and the morphogenesis of hepatocytes and ECs was monitored daily in the living assay. The ECs formed 3D capillary-like structures that extended across an intervening gel to the hepatocyte tissues, while they formed 2D sheet-like structures without hepatocytes.

These results suggest that hepatocytes and ECs have heterotypic interactions across the gel scaffold by diffusional transport.

As we mentioned above, liver has a highly organized structure. Although it was difficult to precisely manipulate cells in conventional culture methods, we can try to reconstruct the liver structure with the developing micropatterning and microfluidic culture systems. In particular, more than three cell types can be cultured in a 3D microfluidic system since multichannel devices have the capacity for seeding different cell types sequentially. For example, the interactions between hepatocytes, HSCs, and SECs need to be elucidated using such microfluidic systems, since HSCs may play an important role in the vascularization process during liver regeneration.<sup>60</sup> When liver is surgically resected, mature hepatocytes in the parenchyma are stimulated to proliferate and form avascular clusters. The hepatocyte clusters are subsequently penetrated by HSCs followed by SECs.

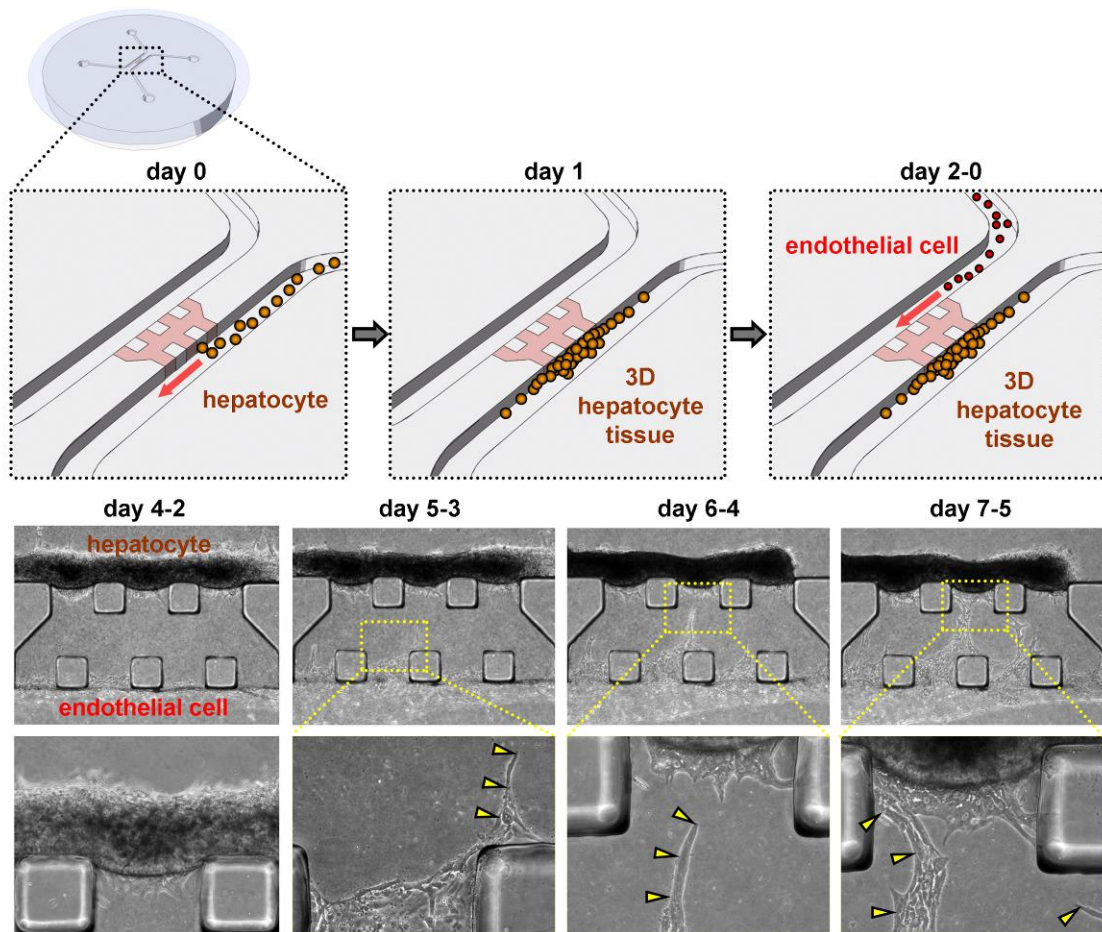


Figure 5. Hepatocyte-endothelial cell co-culture in a microfluidic device. Hepatocytes were seeded into one of the two microfluidic channels (day 0). The device was tilted to allow hepatocytes to accumulate on the side wall of the collagen gel. These hepatocytes were cultured under flow conditions, and formed 3D structures on the sidewall of the gel (day 1). Next, endothelial cells were seeded on the other side of the gel (day 2-0). Hepatocytes and endothelial cells were cultured under static conditions after day 2-0. Some endothelial cells formed vascular sprouts on day 5-3 (arrowheads, day 5-3). These sprouts extended toward the 3D hepatocyte tissues (arrowheads, days 6-4 and 7-5).

## **Tumor-EC Interactions**

Tumor cell invasion into the surrounding tissue and intravasation are critical steps in cancer metastasis. Both steps are regulated not only by the intrinsic tumor cell invasiveness, but also by the tumor microenvironment, including biochemical and biophysical factors, as well as heterotypic cell interactions. Our microfluidic platform fills a critical gap in *in vitro* models of cancer metastasis by combining the interactions of an endothelial cell monolayer with cancer cells within the same 3D ECM matrix, and enabling control of critical microenvironmental cues such as growth factor gradients and matrix stiffness. This provides more physiologically relevant conditions, including both the effects of the acellular and cellular microenvironment components. Furthermore, the modular design allows for the control of these components independently, as well as the design of novel assays for studying the role of biochemical, biophysical and cell-cell interactions in cancer metastasis.

Although, much progress has been made in studies of single tumor cell migration *in vitro*, the role of the vasculature in tumor cell migration and how it may influence intravasation remains poorly understood.<sup>6</sup> It is becoming increasingly evident that apart from the intrinsic motility of tumor cells, paracrine interactions with the endothelial cells may also affect tumor cell migration and intravasation. In order to address this question, we present a tumor-endothelial cell interaction assay, which models the growth of an invasive tumor towards an endothelial monolayer. In this assay, similar to the angiogenesis assay, an endothelial monolayer is seeded in one channel, while the cancer cells are seeded in a second channel (Fig. 6). As a model for highly invasive cancer, the brain cancer cell line U87MG (glioblastoma cells) was used along with primary human dermal microvascular endothelial cells. Contrary to the cancer cells that



form a tumor mass in the channel and subsequently invade into the ECM, the endothelial cells form a continuous monolayer, spanning the bottom, top and side PDMS walls and the collagen gel wall. In order to allow for the formation of this confluent, intact monolayer, the endothelial cells are seeded 24 hours before the tumor cells. Subsequently, the cancer cells proliferate and form an invasive tumor-like structure that begins to invade the ECM after 24 hours. The porous collagen gel that is located between the two cell seeding channels provides a 3D ECM-like matrix for the endothelial cells to form sprouts towards the invading tumor mass. Furthermore, it allows for bi-directional exchange of secreted factors from the tumor cells to the endothelial cells through diffusive transport.

Using time-lapse phase-contrast microscopy, the migration of each cell type into the ECM can be monitored every 24 hours. Figure 6 demonstrates the cell-cell interactions during this experiment; within a time-scale of two to three days the tumor cells form an aggressively growing mass, and at the same time sprouts originating from the endothelial monolayer are also observed. Interestingly, once a few tumor cells initially invade into the ECM, the follower cells appear to migrate along paths defined by these leader cells in the matrix. These leader cells are guided towards the sprouting monolayer and some of them will eventually transmigrate (Fig. 6), while others become oriented towards the sprouting capillaries. Hence, our microfluidic platform presents the opportunity to assess tumor-endothelial cell interactions in a 3D matrix where endothelial cells can form sprouts, across which cancer cells can invade.

The assay design described above may also be used for studying tumor angiogenesis, where the expanding tumor mass serves as the condition channel, as described previously in the angiogenesis section. Using a confocal microscope, the

sprouting of the endothelial cells as well as tumor migration can be monitored and characterized. Apart from the intrinsic reciprocal paracrine interactions between the tumor and the endothelial cells, it is possible to establish external growth-factor gradients which could simulate a third cell type, such as macrophages producing EGF gradients. For these studies the assay design that includes three channels with two pairs of ECM regions was used, so that different conditions could be established across each pair. By seeding the tumor cells in the central channels and applying different conditions across each ECM region, the interactions of the tumor cells with the endothelial cells can be monitored in the same platform under control and test conditions. Hence, this allows for direct and straightforward comparison of the cell migration characteristics, such as migration velocity, persistence time and directionality.

In summary, our microfluidic platform provides a versatile platform for designing assays to systematically quantify the effects of the cellular and non-cellular tumor microenvironment on cancer cell motility. Under a systematic variation of the biochemical (e.g. EGF gradients) and biophysical (e.g. interstitial flow) factors, the results of the tumor-endothelial interaction assay can provide insight into the mechanism of tumor cell guidance towards and transmigration across an endothelial monolayer. Furthermore, the assay enables real-time assessment of tumor cell migration and intravasation in order to develop new metrics of tumor cell invasiveness. Finally, apart from the cancer-endothelial interaction assay presented above, a significant extension of this work would be the incorporation of a continuous flow of circulating tumor cells in one of the microfluidic channels in order to study cancer cell extravasation. This can be achieved by seeding a monolayer of endothelial cells onto the channel and the 3D ECM wall and subsequently introducing the circulating tumor cells

into the channels. This would enable monitoring their arrest at the endothelium and their transmigration from the flow-stream through the endothelium into the 3D ECM.

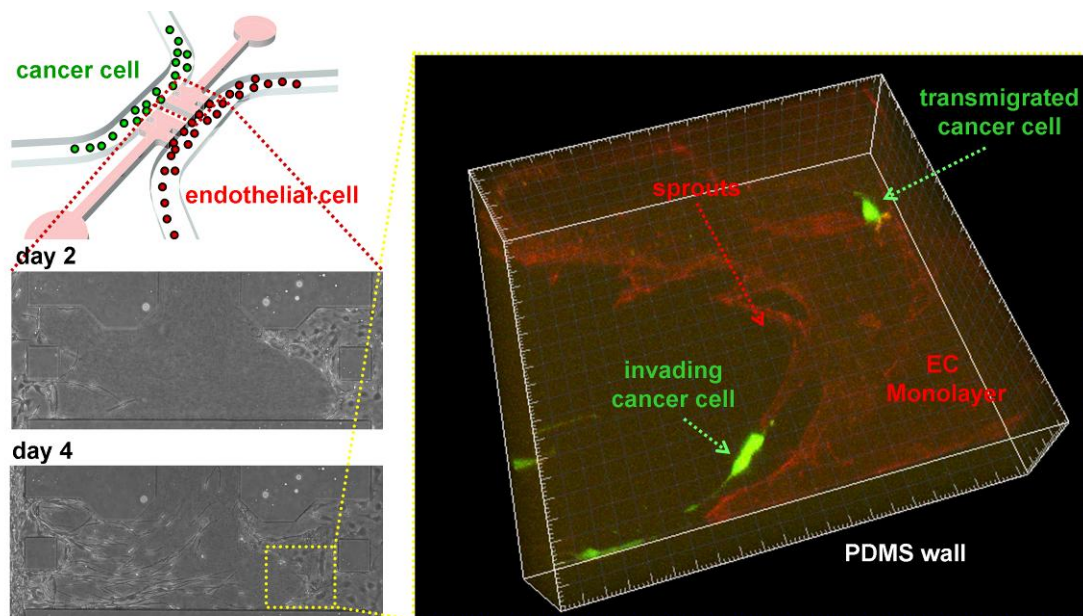


Figure 6. Tumor-EC interaction assay. Tumor cells are cultured in the left channel, while endothelial cells are seeded in the right channel. The growing tumor mass and the endothelial monolayer response were monitored every 24 hours (left: shown at 48 and 96 hrs). Confocal microscopy (right) was used for visualizing the invading and transmigrated tumor cells (green arrows), as well as the sprouting monolayer (red arrow).

## CONCLUSIONS

Microfluidic cell culture holds considerable promise for recreating realistic microenvironments in which to study biological processes involving multiple, interacting cell populations. Many examples have already been reported: for example, studies of cell migration, angiogenesis, interactions between endothelial and tumor cells, and steps toward the development of *in vitro* systems to mimic certain aspects of

organ function. But the potential of these systems is just now becoming widely recognized, and we can expect to see many new applications.

Despite the promise of such microfluidic cell culture assays, there are some disadvantages compared to conventional techniques, and challenges that need to be addressed. For example, cell regions typically have a high surface area to volume ratio, so surface chemistry takes on greater significance. In this context, surface materials and treatments deserve special attention. In addition, although these systems have distinct advantages in terms of their ability to visualize cell growth and function in live-cell assays, the small numbers of cells make the usual biochemical assays difficult at best. New methods will need to be developed that allow for live cell imaging of intracellular events such as the activation of signaling pathways.

The future is bright, however, and microfluidics will likely emerge as a viable platform for a variety of applications ranging from the creation of functional *in vitro* tissues to drug discovery to personalized medicine.

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104. Different from 2-dimensional migration, steps of 3-dimensional cell migration can be arranged in 5 steps; Pseudopod protrusion at the leading edge, formation of focal contact, focalized proteolysis, actomyosin contraction, and detachment of trailing edge.<sup>28</sup> Cells on 2-dimensional flat substrate cannot have epithelial cell polarity, and also often have different patterns of gene expression. Growth, motility, differentiation and morphogenesis are different due to 3-dimensional matrix dependent regulation in 3-dimensional environments.<sup>95</sup> For example, epithelial cells form 3-dimensional lumen structures with epithelial polarity and mesenchymal cells lie in the ECM. 2-dimensional migration models cannot represent the whole 3-dimensional structures and also 3-dimensional matrix-induced morphogenesis such as vessel sprouting and branching. 3-dimensional migration of tumor cell also has diversity of amoeboid migration, mesenchymal single cell or chain migration and other collective movements forming cluster or multicellular strands or sheets.<sup>28</sup> Considering tissue engineering applications used to restore, maintain or enhance tissues and organs, we need a better understanding of 3-dimensional structures and responses of cells.<sup>2,3,34,35,56,62</sup> To engineer living tissues *in vitro*, cultured cell are coaxed to grow on bioactive degradable scaffolds that provide the physical and chemical cues to guide their differentiation and assembly into 3-dimensional tissues.<sup>35</sup>

