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Microfluidic 3D cell culture: from tools to tissue models

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The transition from 2D to 3D cell culture techniques is an important step in a trend towards better biomimetic tissue models. Microfluidics allows spatial control over fluids in micrometer-sized channels has become a valuable tool to further increase the physiological relevance of 3D cell culture by enabling spatially controlled co-cultures, perfusion flow and spatial control over of signaling gradients. This paper reviews most important developments in microfluidic 3D culture since 2012. Most efforts were exerted in the field of vasculature, both as a tissue on its own and as part of cancer models. We observe that the focus is shifting from tool building to implementation of specific tissue models. The next big challenge for the field is the full validation of these models and subsequently the implementation of these models in drug development pipelines of the pharmaceutical industry and ultimately in personalized medicine applications.

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Introduction

Two-dimensional cell culture techniques, in which cells are grown on a flat substrate such as petri-dishes or microtiter plates, are still common laboratory practice. However, over the last two decades, awareness of the relevance of the cellular micro-environment (e.g. the extracellular matrix and the interstitial fluid) increased. This new cell culture paradigm, referred to as 3D cell culture, is rapidly gaining popularity. For example, embedment of cells in an extracellular matrix is associated with more relevant physiological behavior, such as witnessed by apical–basal polarization [1], lumen formation [2], reduced proliferation and increased differentiation [3] and numerous changes in RNA and protein expression [4]. Furthermore, cells cultured in 3D show important hallmarks of tissues *in vivo*, such as for example bile duct formation in liver spheroids [5] and milk protein secretion by mammary epithelial cells [4].

The transition from 2D to 3D culture techniques is an important step in a trend towards ever more physiologically relevant tissue models. However, 3D culture techniques typically do not yet capture the multicellular complexity of tissues, lack vasculature, do not offer precise control over gradients and undergo medium exchange at discrete time points instead of in a continuous manner.

Microfluidic techniques allow spatial control over fluids in micrometer-sized channels that can be explored to extend the physiological relevance of 3D culture models. Early examples demonstrate spatial patterning of adhesion molecules [6] and hydrogels [7,8], which are still used in microfluidic 3D cell culture. Today, the three most important drivers for the use of microfluidic techniques in 3D cell culture are:

(i) The ability of co-culturing cells in a spatially controlled manner
(ii) Generation of and control over (signaling) gradients.
(iii) The integration of perfusion/flow.

Mechanobiological aspects, such as active stretch and tension, is another functional aspect that can be added using microfabrication techniques. Although interesting, it has received minor attention in combination with 3D cell culture, and will therefore not be discussed in detail. The interested reader is referred to a recent review by Polacheck *et al.* [9•].

In this review, we discuss the most dominant and important recent examples of how microfluidic tools were applied to improve 3D cell culture models. Efforts over the last two years will be categorized and discussed in the context of abovementioned drivers. We particularly emphasize the contribution of microfluidics to the unmet needs in 3D cell culture, as well as the role of these models in the drug development pipeline. For reviews regarding manufacturing of microfluidic devices we refer to other publications [10,11]. We observe that the focus is shifting from tool building to more in-depth focus on the development of specific models. The full validation of these models and the symbiosis with recent developments in stem cell niches and induced pluripotent stem cells (iPSc) will determine the trend for the coming years.

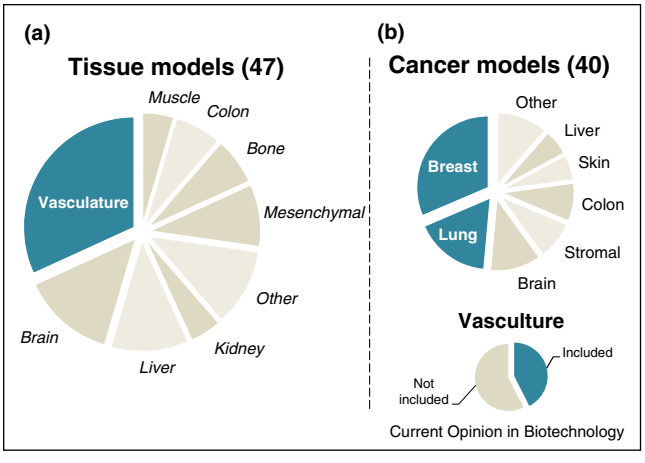
Overview

We inventorized 87 papers that contained the keywords '[*microfluidic OR microengineered*] *AND 3D cell culture*' which appeared on PubMed since 2012. The papers were categorized according to the tissue and organ model addressed and depicted in Figure 1a. Cancer is a particularly dominant field in microfluidic 3D cell culture, and therefore depicted separately in Figure 1b.

As is shown in Figure 1a, most tissue modelling efforts were focused on vascularity, followed by brain and liver tissue models. The striking dominance of efforts in vascular modelling might be explained by the fact that microfluidics is the only platform capable of perfusing such vessels, thereby inducing the vitally important flow and accompanying shear stresses. This, in addition to the co-culture context and relevance of gradients in for instance angiogenesis assays, makes that vasculature models benefit most from the added value of microfluidics [12]. The attention for brain models fits in a wider trend towards attention for stem cell-derived neuronal models for diseases such as Alzheimer and Parkinson. This follows the more generic increase in popularity of induced pluripotent stem cell techniques and progress in controlling the stem cell niche of differentiated tissues.

Cancer is a complex heterogeneous disease and cancer *in vitro* models are driven by the demand for phenotypic screening models. This fits in a trend towards more systemic approaches to therapy discovery and selection, as well as in the trend towards tailoring therapies to individual patient's characteristics, so-called personalized medicine. Breast and lung cancer models comprise half of the developed cancer models (see Figure 1b), correlating with their high incidence in humans. Vasculature is involved in many parts of the metastatic cascade, the spread of cancer cells. Interestingly, many of the recent developed microfluidic 3D cancer models include a vascular component, as shown in the subgraph of Figure 1b. These models include processes such as angiogenesis, migration and intravastion and extravastion, and will be discussed later.

Figure 1



Overview of the modelled organs of 87 articles which combine 3D cell culture with microfluidics since 2012. **(a)** The distribution of different organ and tissue models addressed by microfluidic 3D cell culture. Vasculature was the most modelled tissue, followed by brain and liver. **(b)** The distribution of the recently developed cancer models. Breast and lung tumor models comprise half of the developed models. Almost half of the cancer models include vasculature.

Microfluidic tools for spatially controlled cell culture

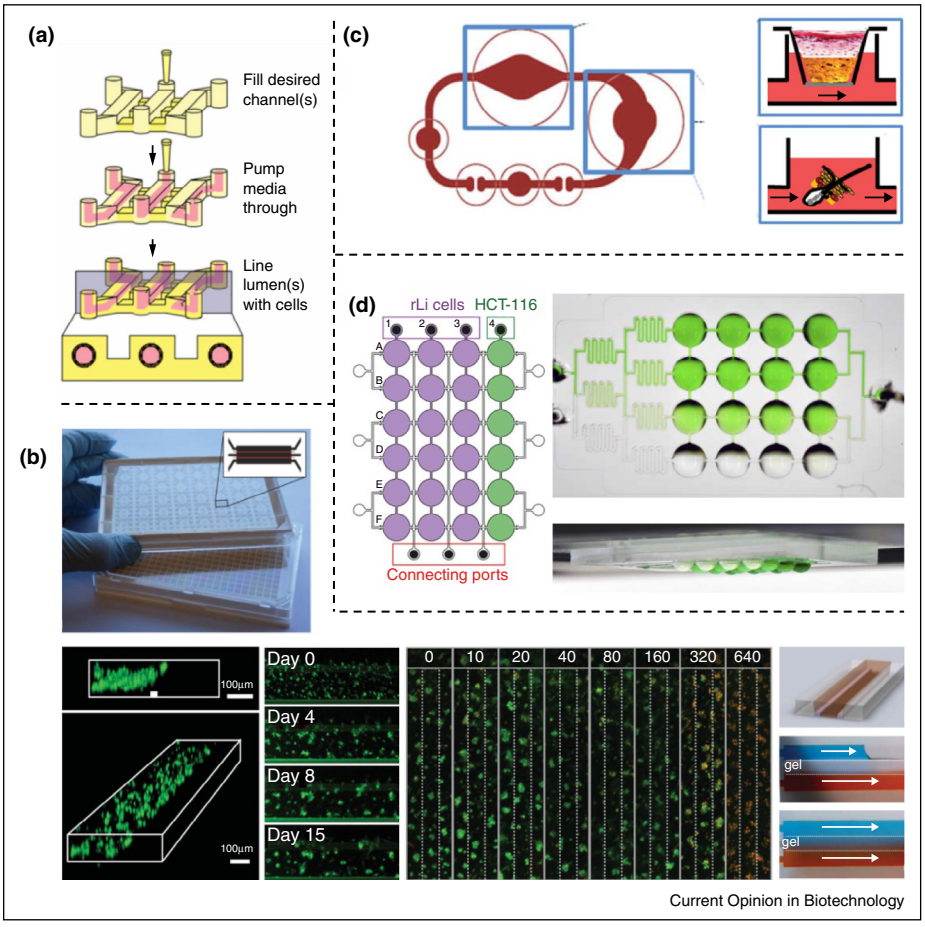
Spatial control is at the basis of many principles found in microfluidic 3D cell culture. It allows patterning of cells and extracellular microenvironment, to create stratified (co-)cultures with basal–apical access, gradient formation and medium perfusion.

In classical culture techniques, the spatial control is usually achieved by a membrane, such as in Boyden chambers [13], to support surface-attached cell growth, and separation of the culture reactor in two compartments. Although typically associated with 2D cell culture, membranes are also widely used in microfluidic chips [14]. A recent trend in microfluidic systems is to use hydrogels to offer cells a more physiologically relevant, three dimensional matrix [15,16]. Hydrogels enable a more relevant environment in which cell can cluster together, without need for surface adhesion. Selective patterning of hydrogels enables co-culture of cells without the need for artificial membranes.

Spatial control over hydrogels is achieved using guiding structures such as ridges, pillars or posts [17–19]. Alternatively, hydrogels can be molded into the right geometry [20••,21•]. Bischel *et al.* show an interesting technique to pattern cells inside a hydrogel, requiring just a few pipetting steps. The hydrogel is introduced into the microfluidic channel. Due to the fluidic properties and differences in viscosity and pressure, a liquid can create a lumen inside the hydrogel. A vessel is formed by introducing a cell suspension, which adheres to the hydrogel [22•] (see Figure 2a).

In the surge towards high throughput, standardized microfluidic platforms, Trietsch *et al.* developed a microfluidic 3D co-culture plate with 96 individually addressable chambers. In this plate, hydrogels are patterned by phaseguides. Perfusion flow was maintained by passive levelling between two reservoirs, thereby eliminating the need for external pumps. The microfluidic channel dimensions were optimized to enable screening using a standard fluorescent microscopy. This was demonstrated by generating a IC_{50} curve of the toxicity of Rifampicin to 3D liver spheroids (Figure 2b) [23•].

Figure 2



Microfluidic techniques for 3D cell culture. **(a)** Cell patterning inside a hydrogel, exploiting the microfluidic properties and differences in viscosity and pressure (see text for more details) [22•]. **(b)** 96 microfluidic culture chambers integrated underneath a microtiter plate. Extracellular matrix gels are selectively patterned in the chamber by phaseguides to obtain a layered profile [23•]. **(c)** A hanging droplet system developed by Frey *et al.* [32]. Since the columns and rows are addressable, flexible co-culture setups are possible. This picture demonstrated the hanging droplet array in combination with a gradient generator. **(d)** A whole tissue perfusion system developed by Ataç *et al.* [29] tissues are isolated and cultured on membrane inserts. Microfluidic channels interconnect the tissue chambers, allowing continuous perfusion and paracrine cell signaling.

Cells can also be spatially controlled without hydrogels, using microchambers or droplets, in which suspended cells settle and cluster to form spheroids [24–27]. Wang *et al.* show an interesting microfluidic device which captures colon spheroids using an microfluidic cell strainer. The captured colon spheroids are then embedded in a hydrogel to provide an extracellular matrix which is crucial for spheroid growth [28].

Co-culture

The ability to spatially control cells paves the way for combining multiple cell types in a way that more faithfully represents the organization of tissues and organs. Using hydrogels, cells can be patterned to mimic the spatial organization found *in vivo*, which is useful to mimic for example the interaction between stromal cells with various tissues. Furthermore, the formation of monolayers against hydrogels allows the study of trans-endothelial migration of (cancer) cells. Microfluidics can also be used to (co-)culture whole tissues and to interconnect them through microfluidic channels for media circulation. For example, Ataç *et al.* show a co-culture of hair follicles with skin tissue slices in a microfluidic Boyden chamber-like system (Figure 2c) [29,30]. Vasculature can be included as well by seeding endothelial cells inside the interconnecting channels [31].

A hydrogel-free co-culture environment can be created using a microfluidic hanging drop system. Frey *et al.* demonstrate a continuously perfused array containing both liver and colorectal cancer (CRC) spheroids. This was used to study drug metabolism and toxicity: the liver spheroids metabolize a chemotherapeutic compound,

thus exposing the CRC spheroids to the metabolized drug (see Figure 2d) [32].

Gradients

In vivo, soluble (bio)molecular gradients are found in many different biological phenomena, such as angiogenesis, invasion and migration. Microfluidic devices have been developed to study angiogenesis and (anti)angiogenic factors [19,22•,33,34], as well as tumor invasion assays [35,36].

Since microfluidics enables spatial control over fluids, the gradients can be precisely controlled. For example, by patterning a hydrogel between to fluids, stable and predictable linear gradients are formed. By altering the channel geometry and applied flow rates, more complex gradient patterns are possible [37].

Han *et al.* studied trans-endothelial migration of neutrophils under influence of a gradient of two chemo-attractants, elegantly demonstrating the power of compartmentalization, spatial control and gradient formation in microfluidic 3D cell culture. They show that the neutrophils respond differently to the two different chemo-attractants and correlate ECM stiffness with migration speed [38].

To integrate these type of assays into the high throughput drug-screening pipeline, Trietsch *et al.* [23•] demonstrate gradient formation within their microfluidic titer plate format. A double perfusion flow was used to generate a gradient over a compartmentalized hydrogel (see Figure 2b). This allows high throughput migration assays, gradient formation in combination with stratified co-cultures.

Instead of using soluble molecules, cells can be used to create gradients as well, resulting in heterogeneous cell densities within a hydrogel. Mahadik *et al.* show a microfluidic device which creates opposing gradients of two cell types [39]. This will be a useful tool to determine for example the optimal cell ratio of niche cells and stem cells.

Perfusion

Perfusion of 3D cell culture is almost exclusively reserved for microfluidic techniques, since the compartmentalized nature of microfluidics allows to perfuse media adjacent to or through a 3D cell culture. Benefits associated with perfusion flow include stable nutrient supply, waste metabolites removal and control of oxygen tension. Perfusion is one of the crucial aspects in vasculature, as it provides shear stress, which affects the cellular morphology and gene expression [40,41].

Vasculature

In the context of 3D cell culture, vasculature models benefit most from the added value of these microfluidics tools including spatial control, co-culture, gradients and perfusion. Vascular models are typically grown by seeding endothelial cells in or against a hydrogel [19,20••,34,37,42–45]. Zheng *et al.* created a perfusable vascular network in microchannels that were molded into collagen (see Figure 2e) [20••]. As a result, endothelial cells are fully surrounded by a natural collagen matrix. Under perfusion with whole blood, authors showed that upon stimulation with phorbol-12-myristate-13-acetate (PMA), long fibers of Von-Willebrand factor (VWF) are secreted. Webs of VWF-fibers form at channel intersections, which were demonstrated to trap platelets more effectively.

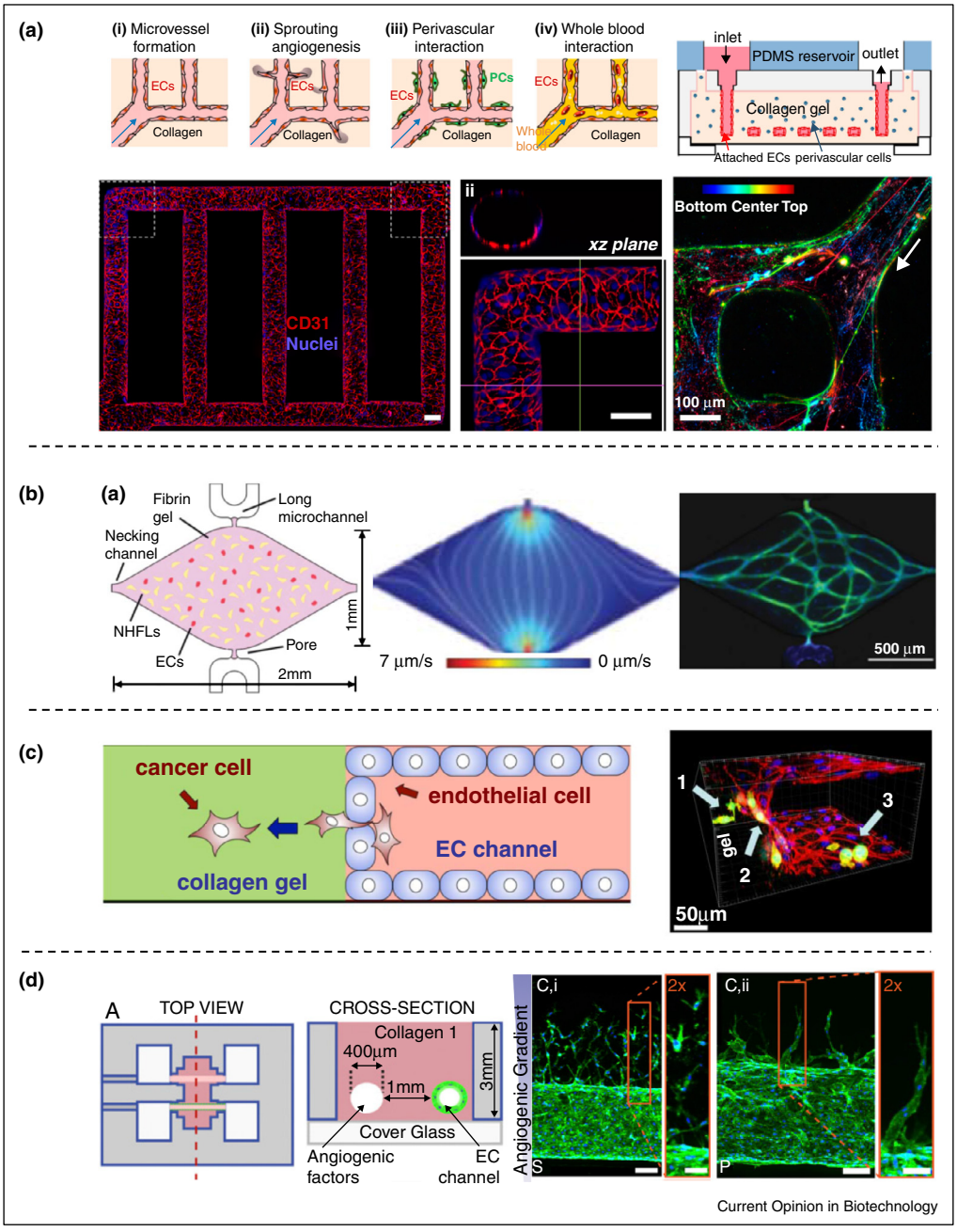
Tourovskaia *et al.* used a similar approach for culturing brain vasculature together with stromal and neuronal cells in an attempt to create a model for the blood brain barrier. Pericytes and astrocytes were mixed with collagen that was injected around needles inserted in a chip. After polymerization, the needles were removed leaving a lumen that was subsequently seeded with brain endothelial cells [44], thereby creating a co-culture of the key cell types that play a role in the blood brain barrier.

Also endothelial–epithelial vessels can be created, as demonstrated by a kidney-endothelial double tubules. A collagen microfluidic structure was used to pattern a tube of Madin-Darby canine kidney (MDCK) cells, next to a tube of human umbilical vein endothelial cells (HUVEC) [45]. Huang *et al.* also show a perfusable, stratified MDCK co-culture with adipose derived stem cells [46], which enhanced cilia formation and increased expression of ion transporters of the MDCK cells.

The predictability of microfluidic flows can be used to control the interstitial flow in the cellular microenvironment, as demonstrated by Hsiang Hsu *et al.* They show a microfluidic device with endothelial cells and fibroblasts embedded in hydrogel. Medium is perfused through the gel from small inlets resulting in spatially defined interstitial flow patterns. The interstitial flow provides mechanical cues that induce vasculogenesis, resulting in *in vivo* like vascular architecture (Figure 3b) [47].

The mechanical cues however can also be transduced by the microfluidic channel geometry, as suggested by a study of the response of endothelial cells on various curvatures [48•]. Within a microfluidic device, HUVEC and brain microvascular endothelial cells (HBMVEC) were grown around small glass rods with a diameter of 10 μm, mimicking the curvature found in microcapillaries. Under continuous flow, HUVECs and brain microvascular endothelial cells (HBMVEC) responded differently. At high curvature, brain endothelial cells resist elongation while HUVECs aligned in the flow direction, suggesting that this phenotype plays a role *in vivo*: by minimizing the elongation, the tight junction

Figure 3



Overview of vasculature models. **(a)** Vasculature *in vitro* model developed by Zheng *et al*. [20••]. A grid layout is patterned in collagen gel and HUVECs are seeded inside. The vasculature shows formation of VWF after perfusion with whole blood and stimulation with phorbol-12-myristate-13-acetate (PMA). **(b)** A microfluidic device consisting of a culture chamber filled with hydrogel containing endothelial colony forming cell-derived endothelial cells (ECFC-ECs) and normal human lung fibroblasts (NHLF). Media is perfused through the gel from small inlets resulting in spatially defined interstitial flow patterns. This provides the cells with mechanical cues, resulting in *in vivo* like vascular architecture (see image, cells labeled with CD31 (green) and DAPI (blue)) [47]. **(c)** A microfluidic *in vitro* model for extravasation, using a co-culture of HUVEC cells and a breast cancer cell line. The breast cancer cells inside the lumen (indicated with 3) extravasate through the endothelial barrier (indicated with 2) into the matrix (indicated with 1) [52]. **(d)** Microfluidic angiogenesis model, using a two channel design with lumen inside a collagen gel. HUVEC cells are seeded and form a vessel, which showed migration (C,i) as well as sprouting (C,ii) of the endothelial cells after stimulation with different pro-angiogenic factors [21•].

length is decreased thereby minimizing paracellular transport, one of the hallmarks of the blood brain barrier.

Cancer models

The metastatic cascade, the way cancer cells spread, is closely linked to the vascular system. Many parts within the metastatic cascade can be studied by co-culturing perfusable endothelial vessels with cancer cells. Using microfluidic 3D cell culture, effects such as angiogenesis [20••,22•,33], migration [49,50], intravasation [51] and extravasation [18,19,44,52] have been studied, and will be discussed in more detail.

Figure 3c illustrates an extravasation setup in which endothelial cells are seeded against a compartmentalized hydrogel. After two days, breast cancer cells (MBA-MD-231) were perfused through the lumen. The breast cancer cells extravasated into the gel and increased the endothelial permeability [52]. Bersini *et al.* modified this setup by seeding human mesenchymal cells (hMSC) into the gel, to create a more specific niche for the extravasating cells and show a significant increase in extravasation compared with the previous setup. Furthermore, the authors show that a gradient of cytokine CXCL5 show similar extravasation compared with the hMSC co-culture. Blocking the CXCL2 receptor, which has a ligand for CXCL5, reduced extravasation [18].

An important driver in tumor migration is interstitial flow [53•,54]. A microfluidic 3D interstitial flow chamber was used to study the migration behavior of MDA-MB-231 breast cancer cells and glioma cells [55].

Many publications show gradient formation of angiogenic factors in combination with perfusable vasculature to study angiogenesis [19,20••,21•,33–36]. Nguyen *et al.* study invasion and sprouting of HUVECs which are exposed to a gradient of various cocktails of pro-angiogenic factors. Interestingly, they found that a gradient of vascular endothelial growth factor (VEGF) alone was not sufficient to trigger angiogenesis. It was found that a gradient of spingosine-1-phosphate (S) triggered single cell migration and phorbol 12-myristate 13-acetate (P) triggered collective cell migration (see Figure 3d). Mixtures of multiple pro-angiogenic factors triggered multicellular sprouting; a hallmark of angiogenesis *in vivo*. This demonstrates the potential for elucidating molecular mechanism of angiogenesis using microfluidic devices.

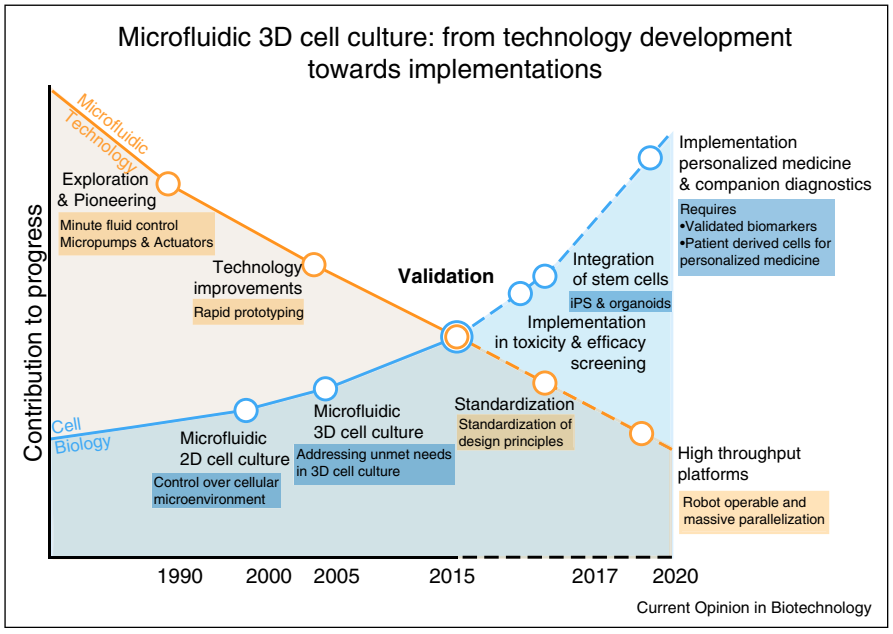
Microfluidics and 3D cell culture: from exploration to validation to implementation

Above examples demonstrate the potential of microfluidic techniques to improve the physiological relevance of 3D cell culture models. We discussed functional additions such as stratified co-cultures, gradient formation, vessel formation and medium perfusion. In addition to the physiological relevance, there are incentives such as improved reproducibility, cost effectiveness and/or ease of handling that may drive the implementation of microfluidics. For example, the reduced dimensions offer advantages such as reduced consumption of expensive cell material, hydrogels and screening reagents. Well defined heights of microfluidic channels dramatically improve imaging quality and speed. Z-stacking with confocal imaging equipment might for many assays not be necessary anymore, as co-culture and migration assays are patterned in the horizontal plane and most cells lie within the same focal plane. Precise metering of liquids with microfluidic techniques enables better quantification of assays.

A model system is only as good as the cells that are used to build it. Human cells are a must for enhanced predictive models. Primary material is physiologically most relevant, but suffers from complex logistics, batch-to-batch variation and often limited life span *in vitro*. Cell lines overcome these problems, but are not always considered good representatives for the *in vivo* situation. iPSc and organoid techniques have rapidly increased in popularity. Organoids are derived from primary stem cells. Stem cells expressing the LGR5 receptor have been identified which have been shown to maintain stemness *in vitro* and differentiate into fully functional intestine, stomach and liver [56–58]. The iPSC techniques allow reprogramming of fibroblasts into stem cells that can be differentiated into various tissues, such as neurons [59], cardiomyocytes [60] and several blood lineage cells [61]. Both cell sources allow to recapitulate various inherited diseases *in vitro*, and to study genotypic differences.

Microfluidic 3D culture models need to be fully validated before they can be applied by a wide range of users in academia and pharmaceutical industry. However, validation is a challenge in its own, and still an issue for many *in vitro* models [62••]. It is widely expected that 3D culture models based on human derived cells are better predictive of clinical outcome than animal tests due to their human origin. Animal tests are thus not suited as a reference model for validation. Retrospective validation based on clinical results for, for example, successful and failed compounds with regards to toxicology, should be used as reference points for validation. However, these data and the relevant biological materials are in many cases not publicly available. An alternative validation strategy is to compare biochemical changes between an *in vitro* model system and clinical studies, such as for example gene expression profiles, enzymatic activities and metabolism. Clinical biomarkers can guide this validation and improve the comparability between organ models and the clinical reference point. To identify and assess such biomarkers, sensitive analytical methods are needed such as sequencing, microarray and/or mass spectrometry techniques. A particular challenge here is the sensitivity of analytical systems as only small

Figure 4



Timeline of breakthrough developments that contributed to microfluidic 3D cell culture. As the microfluidic tools are in place, we are currently at the validation phase, a bottleneck that needs to be addressed before the field can advance and replace current *in vitro* models.

amounts of medium and cells are available from microfluidic cell cultures. We expect important developments in the near future in which microfluidics and Organ-on-a-Chip systems are coupled with mass spectrometry analysis [63].

In our opinion, the various microfluidic designs of available model systems are no longer the bottleneck to the use of microfluidic 3D cell cultures in a wide range of academic and industrial applications. Although complications such as material incompatibility to hydrophobic compounds, as for instance the case for PDMS systems, still persist and many chips still lack a user-friendly interfacing [11], others have addressed these issues in a convincing manner. As microfluidic technology matures, the focus will shift towards biological development and validation of physiologically relevant models (Figure 4). The trend for the coming years will be to use microfluidic 3D cell culture in combination with the recent advances in stem cell biology, such as iPSC [64] and organoid technology. This will allow to take into account differences between patients in various applications: first, novel diagnostic tests to predict treatment outcome for an individual patient; second, supporting clinical trial design; or third, taking the individual differences already into account during drug discovery and developments, both in respect to efficacy and toxicity. Thus, ultimately, we expect that microfluidic humanized 3D cell cultures will play an important role in the development of personalized medicine.

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