Tumor-on-a-chip: microfluidic models of tumor morphology, growth, and microenvironment

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Abstract

Cancer remains one of the leading causes of death, albeit enormous efforts to cure the disease. To overcome the major challenges in cancer therapy, we need to have a better understanding of the tumor microenvironment (TME), as well as a more effective means to screen anti-cancer drug leads; both can be achieved using advanced technologies, including the emerging tumor-on-a-chip technology.   Here we review the recent development of the tumor-on-a-chip technology, which integrates microfluidics, microfabrication, tissue engineering and biomaterials research, and offers new opportunities for building and applying functional three-dimensional (3D) *in vitro* human tumor models for oncology research, immunotherapy studies, and drug screening.   In particular, tumor-on-a-chip microdevices allow well-controlled microscopic studies of the interaction among tumor cells, immune cells, and cells in the TME, of which simple tissue cultures and animal models are not amenable to do. The challenges in developing the next-generation tumor-on-a-chip technology are also discussed.

Key words: tumor-on-a-chip, microfluidics, tumor microenvironment, drug screening

1. Introduction

Cancer remains one of the leading causes of death in the US and many other countries in the world, despite the extensive research and enormous efforts in drug discovery over the last few decades to cure the disease. This is partly due to the high cost of developing a new anti-cancer drug, as well as the need to better understand cancer development and the tumor microenvironment (TME), including the roles of inflammation, different effectors and suppressors of immune responses, the heterogeneity of tumor stroma, and the function of tumor vasculature. To make significant improvements in cancer therapy, it is necessary to develop more effective approaches to screen anti-cancer drug leads and to have a better understanding of TME using advanced technologies, including the organs-on-chips technology [1-5].

To date most cancer research and anti-cancer drug screening have been conducted using cell culture and animal models. While animal models of cancer can provide essential *in vivo* information of tumor growth and response to drug molecules, they could be very costly and the results may have very large variations among the animals used, thus difficult to get relevant statistics. Further, small animal models such as mouse models for cancer studies may not accurately represent what happens in human [6]. On the other hand, two-dimensional (2D) and three-dimensional (3D) cell cultures have been widely used for screening anti-cancer drugs, and studying cell signaling, proliferation, migration, and drug responses including altered protein/gene expression [7, 8]. These *in vitro* models may use co-culturing of multiple cell types in hydrogel matrices and include patient-derived cells [9, 10]. Although cell culture models are low cost, easy to handle, and typically have high repeatability, they may not be able to mimic microenvironment in an organ or an animal, thus are not suitable to study the effect of complex spatial organization and interaction of cells.

As an alternative to animal models and cell culture models to address the complex problem of cancer development and treatment, 'tumor-on-a-chip' technology has emerged recently as a new tool for cancer studies, providing a unique approach which integrates microfluidics, microfabrication, tissue engineering and biomaterials research, possessing the potential to significantly advance our understanding of cancer biology, allowing accelerated and cost-effective drug discovery [4, 11]. As shown in Figure 1, a tumor-on-a-chip system consists of a microfluidic device that has tissue culture, nutrient and small molecule supply and waste removal functions (Figure 1A) [12]. Ideally, a 3D tumor could grow on the chip with a complex tissue structure comprised of tumor cells, stromal cells and blood vessels either self-organized or spatially organized by design, mimicking some aspects of a tumor (Figure 1B) [13]. Examples of first-generation tumor-on-a-chip systems include a chip in which lung cancer spheroids were embedded in micropatterned 3D matrices immediately contiguous to a microchannel lined with endothelial cells (Figure 1C) [4], and a breast tumor-on-a-chip model comprised of the upper and lower cell culture chambers separated by an ECM-derived membrane that mimics a basement membrane *in vivo* (Figure 1D) [13]. Previous reviews in the literature on tumor-on-a-chip technology include the construction of 3D tumor models [14-17], its applications to specific cancer studies such as metastasis [18, 19], and its utilities in drug discovery [20, 21].

This review aims to showcase the recent developments of the tumor-on-a-chip technology to mimic tumor microenvironments for cancer biology studies and biomedical applications. In Section 2, 3D *in vitro* tumor models established on microfluidic chips are reviewed. Specific microdevices mimicking various tumor microenvironments are elaborated in Section 3. In Section 4, examples of tumor-on-a-chip applications are discussed. The challenges in developing the next-generation tumor-on-a-chip technology are summarized in Section 5.

2. 3D *in vitro* tumor models on chip

To characterize and study the invasiveness and detailed cancer biology of different tumors, *in vitro* culture of cancer cells from a tumor sample is routinely used. A 2D monolayer cell culture on thermoplastics is the gold standard for *in vitro* maintenance and multiplication of cells. Although 2D cell cultures have been widely used in various cellular assays (e.g., migration and toxicity assays) to characterize the metastatic property and drug response of cancer cells, 2D cultures cannot recapitulate the 3D architecture of tissue's complexity, biophysical and biochemical property of extracellular matrix (ECM), and cell-cell interactions of human tumors [22-24]. Furthermore, cell cycle, cellular signaling, and drug sensitivity can be different if cell culture is performed in a 3D instead of a 2D microenvironment [25-27]. *In vivo* 3D models using animal xenografts are also popular but suffer from ethical concerns and unable to mimic human-specific biology and physiology. *In vitro* 3D tumor models are created by adapting several 3D tissue engineering methods to construct cells into three-dimensional space and mimic the *in vivo* tumor microenvironment in the body (Table 1). Among them, top down methods use decellularized scaffolds and bottom up methods utilize cells to build up tumor tissues for *in vitro* 3D tumor models. In tumor-on-a-chip systems, 3D tumor tissues are often cultured initially by established tissue culture protocol, then transferred onto the microfluidic chip for analysis. Thus, *in vitro* 3D tumor models can be adopted in tumor biology research and the development of therapeutics for personalized medicine [28, 29]. Next, we discuss common techniques for creating 3D *in vitro* tumor models in details (Figure 2).

2.1 *Ex vivo tumor culture*

Primary tumor tissues from biopsy or surgical resection can be embedded in ECM and cultured as an *in vitro* model [30-34] (Figure 2a). The embedded tumor sections retain the

tumor vasculature, nearby stroma, and the heterogeneity of the tumor cells. Microfluidic technology can be combined with *ex vivo* tumor section culture system for parallel drug sensitivity testing while maintaining continuous control over culture conditions [35].

2.2 *Conventional transwell model*

Transwell inserts (also known as Boyden chambers) are widely used to perform conventional migration, invasion, and transendothelial migration assays, to assess the migration of cancer cells in combination with a chemical gradient. A transwell insert is composed of a polymeric porous membrane to allow cancer cells to migrate through the pores. A transwell insert is routinely used together with a multiwell plate with chemoattractants inside wells. In a migration assay, the ability for cancer cells to translocate through the pores is measured. The invasion assay further characterizes the migration of cancer cells through a 3D ECM layer on the porous membrane. In addition, the transendothelial migration ability of cancer cells can be characterized by using a transwell insert with a confluent endothelial cell layer grown on top of the membrane [36] (Figure 2b).

The transwell assay is usually performed as an endpoint assay since it is difficult to image the kinetic behavior of cells migrating through the pores. Moreover, the steepness of chemoattractant gradient established between the well and inside the transwell insert is difficult to be controlled, making the transwell assay results semi-quantitative. However, transwell assays are quite suitable for more motile or invasive cell subpopulations as they can be recovered after the transwell assay [36].

2.3 *Tumor spheroids*

A tumor spheroid is derived from three dimensional aggregations of cells under non-adherent cell culture conditions [37]. The tumor spheroid resembles a small tumor mass in its

morphology, growth kinetics, nutrient transport, and cell-cell as well as cell-matrix interactions. Thus, the tumor spheroid serves as an excellent *in vitro* 3D tumor model [28, 38].

Tumor spheroids can be generated by using single or multiple cell suspensions from permanent cell lines as well as dissociated cells from primary isolated tumor tissues and organotypic tissues [38]. Several common methods to generate tumor spheroids include suspension culture, hanging drop method, liquid overlay on non-adherent substrates, two phase encapsulation, and assembly by bio-conjugate chemistry or magnetic particles [28]. In the suspension culture, cells are placed in a spinner flask [39] (Figure 2c) or a NASA micro-gravity vessel [40] (Figure 2d) to promote spheroid formation by inducing aggregation. The suspension culture is advantageous in high throughput production of spheroids, but the disadvantages are limited control over spheroid size and uniformity. The hanging drop method utilizes microtiter plate or microstructures to inversely hold a cell suspension droplet [41-47] (Figure 2e). The cells aggregate under gravity and subsequently form spheroids inside the droplet. The hanging drop method is of moderate throughput, but it possesses better control over the size of the spheroid. Microfluidic perfusion networks in combination with hanging drop methods have been utilized for continuous spheroid culture and drug screening [44-47]. Alternative to hanging droplet method, using the liquid overlay method, cell suspension is cultured on non-adherent substrates to produce spheroids [48-50] (Figure 2f). The advantage of the liquid overlay method is its simplicity of operation, but the disadvantage lies in its poor control over spheroid size. Similarly, to produce tumor spheroids by avoiding cell adhesion to cultureware and inducing aggregation, an aqueous two phase system can also compartmentalize cell suspension and produce spheroids without the concern of drying and possible inefficiency in chemical transport and toxicity of an oil phase [51-53]. Three dimensional spheroids can also be formed by assembly of cells using bioorthogonal chemistry [54] or incubation of cells with magnetic particles [55, 56] (Figures 2g, 2h).

Recently, several microfluidic techniques have been developed to create tumor spheroids by either hydrodynamic trapping of cells in stagnation regions or in microwell structures [57-60], aggregating multiple cells in double-emulsions or hydrogel droplets [61-64], or aggregating cells on a digital microfluidic platform [65] (Figure 2i). The advantages of generating spheroids by microfluidics include control over spheroid size with continuous perfusion, as well as real time and *in situ* observation of spheroid formation kinetics. However, spheroids produced in some microfluidic models are difficult to be retrieved for off-chip analysis [57, 63, 66].

2.4   *3D cell culture in 3D matrices*

Tissue engineering methods have been adopted to create 3D tumor models. A scaffold is a biocompatible and chemically stable extracellular support structure serving as an instructive support for cell attachment, growth, and morphogenesis into tissues [67] (Figure 2j). A porous scaffold can be made from decellularized tissues or from fabrication of several natural ECM proteins or biocompatible polymers such as collagen, hyaluronic acid, silk protein, polyethylene glycol (PEG), and polylactic acid (PLA) [68, 69]. The scaffold is commonly prepared by freeze drying, electrospinning, phase separation, and microscale macromolecular self-assembly [70-74].

Tumor cells cultured in scaffolds showed less sensitivity to chemotherapy and yield tumors with more invasive phenotypes [70, 75-77]. While the porous scaffolds have the mechanical and chemical characteristic of ECM for 3D tumor cell culture, the disadvantages include lack of vasculature structure in fabricated scaffolds that hindered perfusion for long term culture, as well as poor control on cell placement positions inside the scaffold.

Alternative to scaffolds, a bottom-up approach using cells or few-cell spheroids as building blocks has emerged, inspired by the embryonic developmental processes [78, 79] (Figure 2k).

Hydrogels as extracellular matrix support are embedded with cells or few cell spheroids as building blocks (also known as bioinks) [80, 81]. Several natural polymers such as collagen, fibrin, Matrigel®, hyaluronan, chitosan, gelatin, and alginate, as well as synthetic polymers such as PEG can be used to create property-controlled hydrogel matrices. The bioink containing multiple cell types and multiple ECMs can be printed at high density into large scale tissues and organs through the layer-by-layer additive bioprinting. The cell positions in three dimensions can be automatically and precisely controlled using bioprinting to create multicellular tissue with vasculature mimicking the *in vivo* tissue hierarchy and the microenvironment [80, 82, 83]. Common bioprinting methods include inkjet printing [84, 85], microextrusion printing [86, 87], laser-induced transfer printing [88], and stereolithography [89, 90].

2.5 *Microfluidic tumor-microvascular model*

The vasculature plays a pivotal role in tissue engineering and tumor biology [91]. Tissue engineering with vasculature is important for 3D persistent tissue culture. Moreover, the growth and dissemination of cancer requires growth of new vasculatures for nutrient transport [92]. Many cell types in the vasculature such as endothelial cells interact with cancer and modulate the tumor microenvironment as well as the cancer phenotype [93]. Conventional transwell assay, tumor spheroids, and scaffold approaches share the disadvantage of their inability to incorporate tumor-vasculature interactions in the culture. Using microfluidic technology, capillary lumen structures have been fabricated to mimic the microvasculature in tissues [94]. Common methods to create capillary lumen structures as microvasculatures include molding the capillaries in hydrogels by needles or rods [95-98], by photoresists [99-101], by sacrificial carbohydrates [102], or creating lumens based on viscous fingering instabilities [103, 104]. Alternatively, an endothelial vascular network as the microvasculature can be formed by endothelial sprouting in hydrogels [105-112], monolayer

on ECM hydrogel [113-115] or on a porous membrane [116, 117], and monolayer in microchannels [118, 119] (Figure 2l).

Creating a functional microvasculature network together with 3D tumor model is essential to recapitulate the tumor microenvironment *in vitro*. By using a microfluidic perfusable platform to co-culture vasculature and cancer cells, it allows better kinetic examination of important cancer progression stages such as angiogenesis, intravasation, and extravasation in a controlled microenvironment [99, 110, 112, 120, 121]. Future challenges for microfluidic tumor-vasculature model include validation of the platform to clinical tumor tissues and increase complexity of the emulated microenvironment, such as chemical gradients and fluid flow at biological relevant speed and rhythms. Tumor microenvironments are complex and each component within often interact and affect one another. Current efforts have focused on mimicking specific tumor microenvironment to answer different biological questions. A microfluidic platform with active control components such as microvalves and micropumps can be programmed to recapitulate multiplex physical and chemical gradients together with multiple cell types to better mimic the complex microenvironment of a tumor. However, the design, experiment, and analysis on such platforms still pose great challenges that yet to be overcome.

3.   Mimicking tumor microenvironment using microdevices

Cancer is a complex and heterogeneous metastatic disease modulated by genetic, epigenetic, and cellular signaling influenced by its surrounding stroma. The cancer cells grow uncontrollably into a primary tumor and interact with the supportive and immune cells as well as the biochemical and biophysical components of ECM in the nearby stroma. Within the tumor microenvironment, three aspects are important: (1) hypoxia in the necrotic core of primary tumor tissue further drives metabolic shifts of cancer cells in the peri-necrotic niche;

(2) new vasculature growth is induced by the tumor and tumor-associated stroma for nutrients in the peri-vascular niche; (3) cancer cells interact with stroma to evade the immune system and adopt invasive and migratory phenotype to metastasize to distant tissues in the metastatic niche [93, 122] (Figure 3).

In the peri-necrotic niche, the metabolic state of cancer cells is reprogrammed under hypoxia and ischemia due to an increase in the tumor mass. A necrotic microenvironment with dramatically low oxygen and nutrient concentrations as well as high acidity further induces the heterogeneity within cancer cell population and promotes cancer cells survival in the harsh environment, as well as their metabolic resistance to many cancer therapeutics [123, 124].

Within the peri-vascular niche, by cross-talking with stroma, the cancer cells also induce outgrowth of new vasculatures (angiogenesis) and new lymphatic vessels (lymphangiogenesis) for nutrient and gas transport to enable cells survival and proliferation [125, 126]. However, the tumor vasculatures are often immature and leaky in comparison to the normal vasculature [127]. The peri-vascular niche also overlaps with the metastatic niche. New vasculature allows dissemination of cancer cells as they shed to circulating tumor cells, and among them tumor initiating cells can grow into secondary metastasis when seeded in distant tissues.

The metastatic niche must be developed for invasive cancer cells to shed from the primary tumor, invade through the basement membrane into the stroma, intravasate into nearby vascular or lymphatic vessels, travel and survive in the circulatory system, extravasate into a distant tissue site, and form new micro-metastasis in new sites [128]. In some forms of cancer, cancer cells can also invade the nervous system during the process termed as peri-neural invasion, which is a contributor to tumor-related pain [129]. The complex sequential process

that cancer cells undergo is also known as a metastatic cascade [130]. Recently, the theory of tumor initiating cells or cancer stem cells as a rare group of circulating tumor cells suggest that the microenvironment is important for cancer stem cells to seed in distant tissues and form new metastasis [128].

Microfluidic platforms allow recapitulation, manipulation, and observation of cancer cell responses in tumor microenvironment on a chip. An *in vitro* model recapitulating the cancer cells as well as its microenvironment can enable more biomimetic and clinically relevant outcome to accelerate our knowledge in tumor biology and improve cancer therapeutic development. In this section, we briefly review the microdevices developed in the past few decades to study different tumor microenvironments, including peri-necrotic niche, peri-vascular niche, and the metastatic niche [122, 131, 132].

3.1   *Peri-necrotic niche: modeling hypoxia and necrosis*

In most tumor types, hypoxia is a mediator of tumor progression and therapeutic resistance [123]. As the primary tumor grows and its hyper-proliferating area increases, an imbalance between the hyper proliferative cancer cell growth and nutrient as well as the gas supply from the vasculature causes ischemia in the local tissue [123]. The new vasculature to deliver more nutrient and gas is induced by the perivascular niche and in part by the hypoxia. However, the new vasculature is often abnormal and fails to rectify the nutrient deficit. The persistent hypoxia in the tumor have several effects such as selection of survival cancer cell genotypes, up-regulation of pro-survival gene expressions, metabolic switches into anaerobic glycolysis, epithelial-mesenchymal transition (EMT), and therapeutic resistance [133]. Thus, creating an *in vitro* platform to recapitulate the hypoxia in an *in vivo* tumor microenvironment is very important.

In conventional tissue culture laboratory, precise control over gas condition is challenging due to continuous oxygen diffusion into the culture medium in ambient air [124]. A CO_2 incubator equipped with additional nitrogen gas mass flow controller can regulate the oxygen concentration within the incubator, but the oxygen gradient is still different in comparison to oxygen tension in the tissue. Alternatively, biochemical induction of key transcription factor of cellular hypoxia response such as hypoxia inducible factor (HIF) can be done to induce cellular signaling pathways in the hypoxia condition. The biochemical induction limits the spectrum of hypoxia study to dedicated signaling pathways [124].

Alternatively, with microfluidics, the gas permeability of the chip material provides the advantage of creating a hypoxic microenvironment to simulate the peri-necrotic niche. Poly(dimethylsiloxane) (PDMS) is a biocompatible silicone rubber with high gas permeability and it has been a popular material for microfluidic chip fabrication by using soft lithography techniques [134, 135]. Low oxygen environment or an oxygen gradient can be created by flowing different gases, gas-equilibrated liquids, or oxygen scavengers in microfluidic networks [136-138]. Using poor gas-permeable thermoplastic as microfluidic chip material or embedding a thin thermoplastic sheet can also improve the control over the gas environment inside the chip [138-140].

On 2D microfluidic platforms, Zhang *et al.* used SUM159 breast cancer cells to demonstrate increased migration in mesenchymal mode as well as production of lactate under hypoxic condition [141]. The acidic microenvironment derived from the metabolic reprogramming is also a factor for cell migration. Neutralization of the environmental acidity can inhibit the migration velocity of cancer cells and simultaneously improve the efficiencies of therapeutics targeting HIF-1 α, colony stimulating factor 1 receptor (CSF-1R), and C-C chemokine receptor type 4 (CCR4) [141]. These results demonstrate the importance of oxygen concentration as well as the pH level in the microenvironment to regulate the migration

potential of cancer cells. Other 2D microdevices can create stable oxygen gradients generated by oxygen scavengers, which become very useful to screen for cell survival and drug response under different oxygen concentrations [66, 138, 140, 142] (Figure 4A).

The response of cancer cells to hypoxia environment in 3D can also be examined by cell embedded hydrogel models. Xu *et al.* demonstrated that the proliferation and invasion of glioblastoma U87MG cells under hypoxia conditions [143]. By flowing normoxia gas in one control channel and hypoxia gas in another near the PANC-1 pancreatic adenocarcinoma cells, Acosta *et al.* showed that hypoxia generated a more aggressive phenotype invading into the collagen gel [144] (Figure 4B).

In addition, microfluidic platforms have been used to exam kinetic formation of necrotic core of a 3D cell embedded hydrogel tumor model [145, 146]. Ayuso *et al.* developed a 3D cell embedded hydrogel system to observe the kinetic formation of necrotic cores in HCT-116 colon cancer cell model as well as U-251MG glioblastoma cell model over a 6-day period. Furthermore, real-time dynamic changes of oxygen and glucose concentrations, cell proliferation, apoptosis, reactive oxygen species formation, and drug response can all be studied *in situ* on chip [146] (Figure 4C). Co-culture multiple cell types with oxygen control is also possible with microfluidic platforms. Lin *et al.* demonstrated that both cell migration and $VEGF_{165}$ and HIF-1α were upregulated in CaSki cervical cancer cells under hypoxia conditions [147] (Figure 4D). Similar response was also observed with U87 glioblastoma cell in an alginate hydrogel [148]. Expressions of VEGF provide evidence that cancer cells under hypoxic environment are stimulated to induce angiogenesis and that there can be cellular signaling cross-talk between peri-necrotic niche and peri-vascular niche. One imperative future direction is to develop more complex microdevices to recapitulate multiple microenvironments for detailed kinetic analysis of signaling crosstalks between various microenvironments, such as elucidation of the interdependency of necrosis and neo-

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angiogenesis in the crosstalk of peri-necrotic niche and peri-vascular niche. Within the peri-necrotic niche, it has been challenging in validating the *in vitro* necrotic tumor model to tumor lysis and to incorporate stroma to investigate tumor-stromal cell interaction. Tumor lysis is the rapid death of large population of cells that causes sudden metabolic disturbances, leading to tumor lysis syndrome (TLS). TLS contributes to high mortality of cancer. It can happen spontaneously due to tumor necrosis or be initiated from anti-cancer therapies [149]. Although 3D tumor spheroids on chip developed recently exhibit necrotic cores as a micro-tumor model [11], validation of this micro-tumor model with a human tumor and its tumor lysis kinetics remains a challenge. Moreover, incorporation of chemical gradients and co-culturing stroma cells such as fibroblasts, macrophages, and natural killer cells to observe how necrosis contributes to stroma remodeling and chronic inflammation remains a difficult task [150]. Novel microdevice design integrated with biosensors and active flow control components such as microvalves is necessary to address these technical challenges.

3.2 *Peri-vascular niche: modeling angiogenesis and quiescence of cancer cells*

As a tumor grows and demands more nutrients for proliferation and survival, the tumor attracts neovascularization of blood vessels and lymphatic vessels through angiogenesis and lymphangiogenesis [125, 126]. It has also been suggested that endothelial cells in peri-vascular niche can regulate quiescence of cancer cells as well as emergence after latency [151]. Using microfluidic technologies and tissue engineering, *in vitro* platforms with tumor and vasculature interactions can be developed and used to improve contemporary anti-angiogenic therapy. Several hydrogel microdevices focused specifically on angiogenesis induction by cancer cells in the co-culture configuration. Chung *et al.* showed sprouting of endothelial cells into collagen hydrogel by VEGF gradient as well as by MTLn3 rat mammary adenocarcinoma cells [152] (Figure 5A). Cross *et al.* also demonstrated formation and lumen structure and invasion of hydrogel of human umbilical vein endothelial cells when

co-cultured with an oral squamous cell carcinoma cell line, OSCC3 [99]. Patra *et al.* showed that when co-culturing HUVEC cells with HepG2 hepatocellular carcinoma cells in tumor spheroids, HUVEC cells migrated outwards to proliferative edge and formed lumen-like structures under stimulation of pro-angiogenic factors [153]. Liu *et al.* used a 3D hydrogel microfluidic device to study angiogenesis induction by salivary gland adenoid cystic carcinoma and oral squamous cell carcinoma cells [154]. Both cell lines can induce strong angiogenesis and the angiogenesis can be inhibited under anti-angiogenic therapy. Aside from studying angiogenetic sprouting, Kim *et al*. demonstrated that a perfusable microvascular network could be created on chip as a vasculogenesis model [110] (Figure 5B). Instead of generating microvascular networks in a hydrogel, Bischel *et al.* and Nguyen *et al.* reported methods to pattern endothelia in a capillary lumen structure as a model of artificial blood vessel and angiogenesis assay [96, 104] (Figure 5C).

In the peri-vascular niche, aside from signaling between cancer cells and endothelial cells, other cell-cell interactions and physiochemical factors in the stroma also influence the angiogenesis. Using a multi-culture microdevice, Theberge *et al.* demonstrated that the microenvironment would change when macrophages interacted with endothelial cells and fibroblasts [155]. In the presence of macrophage with fibroblast and endothelial cells, expressions of several pro-angiogenic factors such as HGF, VEGF, interleukin-8, and anti-angiogenic factor matrix metalloproteinase-12 all increased. Angiogenesis are promoted but the endothelial tubules are abnormal due to the presence of other anti-angiogenic factors that are also secreted by macrophages. This observation supports our current knowledge that stroma cells in the microenvironment are also important in regulating the leaky vasculature cancer phenotype. These reported investigations demonstrate that microfluidic platforms offer new opportunities to recapitulate all the microenvironment components *in vivo* to yield physiologically and clinically results in an *in vitro* assay.

Aside from the biochemical factors and cell-cell interaction in the stroma that can affect angiogenesis, it has been found that the interstitial flow and the shear stress also regulate the sprouting of microvasculatures. The advantage of microfluidic models over other conventional 3D tumor models is their capability to create a perfusable vasculature with precise control with flow manipulations. Song and Munn showed that both interstitial flow and VEGF gradient regulate the angiogenic sprouting and vascular dilation on a tumor-microvascular-on-chip [156]. Song *et al.* further demonstrated interstitial flow enhanced anastomosis, achieving perfusion by connecting multiple vascular sprouts [157].

In addition, the shear stress acting on endothelial cells can also regulate barrier function and induce expression of pro-angiogenic factors, such as VEGF [158]. Buchanan reported increased secretion of pro-angiogenic factors when endothelial cells were co-cultured with MDA-MB-231 breast cancer cells [159, 160]. However, higher shear stress (10 dyne/cm^2) applied on endothelial cells may increase perfusion and decrease secretion of several proangiogenic factors, as well as down regulate HIF-1α. These results indicate that the interstitial flow, biochemical factors, and cell-cell interactions all contribute to the regulation of angiogenesis in the tumor microenvironment. Using microfluidics, a perfusable and controllable platform supporting kinetic analysis of multiple cell co-culture is a promising approach to understand the pivotal roles of each factor and their interactions in regulating tumor angiogenesis. Testing the effectiveness and response to novel anti-angiogenic therapeutic tools using the tumor-on-a-chip platforms could provide detailed kinetic analysis and clinical relevant results.

The key challenge of adopting peri-vascular niche is to incorporate multiplex chemical, physical, and gas gradients (oxygen and nitric oxide) to elucidate its interplay with the peri-necrotic niche. The interdependency between necrosis and neo-angiogenesis is essential for understanding the growth of solid tumor and remodeling of the tumor microenvironment

[161]. To identify the essential features in recreating an *in vitro* perivascular niche, a high-throughput microdevice is required to study microvasculature functions under different combinations of chemical and physical factors. The factors include but are not limited to pro-angiogenic growth factors, stiffness of stroma, shear stress of interstitial flow, and concentration gradients of oxygen and nitric oxide.

3.3   *Metastatic niche: modeling tumor-stroma interaction and metastasis*

In a metastatic niche, cancer cells adapt into invasive and migratory phenotypes, shed from primary tumor, intravasate, extravasate, and colonize in distant microenvironment through the metastasis cascade. Many microfluidic devices have been developed to inspect each process in the metastatic cascade.

First, the cancer cells must locally invade into nearby stroma. Chung *et al.* demonstrated the invasion of MtLn3, U87MG, and 10T 1/2 cancer cells into collagen hydrogels [152]. In microdevices, by taking advantage of the laminar flow and limited mass transport at microscale, stable chemical gradients can be established to investigate chemotactic invasion of 3D cancer model that is difficult to achieve by the conventional macroscale methods. Liu *et al.* studied how MCF breast cancer cells embedded in the basement membrane extract hydrogel are guided by epidermal growth factor (EGF) to invade the matrix [162]. Multiplex chemical gradients can also be easily established in a 3D microfluidic model. Kim *et al.* showed stromal cell derived factor-1α (SDF-1α) and EGF cooperatively modulated the migration of MDA-MB-231 cells [163].

Microfabricated porous microdevices can also be used to select and examine migratory cancer cells from tumor spheroids guided by EGF gradients similar to that in a conventional transwell assay. Using such devices, Kuo *et al.* found decreased EpCAM expression in migratory cells, suggesting that the cells underwent the EMT and gained invasive properties

[164, 165].

The second stage for metastasis is for cancer cells to adhere to endothelium and intravasate into the circulatory system. Song *et al.* developed a microfluidic platform to culture uniform endothelium on a porous membrane to allow chemical transport and study how MDA-MB-231 cells adhere to the endothelium through CXCL12-CXCR4 dependent signaling [116] (Figure 6A). Zervantonakis *et al.* created a microfluidic tumor-ECM hydrogel-vascular interface model to study how HT1080 fibrosarcoma cells interacted with the endothelial monolayer [166] (Figure 6B). While the fibrosarcoma cells have the ability to intravasate across the endothelium, when macrophages are present at the endothelium, macrophages can secrete TNF-α and increase endothelial permeability. As a result, the fibrosarcoma intravasation through the endothelium is increased. Such 3D microfluidic models combined with high resolution microscopy enable real time observation of cancer metastasis kinetics and further capture important parameters determining the microenvironment. Using a similar approach, Lee *et al.* demonstrated that TNF-α also promoted the intravasation of MDA-MB-231 cells [167].

The intravasated cancer cells enter the blood vessel and become circulating tumor cells (CTCs) that travel throughout the body in the circulatory system. The CTCs have been a very active topic for the role in metastasis and the clinical potential as a diagnostic and prognostic tool [168]. While the amount of CTCs is very low in peripheral blood, it is hypothesized that cancer stem cells or tumor initiating cells can seed in distant tissues and grow into secondary tumors [169]. Many microfluidic platforms have been developed for capture and analysis of CTCs, more dedicated articles can be found in the literature [170-172].

At distant sites, the circulating tumor cells need to extravasate through the endothelium and settle in the new microenvironment. Zhang *et al.* demonstrated that chemokine CXCL12

could stimulate salivary gland adenoid cystic carcinoma cells to extravasate through HUVEC endothelium [119]. The stimulated extravasation can also be inhibited by CXCR4 antagonist AMD3100. Chen e*t al.* employed a microvascular network in hydrogel and loaded MDA-MB-231, HT-1080, and MCF-10A cells by perfusion [109] (Figure 6C). The extravasation events (transendothelial migration) of the cells from the microvascular network into hydrogel can be tracked via time-lapsed microscopy. Interestingly, different cancer cell subpopulations exhibit different migration capabilities. Trapped cells as well as clustered cells showed much higher rate of migration into the ECM. Activation of tumor integrins β1 was found to be necessary for both extravasation and bone marrow colonization using the microvascular network microdevice [173]. Several microfluidic models also employed the ECM hydrogel-endothelium monolayer interface models commonly used in intravasation to study cancer extravasation by seeding cells in different microfluidic channels [112, 174, 175].

In the metastatic niche, other stromal cells and biophysical components also influence cancer cells' invasiveness. Multiple cell co-culture microdevices have been developed to study the effect of cell-cell interactions such as autocrine and paracrine signaling on the invasiveness of the cancer cells. Small vesicles containing nucleic acids and proteins (termed exosomes) may be the carriers to carry signaling molecules between the cancer and stromal cells [176]. Hsu *et al.* developed a 2D three-chamber PDMS microfluidic chip with microvalve control to selectively flow the conditioned medium of fibroblast, macrophages, and CL1-0 lung cancer cells to investigate how paracrine signaling from tumor stroma affected cancer cell invasiveness [177] (Figure 6D). Lung cancer cells release TGF-β1 to transform fibroblasts into myofibroblasts and in return promote the migration speed of cancer cells. However, macrophages can immunomodulate the myofibroblasts and the cancer cell migration speed decreases in the macrophage-pretreated and myofibroblast conditioned media. Interestingly, instead of pretreatment, direct combination of macrophage conditioned medium and

myofibroblast conditioned medium resulted nearly three-fold increase of lung cancer cells'

migration speed [177]. Similar to other tri-culture microfluidic models, these results imply

that the responses of cancer cells influenced under multiple factors can be quite complex and

diverse [155, 177].

Multiplex 3D co-culture microdevices also serve as useful tools to investigate how the stroma

interacts and modulates cancer cells. A breast cancer-on-a-chip device developed by Choi *et*

al. recapitulate the mammary duct and stroma as well as tumor spheroid in one microdevice

model [13].   Jeong *et al.* used multiple hydrogel chambers embedded with tumor spheroids

and fibroblasts to show that cancer associated fibroblasts promote cancer cell proliferation

and drug resistance [178]. Liu *et al.* developed a four-chamber co-culture microdevice to

simulate the microenvironment of bladder cancer with T24 cancer cells, macrophages,

fibroblasts and HUVECs embedded in hydrogel [179]. The bladder cancer cells grew into

reticular structure and stromal cell phenotype changed despite the lack of 3D tissue hierarchy

in the system. Bischel *et al.* patterned a 3D lumen structure in a microdevice by viscous

fingering method and successfully verified that the invasion of ductal carcinoma in situ

(DCIS) of breast cancer cells was induced by mammary fibroblasts [180] (Figure 6E). By

using second harmonic imaging, increased collagen modifications were found near the

invasive region, suggesting that the extracellular matrix was remodeled by invasive cancer

cells.

Aside from cell-cell interaction in the metastatic niche, physical factors such as interstitial

flow and mechanical stimulation can regulate invasiveness of cancer cells. Polacheck *et al.*

developed a microfluidic culture chip to apply a stable interstitial flow to MDA-MB-231 cells

embedded in a collagen hydrogel [181]. Cancer cells at different densities responded to

interstitial flows differently. At low cell density, cells migrated with the interstitial flow, and

the migration was dependent on CCR7 signaling. When CCR7 signaling was blocked, the

migration directionality was reversed. Jeon *et al.* demonstrated that the presence of interstitial flow in a microvascular network reduced the extravasation of cancer cells and decreased the permeability of vasculature [112]. By applying cyclic tensile strain on myofibroblasts in a PDMS microdevice, Huang *et al.* showed that tensile strain reduced the ability of the myofibroblast to accelerate cancer cell migration [182]. The effect of cyclic tensile strain is also modulated by IL-1 β that are secreted by other cells in the stroma, which implies the complicated interaction between cancer cells and different stromal cell types in the microenvironment. Stiffness of ECM and stromal cells can also regulate the invasiveness of cancer cells [183, 184]. Finally, transepithelial potential differences in tissues can generate physiological electric field and guide the migration of cancer cells through electrotaxis [185-187]. In short, many metastatic niche studies verified that invasive cancer cells could interact and modulate with biophysical and biochemical properties of the stroma, as well as with all the cellular components in the complex microenvironment. Similar to the challenges in mimicking tumor microenvironments, incorporating multiplex chemical, physical, and cell factors in a metastatic niche is critical in order to create a reliable *in vitro* micro-tumor model and investigate how each component contributes to the modulation of the metastatic cascade.

4. Applications of tumor-on-a-chip technology

The development and application of tumor-on-a-chip technology has the potential to address many important biological questions by replicating major aspects of the tumor structure, microenvironment and tumor biology. For example, a tumor-on-a-chip system may allow us to study the complexity of cancer growth and progression in a controlled fashion, capture and analyze spatiotemporal dynamics of tumor cells interacting with stromal cells, immune cells and other cells in the blood, and perform high-resolution imaging to understand some of the molecular and cellular mechanisms of tumor growth and metastasis. Tumor-on-a-chip

approaches may allow the use of patients' own tumor cells to determine how they respond to anti-cancer drug or immunotherapy and to better predict cancer aggressiveness, achieving the best possible clinical outcome by extending survival and reducing the chances of relapses and emergence of drug-resistant tumors. Although the technology is still in its early stages, the current designs of microfluidic tumor-on-a-chip systems have already shown promises in growing simple 3D tumors and having good controls over the tumor microenvironment. Some of the applications include multiplexed drug screening, transport of nanoparticles, transcription analysis, proteomic analysis, and metabolic changes in cells.

4.1   *Multiplexed drug screening*

Conventional pre-clinical drug screening is expensive and time-consuming, and requires large number of cells. Recent advances in microfluidics technology have enabled cost-effective high-throughput screening. Aside from having a lower cost and faster processing speed, microfluidics chips require a much smaller sample volume. Furthermore, these chips can be customized to monitor the effects of anticancer drugs on any number of parameters, including cell migration [188]. Specifically, Zhang *et al.* developed a microfluidic device with 3120 different microchambers in which cell density was varied throughout the chambers, and the average migration velocity and the percentage of migrating cells were quantified. This device can create chemical gradients of multiple anti-tumor drugs and generate multiplicates of sample data on a single chip to specifically monitor the mesenchymal migration and survival of tumor cells upon exposure to drugs that inhibit cell migration, including axitinib [189]. In a study by Sobrino *et al*, vascularized microtumors were created on a PDMS membrane to study the effects of vascular targeting agents, such as Apatinib and Linifanib [132] (Figure 7A). A key drawback to this approach is the absorption of the agents in question by the PDMS membrane. Further work is needed to determine the effects of the partition coefficients of various types of drugs in different types of microfluidic platforms.

Choi *et al.* explored the tumor microenvironment as a crucial regulator of tumor progression by designing a microchip with two microchannels surrounding a basement membrane with epithelial and stromal cells to simulate pre-invasive breast cancer lesions. Tumor spheroids were cultured on top on the epithelial cell layer [13] (Figure 7B). This model replicates the 3D microarchitecture *in vivo* and enables simulation of physiological delivery of intravenously administered paclitaxel by continuous flow through the lower microchannel [13]. This device can be scaled up easily for multiplexed screening of drug molecules based on their efficacy and safety, and the platform is flexible enough to be used for models of other types of cancer.

4.2   *Transport and delivery of nanoparticles*

Microfluidic systems can be used to evaluate nanoparticle transport *in vitro* and optimize nanoparticle designs by selecting the right size, shape and surface chemistry, such that the nanoparticle systems identified would have higher rates of success in drug delivery or *in vivo* imaging, thereby reducing the number of costly animal studies [190]. Recently, Albanese *et al.* designed a tumor-on-a-chip microfluidic model to study how nanoparticles were transported in the 3D tumor spheroid. They showed that flow rate affected the accumulation of the nanoparticles in the *in vitro* spheroid model [11]. Kwak *et al.* developed a tumor-microenvironment-on-chip (T-MOC) model to recapitulate the complex transport of drugs and nanoparticles within a 3D model of breast cancer and endothelial cells [191]. They could quantify the effects of nanoparticle size on the extravasation and interstitial diffusion. There was a significant decrease in both parameters between the 100 nm and 200 nm nanoparticles [191]. Bagley *et al.* demonstrated the use of plasmonic nanoantennae to enhance transport into a model of ovarian cancer via heat generation. They also used temperature-controlled microfluidic devices to measure diffusion of the nanoparticles *in vitro* [192]. The use of microfluidic devices to aid in the rapid development of translatable nanoparticles for tumor

microenvironment studies is a very active and promising area of research.

4.3   Analysis of transcription

Using droplets in a microfluidic platform is an effective way to conduct transcription analysis on the level of single cells. Zhang *et al.* developed a microfluidic device for performing single copy RT-PCR (Reverse Transcription Polymerase Chain Reaction) using agarose droplets, which contained both sample and RT-PCR reagents [193]. The platform was validated by showing significant differences in expression of the EpCAM cancer biomarker gene between different types of cancer cells [193]. Microfluidic droplets were also used in a separate study by Hayes *et al.* to evaluate extracellular matrix gene expression levels in patient samples of colorectal cancer in order to find a potential correlation between differential expression and metastatic potential [194]. A study by Jang *et al* demonstrated that a droplet-based model of microtumors can be used effectively to analyze the gene expression of markers related to the EMT [195]. Developing high-throughput single cell analytical techniques and using patient samples to find correlates or clusters of genes of interest has the potential to greatly expand the number of therapeutic targets currently known to us.

4.4   Proteomic analysis

Quantitative analysis of the cancer proteome has the potential to have a tremendous impact on not only molecular diagnostic technology, but also on discovering novel therapeutic targets. In a study by Sun *et al.*, a microfluidic cytometry imaging system was developed that is capable of quantitative, single-cell proteomic analysis in both cultured cell lines and patient samples, using as little as 1,000 cells. Its clinical application was demonstrated by analyzing four proteins within the mTOR signaling pathway using human brain tumor samples, and comparing the results to that using well-established clinical immunohistochemical (IHC) protocols. The IHC findings corroborated the single-cell analysis in all but one case [196]. In

a study by Jeong *et al*, human colorectal cancer cells were co-cultured with fibroblasts on a PDMS microfluidic chip, which was then used to quantify the level of proteins involved in angiogenesis, apoptosis, and cell motility [178] (Figure 7C). On a larger scale, Xu *et al* designed a biomimetic multi-organ microfluidic chip to assess changes in the expression levels of CXCR4, RANKL, and other markers in the various 'distant organs' after tumor cell invasion [197].

4.5   *Analysis of metabolites and energy metabolism*

Cancer cells tend to continuously multiply without cell cycle check, thus understanding the mechanism of cancer cell energy metabolism is critical to both basic cancer research and cancer therapeutics. Microdevices are well suited to study tumor cell energy metabolism by controlling both the oxygen supply and nutrient depletion to the cells. Xu *et al.* designed a 3D microfluidic chip to study the energy metabolism in tumor-associated fibroblasts and bladder tumor cells, specifically measuring lactic acid concentration and mitochondrial-related gene expressions [198]. Culture media were perfused through the microfluidic channels which contained fibroblasts or bladder tumor cells or both. The conditioned media of co-cultured cells had the highest lactate concentration, suggesting that the aerobic glycolysis increased under the co-culturing condition. This microfluidic platform provided a unique non-contact co-culture condition to investigate energy metabolisms between different cell types. Zhu *et al.* also examined cancer cell metabolism using a microfluidic tumor-endothelial cell co-culture system. Lactic acid and mitochondrial protein levels were measured and found to increase in the co-culture group [199]. Similar approaches can also be adopted to screen for drug resistance, or investigate energy metabolism in regenerative medicine [200, 201].

Since cancer cells that release high levels of lactate correlate to increased metastasis [202], microdevice has been developed to perform single cell analysis of metabolites. Mongersun *et*

al. developed a droplet microfluidic platform that quantifies the lactate release rate down to single-cell resolution. The PDMS/glass microfluidic chip with the flow focusing design produced droplets containing single cancer cells and allowed real time monitoring of lactate release within each individual droplet [203]. Performing metabolic analysis on single populations of tumor cells can yield significant insight into the mechanisms of tumor heterogeneity and energy metabolism reprogramming, both are important for predicting cancer metastatic potential as well as drug resistance [204].

5. Conclusions and future perspectives

To fully realize the potential of tumor-on-a-chip approaches, a number of key questions must be addressed. For example, how to model the mechanism of intravasation and extravasation using such a system; how to allow tumor-associated tissues mature in a chip with respect to self-organization, if there is a minimum number of components needed to construct a tumor-on-a-chip system that allows a tumor to grow on a chip. Clearly, different approaches need to be developed to quantitatively analyze tumor-matrix interactions (including, e.g. matrix remodeling and growth factors) in order to understand the enhanced permeability and retention (EPR) effect as well as the phenotype of dormancy. It is also very important to reflect the heterogeneity and evolution present in the tumor by using a tumor-on-a-chip system. As a control, we need to have both cancerous and normal tissues grown on a chip in order to compare different features. It is also possible to use tumor-on-a-chip approaches to study tumor-immune response as how bacteria and virus trigger oncogenesis.

Tumor-on-a-chip systems may have different designs and complexities, depending on the medical relevance and biological question(s) to be addressed. It is necessary to avoid constructing oversimplified or overcomplicated systems, and have sufficient complexity driven by need. Accordingly, a tumor-on-a-chip system may include one or more of the following considerations: (a) structural features including 3D tumor constructs and

microfluidics designs; (b) biomechanical and kinematic parameters such as matrix stiffness and anisotropy, cell adhesion, and flow conditions; (c) cell types and sources, including patient cells, cell lines, stromal cells, stem cells and progenitor cells; (d) cell metabolism, culture media transport, waste removal and cytotoxicity; (e) physiological levels of concentration and concentration gradients of circulating factors. To accurately capture important features of a tumor, it may also be necessary to consider metastatic sites, recapitulation of cancer-immune cells interactions, and integrate real-time, on-chip monitoring of relevant biophysical and biochemical parameters. While preformed scaffolds for tumor structure and organization have certain advantages, the self-organized tumor structure through evolution of cell-cell interaction may provide a better model for tumor-on-a-chip platform. It is likely that different tumor-on-a-chip systems with different features and complexities are needed for different cancers and/or address different questions.

The emerging tumor-on-a-chip technology has the potential to transform the fields of oncology and cancer biology. However, there are roadblocks in technology development, including design, optimization, analysis, and validation. Consistency of device properties (such as its biocompatibility, fit for purpose, ease of handling, and mechanical properties) relies on material choices. Most devices have been built on PDMS-based substrates, which have been outstanding for studies on biological mechanisms, but have severe limitations when used with hydrophobic drugs. Other moldable and printable surrogates must be explored to overcome this limitation such as off-stoichiometry thiol-enes [205], epoxy resin [206], and perfluorinated polymers [207]. Systematic manipulation and automation of the physical and chemical parameters within the microfluidic device will require integration of micro-device printing experts with polymer chemists and material scientists. Material choice and user operability are chief concerns when considering the scalability of the device and GMP (good manufacturing practice) development. Further, it is important to establish the

shelf-life (longevity post-manufacturing and pre-utility) and sustainability (e.g., duration of cell culture and waste removal in the system) of tumor-on-a-chip devices.

Although the potential of tumor-on-a-chip systems as cancer research tools has been demonstrated through proof-of-concept reports, major challenges for translating the technology to clinical practice remain, including the validation of device functionalities by comparing with well-established *in vivo* tumor models, and the correlation of the results obtained using tumor-on-a-chip systems with clinical tumor tissues. Tumor-on-a-chip system has the unique advantages of precisely manipulating the physical and chemical factors in the tumor microenvironment, co-culturing stromal cells with cancer cells, providing optical window for real time observation of molecular and sub-cellular processes through microscopy, and integrating with biosensors for quantitation [208]. Tumor-on-a-chip system can be superior over animal xenograft models concerning physiochemical differences, biological variation, cost, and ease of statistical analysis. To fully realize the potential of the tumor-on-a-chip technology, it is essential for researchers in biomedical engineering, material science, biophysics, cell biology, and oncology to make concerted efforts in designing and optimizing tumor-on-a-chip systems for cancer research, drug discovery, and in translating the technology to clinical use [209].

Tables

Table 1. Comparison of *in vitro* tumor models

3D tumor models	Processes	Advantages	Disadvantages
Transwell assays	Migration, invasion through ECM, transendothelial migration	Recovery of motile cell population, easy to perform	No control over gradient, endpoint assay, inability to create multiplex gradient, no cell-cell interaction
Tumor spheroids	Mimicry of tumor mass in 3D	A micro tumor with 3D structure, necrotic core, and nutrient transport property; perfusable with microfluidics	No vasculature on spheroids
Ex vivo tumor section	Direct in situ analysis on in vitro cultured tissue	Retains primary tumor and stroma	Require primary tumor tissue for every experiment
Scaffold	Solid extracellular support for 3D cell culture	A characterized ECM structure for 3D cell culture	Difficult to uniformly distribute cells in scaffolds. Difficult to perfuse the model
Bio-ink 3D printing	Layer-by-layer construction of cells	Printing multiple cell types and ECMs; high spatial precision	Specific bioink formulation is needed for optimal cell survival
Microfluidic microvascular model	Patterning microscopic vascular capillary	Perfusable model, microscopic observation for kinetics, incorporation of gradients	Size limited to small tumors

Figure Captions

Figure 1. **The concept of tumor-on-a-chip**. (A) A microfluidic device that has tissue culture, nutrient and small molecule supply and waste removal functions for growing tumors on a chip. Adapted from [12]. (B) The ultimate goal is to grow 3D tumor on chip with a complex tissue structure consisting of tumor cells, stromal cells and blood vessels. (C) An example of tumor-on-a-chip in which lung cancer spheroids were embedded in micro-patterned 3D matrices immediately contiguous to a microchannel lined with endothelial cells. Reprinted with permission from Macmillan Publishers Ltd. (D) The physiological microarchitecture is recapitulated in the breast-cancer-on-chip microdevice with two cell-culture chambers separated by an ECM-derived membrane. Adapted from [13] with permission from the Royal Society of Chemistry.

Figure 2. **Existing techniques to create 3D *in vitro* tumor models.** (a) An *ex vivo* tumor culture based on a tumor tissue section. (b) Single tumor cells embedded in hydrogel on transwell insert is one of the earliest 3D model that can also characterize the invasiveness. Tumor spheroids can be prepared from dissociated cells from tumor or circulating tumor cells (CTCs) by (c) spinning mask method, (d) NASA microgravity apparatus, (e) hanging drop method, (f) liquid overlay method, (g) magnetic levitation after cells are incubated with magnetic nanoparticles, (h) bio-orthogonal chemistry, and (i) microfluidic methods such as flow focusing, droplet microfluidics, and digital microfluidics. Alternative to spheroids, 3D tumor models can be fabricated by seeding cells in artificial 3D matrices. (j) Cancer cells can be seeded in fabricated scaffolds. (k) Cell-embedded bio-ink can be printed as building blocks for tissues. (l) Microfluidics-microvascular model uses a microdevice to model a multiple tissue type microenvironment. Modified from [28, 29].

Figure 3. A tumor microenvironment of solid tumor consists of peri-vascular niche, metastatic niche, and peri-necrotic niche. Hypoxia is a result of growth/nutrient imbalance induce metabolic reprogramming in the peri-necrotic niche. Tumor induces angiogenesis and lymphangiogenesis in effort to gain more nutrient and access to circulatory system in peri-vascular niche. Cancer cells shed, invade, intravasate, extravasate to metastasize to distant tissue and create secondary tumors. The tumor microenvironment is complex and composed of cell-cell interaction and biophysical as well as biochemical interactions between the tumor and the stroma. Modified from [128, 132].

Figure 4. Microdevices to model the peri-necrotic niche. (A) (i) The cross-sectional view of oxygen gradient generating microfluidic chip by Chen *et al*. (ii) A549 cell culture for 6 hours with and without oxygen gradient (hyperoxia). Hyperoxia induced cell death is visible on the right side of the channel. (iii) A549 cell culture under 4 hours 1mM hypoxia dependent anti-cancer drug Tirapazamine (TPZ) with and without oxygen gradient. Increased efficacy of TPZ is seen on the left side. Adapted from [66] with permission of The Royal Society of Chemistry. (B) (i) A microfluidic chip by Acosta *et al*. to create oxygen gradient by diffusion of gas between two gas supply channels. (ii) The predicted oxygen concentration gradient at steady state within the cross-section of microfluidic device. (iii) Increase invasion into collagen hydrogel by PANC-1 cancer cells under hypoxia condition. Reprinted from [144], with permission of AIP Publishing. (C) (i) A microdevice by Ayuso *et al*. to provide nutrient and oxygen gradient across it. (ii) Increased hypoxia in HCT-116 colon cancer cells imaged by hypoxia-sensitive dye. [146]. (D) (i) Schematic diagram of an integrated microfluidic oxygen gradient generator by Lin *et al*. (ii) cell migration of CaSki cancer cells and HUVEC endothelial cells under 5% O_2 concentration for 2 days. Adapted from [147].

Figure 5. Microdevices to model the peri-vascular niche. (A) (i) Schematic for a 3D microfluidic chip with scaffold channel for hydrogel patterning and flow channels to pattern

different cell types for study of angiogenesis and invasion. (ii) Angiogenesis of endothelial cells in the middle toward MTLn3 cancer cells and invasion of cancer cells towards the vasculature. Adapted from [152] with permission of The Royal Society of Chemistry. (B) (i) Microfluidic chip design for creation of microvascular network and angiogenic sprouting. (ii) (top) Immunofluorescence staining of a fully functional microvascular network with endothelial cells, pericytes, cancer cells, and leukocytes (bottom) Immunofluorescence staining of the angiogenic sprouting model. Adapted from [110] with permission of The Royal Society of Chemistry. (C) (i) Device schematic of a microdevice holding 3D hydrogel with microchannels. (ii) Angiogenic sprouting of endothelium towards different angiogenic factors [96].

Figure 6. Microdevices to model the metastatic niche. (A) A microfluidic vasculature with region-specific activation of endothelium for cancer cell adhesion analysis [116]. (B) (i) A microfluidic tumor-vascular interface model. (ii) Invasion of the endothelium by fibrosarcoma cells [166]. (C) (i) A microfluidic microvascular network platform. (ii) The extravasation dynamics of MDA-MB-231 cells. Adapted from [109] with permission of The Royal Society of Chemistry. (D) A microdevice to study how the paracrine signaling between macrophage, lung adenocarcinoma cells, and myofibroblasts can affect the invasiveness of the cancer. Adapted from [177] with permission of The Royal Society of Chemistry. (E) The tubeless lumen model to study invasive transition of MCF10aDCIS ductal carcinoma by mammary fibroblast [180].

Figure 7. Microdevices for tumor-on-a-chip studies. (A) Establishment of Vascularized Micro-Organs (VMOs). (i) A schematic depicts the microfluidic platform of the VMO, which consists of a thick layer of PDMS with patterned tissue chambers and microfluidic channels, bonded on top of thin PDMS membrane and a glass cover slip. The cell-ECM suspension is injected through the gel-loading ports at either end of the tissue chamber. The four media

reservoirs are attached to the inlets and outlets of the microfluidic channels. (ii) A depiction of a representative tissue chamber at day 7 with a fully developed vascular network. Transduced endothelial cells, shown in red, are migrating out and fusing with microfluidic channels. This platform is used later in the study to establish a human colon cancer micro-tumor. Adapted with permission from Nature Publishing Group. (B) A human breast cancer-on-a-chip. (i) A depiction of DCIS (carcinoma) in a mammary duct, with basement membrane, epithelium, stroma, and capillary blood flow. Adapted from [13] with permission from the Royal Society of Chemistry. (ii) An image showing the interaction between breast cancer cells and an artificial microvessel embedded in a microfluidic tumor-on-a-chip device. Adapted from [98] with permission from the American Association for Cancer Research. (C) Design of microfluidic chip for tumor spheroid-fibroblast co-culture. This chip is used for 3D co-culture of human colorectal cancer cells and fibroblasts. There are four units per chip and 7 channels per unit for loading with either cells or media. The bottom-left shows in detail a cell-loading channel. Adapted from [178] with permission.
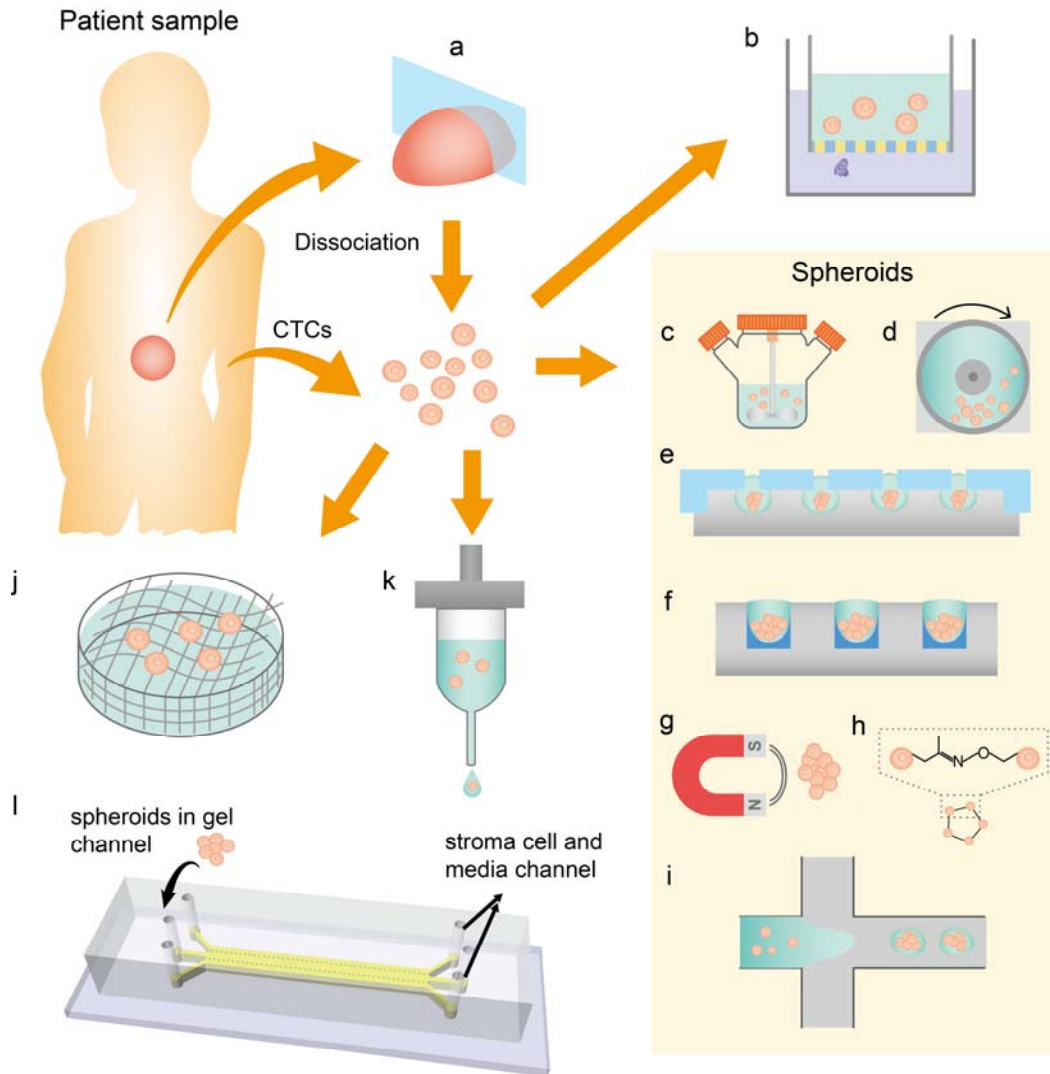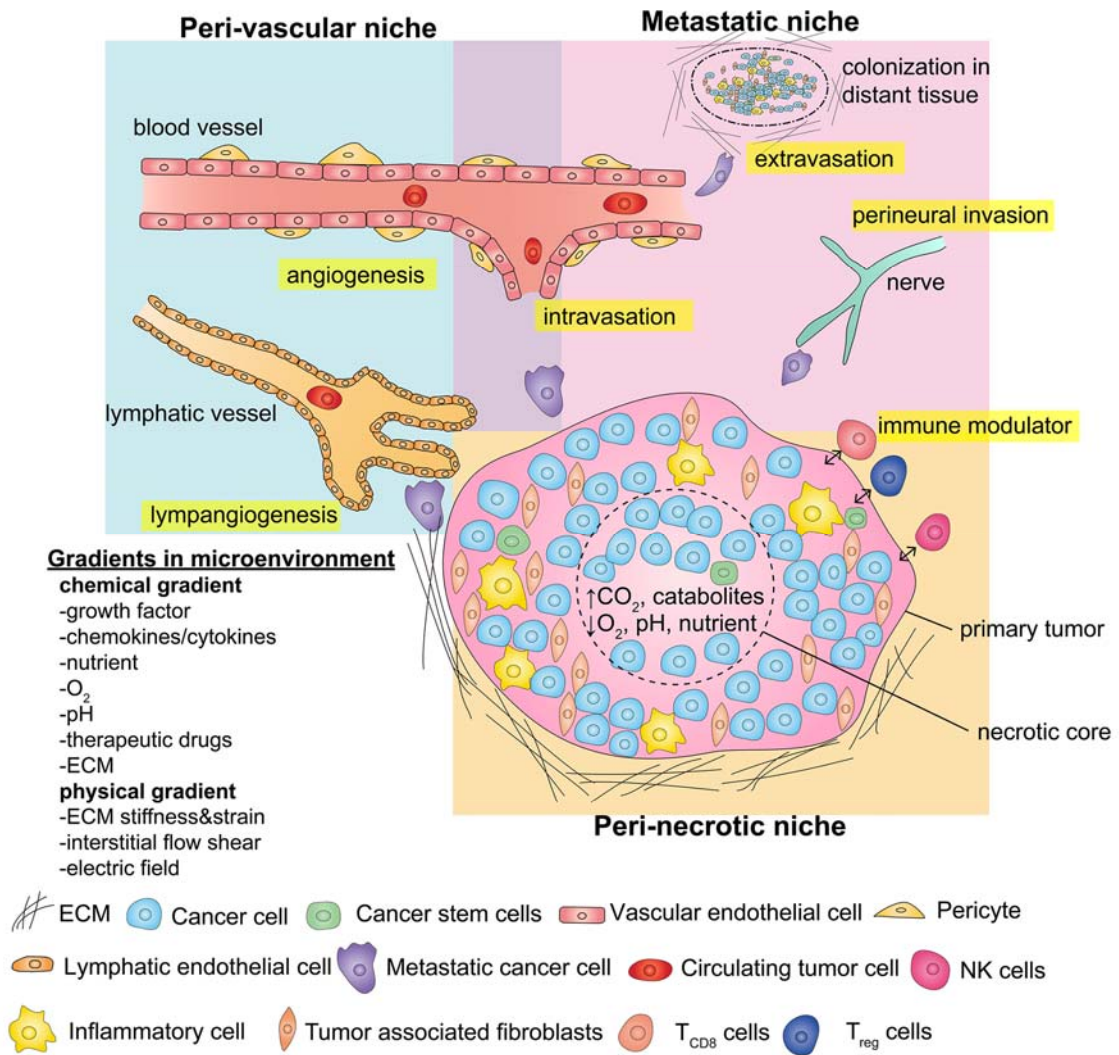
Figures

Figure 1
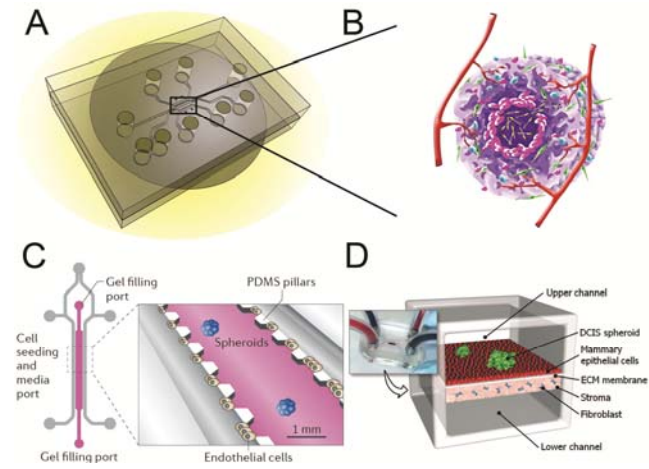
Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

PDMS top channel layer
Polyester membrane
PDMS bottom channel layer

Cancer cells
Endothelium
Flow
Chemokine

B (i)

(ii)

C (i)

HUVEC
Media Channel
NHLF

HUVEC
NHLF
Media Reservoirs

(ii)

0:00 0:30 1:00
1:30 2:00 2:30
3:00 3:30 4:00

D

Left 1
Left 2
Left 3
Bottom 3
Bottom 2
Bottom 1

Fibroblast MRC-5
Ma
Microvalves
Cancer cell CL1-0

Right 1
Right 2
Right 3

E

□ ECM hydrogel
✳ HMF
● DCIS lumen model

Figure 7

A (i)

PDMS Device
PDMS Membrane
Glass Cover Slip
Tissue Chamber
Media reservoir
Microfluidic channel
Gel loading port

(ii)

EC

B (i)

Capillary
Blood flow
Mammary duct
Stroma
Mammary epithelium
DCIS
Basement membrane
Fibroblast

(ii)

100 μm

C

77 mm
55 mm

1000 μm
90 μm
210 μm
135 μm
190 μm
Collagen matrix-filled cell channel (tumor or fibroblast)

① ② ③ ④ ③ ② ①

Medium   Fibroblast   Medium   Tumor   Medium   Fibroblast   Medium
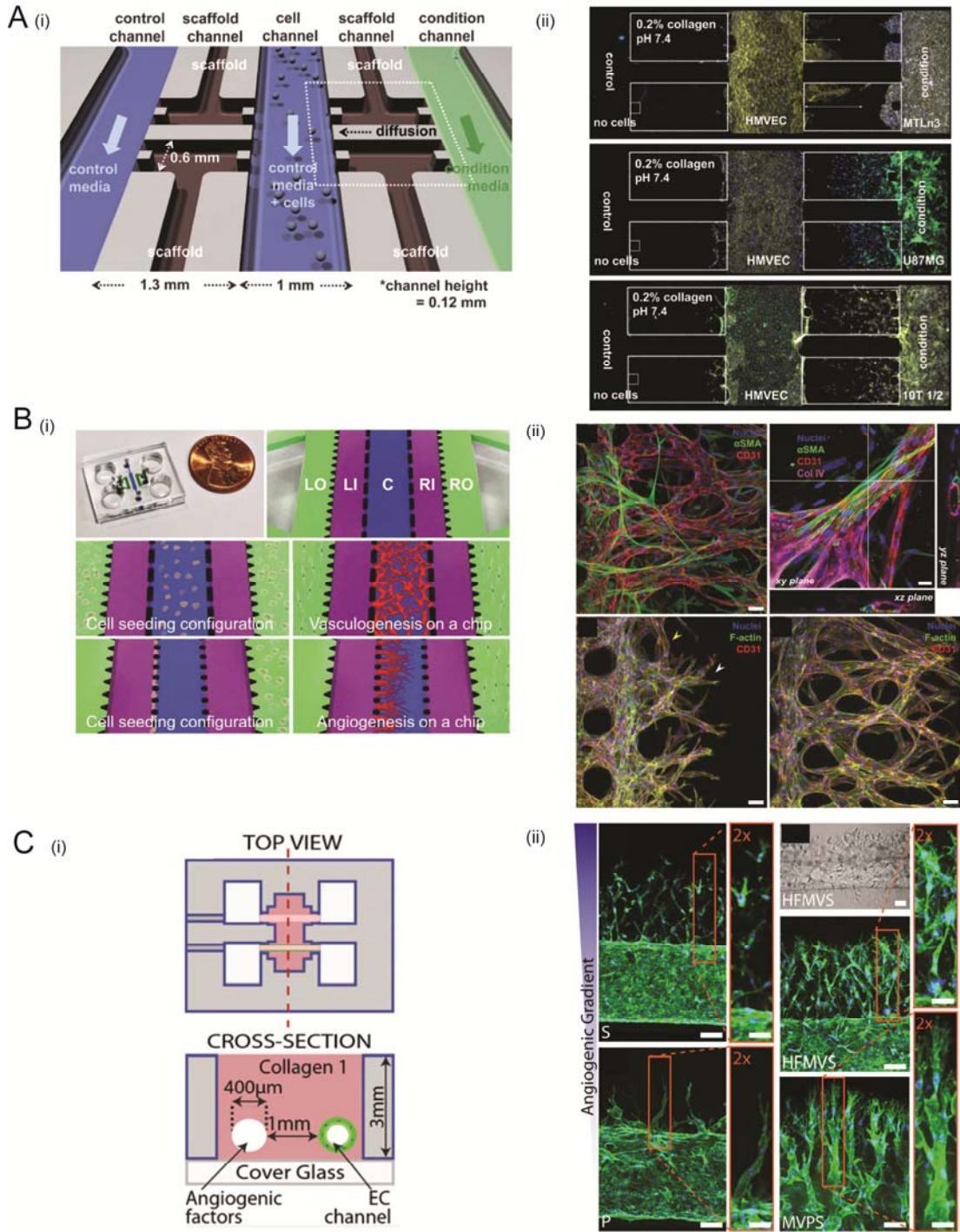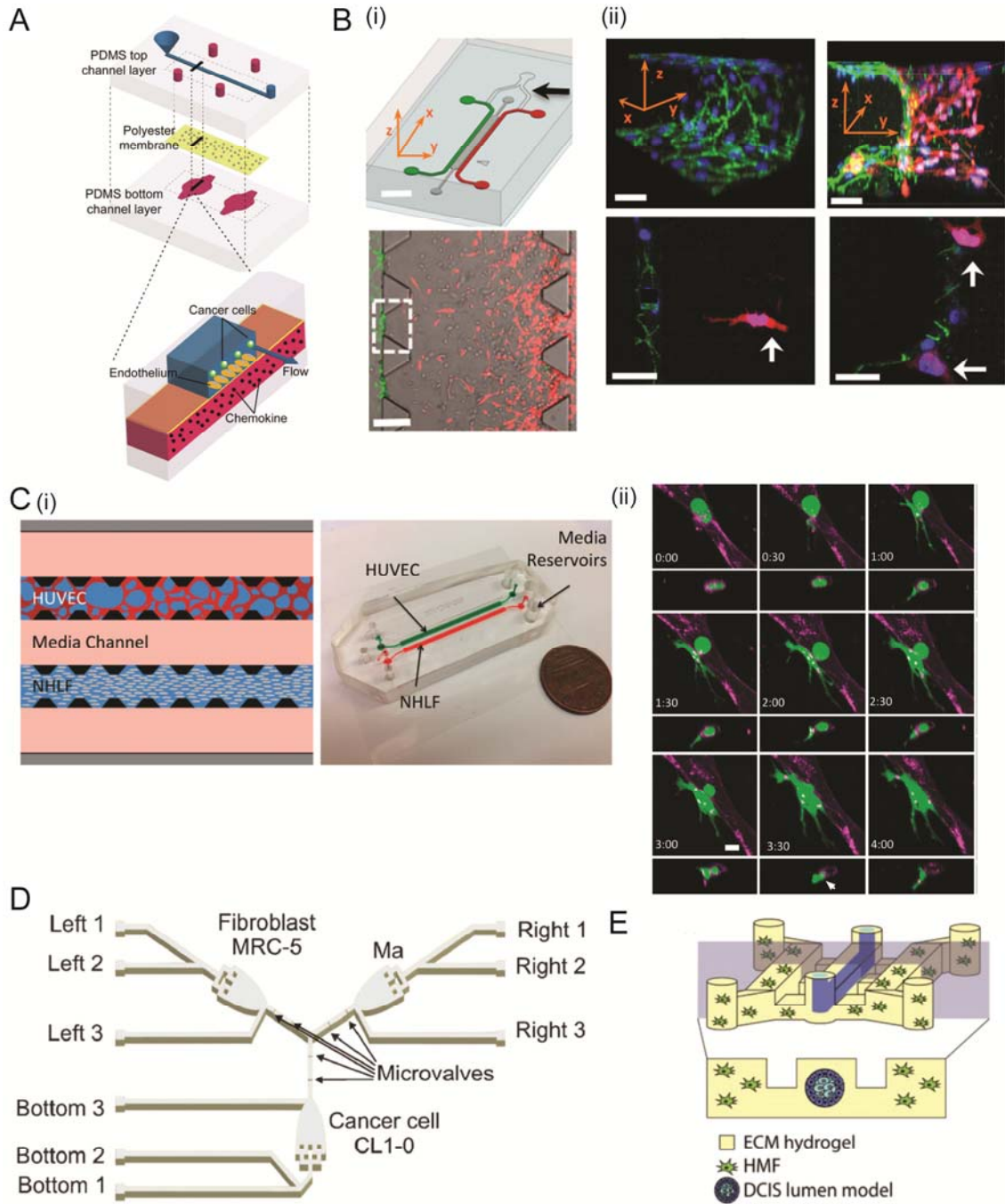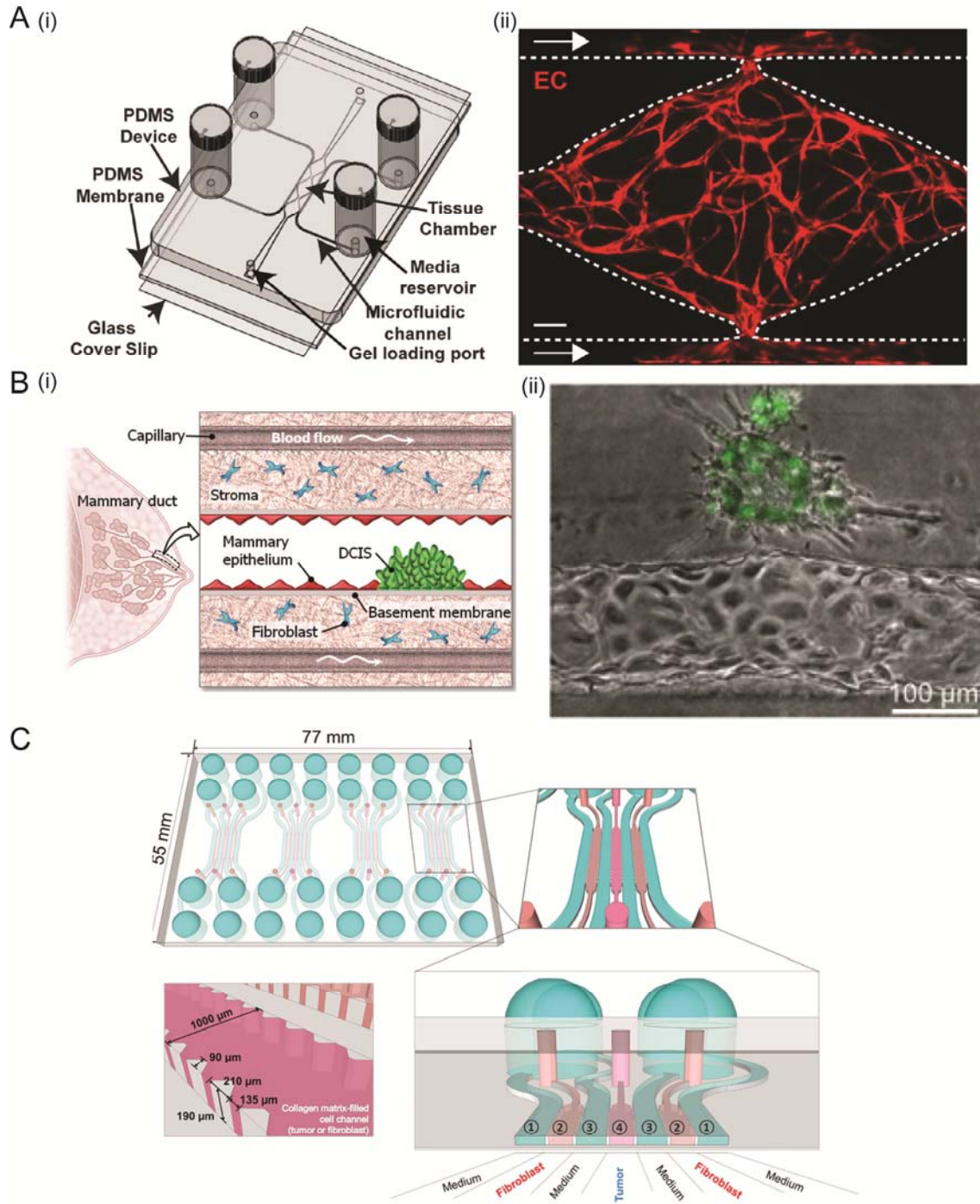
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