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Recent advances in microfluidic technologies for cell-to-cell interaction studies

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Microfluidic cell cultures are ideally positioned to become the next generation in vitro diagnostic tools for biomedical research, where key biological processes such as cell signalling and dynamic cell-to-cell interactions can be reliably analysed under reproducible physiological cell culture conditions. In the last decade a large number of microfluidic cell analysis systems have been developed for a variety of applications including drug target optimization, drug screening and toxicological testing. More recently, advanced *in vitro* microfluidic cell culture systems have emerged that are capable of recapitulating the complex three-dimensional architectures of tissues and organs, thus representing valid biological models to investigate mechanism and function of human tissue structures as well as to study the onset and progression of diseases such as cancer. In this review, we present the most important developments in single-cell, 2D and 3D microfluidic cell culture systems for studying cell-to-cell interactions published over the last 6 years, with focus on cancer research and immunotherapy, vascular models and neuroscience. Additionally, the current technolgical development of microdevices with more advanced physiological cell microenvironments interconnecting multiple organ models, so-called body-, human- and multi-organ-on-a-chip, are reviewed.

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Introduction

The interaction between cells is an essential feature in multicellular organisms, crucial for the development and for a physiological functioning at tissue level. Cell-to-cell interactions appear direct, such as stable cell-cell junctions organizing cell layers in tissue, or indirect, as when cells communicate by secreting signalling molecules. Cells interact between the same phenotype as well as between cells of different phenotypes. It is of highly importance to understand the phenomenon of cellular interactions on order to gain knowledge of several biological functions including cancer development and migration, wound-healing and stem cell development.[1, 2] This information can be translated into applications such as drug screening and tissue engineering. To establish assays for cell-to-cell interaction studies, co-culture of two or more cell types are conducted. The standard method for co-culture is by directly adding different cell types into the same culture well, or for example by culturing cells in Transwell® systems, consisting of two compartments separated with permeable membrane within each well. Although the culturing of cells on flat surfaces are favoured due to the simple approach, the models are based on 2D monolayers of cells which deviate from cells *in vivo* in qualities such as cell morphology, physiology, and gene expression. The microenvironment of most cells in living tissue is the extracellular matrix (ECM), a surrounding of a complex molecular composition and fibres, providing structural support and thereby allowing cells to grow three-dimensionally.[3, 4] In approaches to mimic the ECM *in vitro*, cells are cultured in 3D gels or matrices. However, it is challenging to create a well-controlled microenvironment with dimensions corresponding to tissue structures *in vivo*, and thus the cellular morphogenesis will still differ from native tissues.[5] To develop more physiological relevant co-culture cell models, microfluidic and organ-on-a-chip systems are used as advanced tools for cell-cell interaction studies.

For over two decades, the application of micromachining technologies for biomedical research has led to the development of miniaturized assays for advanced in vitro cell analysis, so-called cell-based microfluidic platforms. To date a variety of fabrication methods for microfluidic systems are utilized including etching techniques, photo-/e-beam lithography, embossing, replica moulding, laser photo-ablation, as well as 3D printing as an additive manufacturing technique due to the recent affordability in prize. [6, 7] Selection of the right fabrication method is mostly determined by existing infrastructure (technology and equipment), fabrication speed, desired resolution, and fabrication material. With fabrication techniques originated from the microelectronic industry the early devices constituted of glass-, silicon- and photopolymer-based microfluidics. Particularly glass was a favoured material due to compatibility with biomedical applications. However, micromachining of glass and silicon wafers involve the use of cost-intensive techniques , and require clean room infrastructure.[8, 9] Therefore, a diverse set of rapid and cost-efficient fabrication techniques for microfluidics applicable for various materials, such as hydrogels, thermosets and thermoplastics, are increasing in popularity.[10-15] With the emergence of inexpensive soft lithography as technique for molding of microchannels the cost as well as availability of microfabricated devices has improved.[16] Since then, microfluidic devices provide powerful tools for biological and chemical studies and are spreading rapidly.[17-19] Integrated systems combined of microchannels with pumps, valves , filters and sensors are referred to as 'lab-on-a-chip' (LOC) or 'Micro Total Analysis Systems' (μTAS) systems.[20] The introduction of microfluidics to life sciences enabled to address crucial limitations of standard assay formats including temperature control, gas control, precise control over geometry, nutrient supply, waste removal, chemical administration, assay parallelization as well as cell culture automation. Initially, cell-based microfluidics were developed for counting and analysing cells in miniaturized flow cytometers, systems which are commercially available today[21]. Microfluidic devices for cellular studies have been further established to investigate cell transport and cultivation, in the absence and presence of concentration and temperature gradients or shear force conditions[22]. Overall, microfluidic systems have been used to perform cell sampling, cell trapping, sorting, patterning, capture, drug administration and multi-parameter cell analysis[23-26]. Microfluidic systems can provide defined and reproducible stimulation scenarios that allow the reliable investigation of cell behaviour in an environment that mimics mechanical forces within living tissues. In particular mechanical strain, which represents an important factor that can trigger stem cell differentiation in vitro [27-31], can be effectively achieved by using elaborate micro-scale systems with various techniques[32-35]. However, in the human body individual cell types are spatially arranged in three dimensions with high precision, constantly interacting and responding to adjacent cells. Since dynamic, yet controlled, cell-to-cell interaction play a key role in the maintenance of tissue function, regeneration and repair, co-culture systems have been established as an indispensable tool to investigate the dynamic interplay between homo- as well as heterotypic cell populations. Therefore, microfluidics underwent the transition from 2D monolayer cell culture to 3D cell culture and thereby advancing the technology into more physiologically relevant, *in vitro* models, including bio-engineering methods such as cell-laden scaffolds and tissue spheroids on-chip. A further trend is to integrate complex co-cultures rather than single cell populations. For instance, co-culture systems can foster cell-to-cell interaction to improve cell function, regeneration and differentiation capacity, and activation of immune cells. [36-40]

In this review, we report on the latest progress on microfluidic devices developed to study interactions between heterotypic cell populations, except for neurobiology where also homotypic interactions between single neurons are described. A more comprehensive review describing homo- and heterotypic cell-cell interactions on chip can be found elsewhere.[41] We begin by introducing 2D models for investigation of cancer development and progression, and continue with more complex 3D models for studying of cancer biology and drug testing, neurobiology, and engineering of vascular models. Further, we shortly review recent devices for analysing cell-cell interactions at single-cell level. In the last section we examine recent advances in multi-organ-on-a-chip and body-/human-on-a-chip systems with respect to interaction of cell co-cultures. The reviewed literature was strictly selected after the criteria recency (>2010) and significance with respect to cell-to-cell interaction and cellular cross-talk (>1 cell type).

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1. Microfluidic models for cancer biology, cancer immunology and cancer therapy

The immune system functions as regulatory authority and responsible for stable maintenance of human physiology. The study of immunology using in vitro models has so far given the opportunity to get more insight into the complex process of immune response and many immune-related diseases. Microfluidic systems have been used as miniaturized in vivo-like physiological models that mimic cell-to-cell interactions and simulate the human metabolism in health as well as disease. In particular, two main application fields for such microsystems are immunology and cancer research. Even though immune response during inflammation and allergy is of great interest and therefore has been extensively analysed using microfluidic devices, however, the application of microfluidics in this field mainly focuses rather on the response of immune cell populations to pro- and anti-inflammatory soluble factors than actual cell-to-cell interaction studies. Therefore, more detailed information on such microfluidic models for immune-mediated cell motility studies and allergy-on-chip can be found elsewhere and are not reviewed here.[42] However, cancer biology encompasses of a broad range of research disciplines that share a common goal in establishing more *in vivo*-relevant tumour tissue models for improved drug development, lead optimization studies and screening efforts. An important aspect of microfluidic tumour research is concerned with understanding cell-to-cell interactions between tumour cells and various target cell types including stromal, endothelial as well as immune cells under physiological relevant conditions.[2, 38, 43] Over the years, a variety of advanced microfluidic *in vitro* tumour models have been established for (i) two-dimensional cultivation of tumour cells and (ii) formation as well as cultivation of three-dimensional tumour structures (e.g. cell-laden hydrogels or spheroids).[5, 44-47] More recently, the immunology of cancer has gained momentum due to the emergence of cell-based immunotherapy as promising complementary anticancer treatment strategy. In the following subsections bioengineering of cancer microenvironment on-chip as well as recent applications of novel microfluidic cancer models for the study of immune and cancer cell crosstalk, as well as chemotherapy and immunotherapy are discussed in more detail.

1.1 Recent advances in two-dimensional cell migration models for cancer research

The application of two-dimensional microfluidic cell culture systems capable of monitoring the interaction of stromal with tumour cells is of particular importance in understanding cancer development and progression. To enable microfluidics to act as promising tool for such studies, a lot of research was focussing on compartmentalization of co-cultures in the form of separated microfluidic chambers as well as cellular patterns. To gain a deeper understanding of site-directed cancer cell migration, Ma and co-workers developed a microfluidic device that promotes indirect cell-to-cell interactions.[48] As shown in Fig. 1A, an interconnected microfluidic channel network allows for the cultivation of various co-culture systems to study e.g. the interactions of human embryonic lung fibroblasts (HFL-I) with either carcinoma cells (HepG2, ACC-M and ACC-2) or normal epithelial cells (GES-1). Results of this study revealed that site-directed migration and trans-differentiation of embryonic fibroblasts exclusively takes place in the presence of carcinoma cells. Similar microdevices based on interconnected cell culture chambers have also successfully been employed in a variety of cancer cell migration assays and motility studies as well as chemotherapy screening applications, however the basic biochip functions remain similar.[49, 50, 51] To generate spatially-resolved cell culture compartments that separate cancer cells from stromal cells in a more refined approach, Menon and co-workers integrated anisotropic cell culture surfaces based on tuning of surface wettability to study induction of reactive oxygen species (ROS) in stromal cells (Fig. 1B).[2] With this technology, it is possible to control the time point for cell co-culture to be initiated be simply injecting media through the hydrophobic centre compartment thus interconnecting the lateral chambers and commencing both, direct as well as indirect cell-to-cell interactions and cell migration. The authors demonstrated that during co-culture of HS5 bone marrow stromal cells and HuH7 liver tumour cells within the microfluidic device the generation of ROS was increased 4-fold, leading to ROS-induced stromal cell death. At day 9 of on-chip co-culture, transformation of highly aggressive metastatic HuH7 tumour cells was observed, indicating that the ROS-concentration influences the rate of tumour invasion and progression.[52] Another two-dimensional co-culture system for was recently established to investigate the interaction of tumour cells with immune cells (Fig. 1C).[43, 53, 54] In this study protein micropatterning based on a laminar flow patterning technique was used to established an anisotropic nano-biointerface that enables guided cell adhesion and direct cell-to-cell contact between adherent, anchorage-dependent cell types and surface-activated immune cells. As application scenario, Liu *et al.* established a microfluidic co-culture model for anticancer drug screening of different chemotherapeutics by mimicking a bladder cancer microenvironment.[55] This microfluidic device consists of four cell culture chambers separated by hydrogel barriers to allow diffusion of nutrients and soluble factors (Fig. 1D). The authors showed that the interaction of cancer cells with stromal and immune cells displayed good analogy to *in vivo* bladder cancer pathology including macrophage migration towards cancer cells, phenotypic alterations of stromal cells as well as formation of vascular-like tumour cell tubes. Tumour expansion, invasion as well as metastasis depend on complex direct and indirect cell-cell interactions between cancer cells and the hosts immune system. To study immune-surveillance, which is a complex cross-talk between cancer cells and the immune system, Businaro *et al.* (Fig. 4E) employed a device similar to Ma *et al.* to analyse the influence of interferon regulatory factor 8 (IRF-8), which plays an important role in the induction of competent immune responses and also is a tumour suppressor.[56, 57] The authors showed that knock-out of IRF-8 in spleen cells inhibits cell motility and interaction with B16 melanoma cells as well as tumour suppression thus increasing melanoma extravasation rate whereas wild-type (WT) cells remain their tumour suppressive character.

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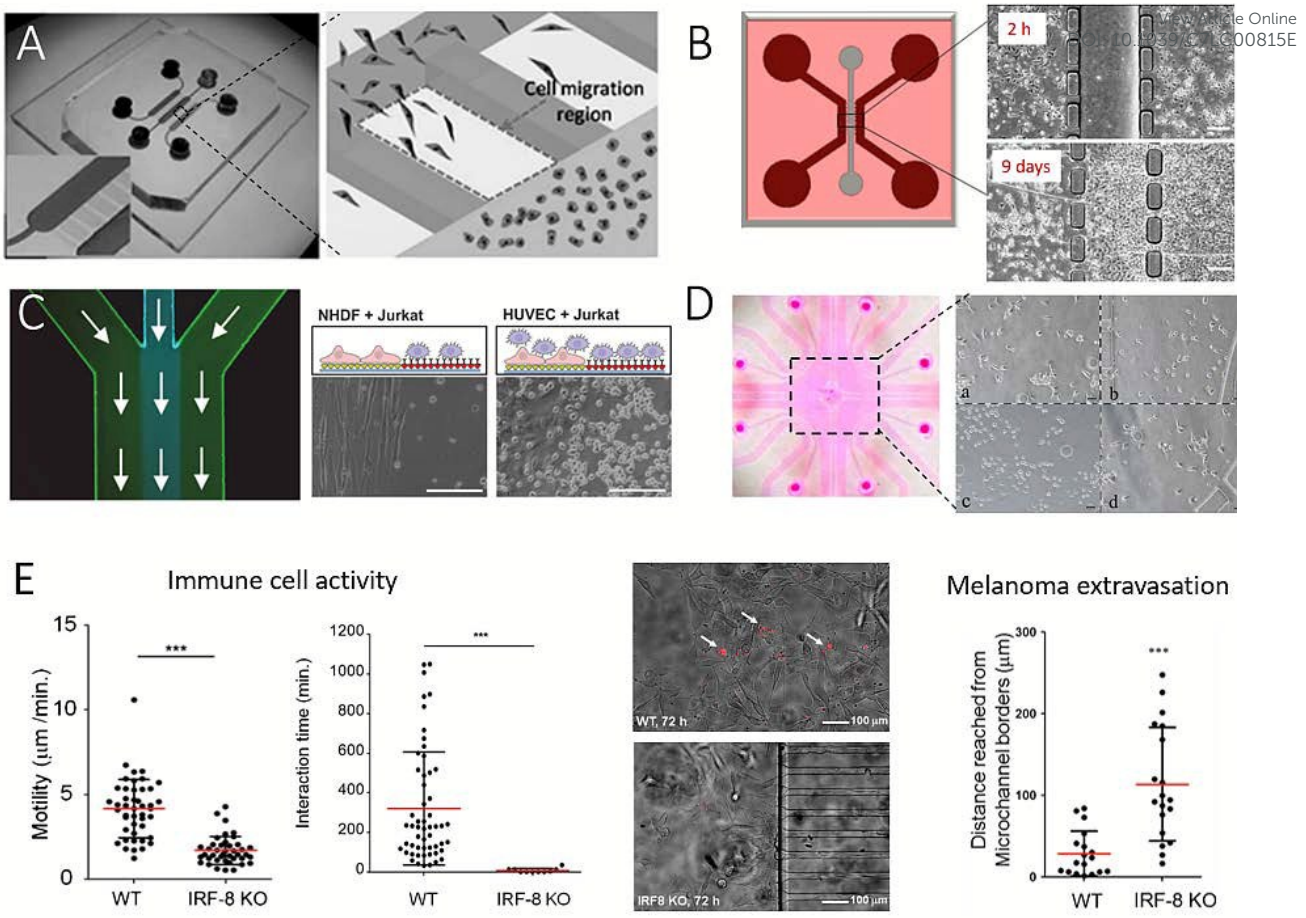

Fig. 1 Examples of microfluidic devices with inter-connected cell culture chambers for studying of cell-to-cell interaction between 2-dimensional layers of tumour cells and various types of mammalian cells. In (A) Ma *et al* observed interactions between fibroblasts and tumour cells. In (B), Menon *et al* studied cell migration and cellular interaction between bone marrow stromal cells and a liver tumour cells, scale bar 20 µm; (C) By patterning a microfluidic channel with different anisotropic crystalline protein nanolayers, Rothbauer *et al* could co-culture and study the interaction between cancer cells and immune cells, scale bar 200 µm; In (D) *Liu et* al could mimic the microenvironment in a bladder by co-culturing four different cell types in cell culture chambers separated by hydrogel barriers. (E) Businaro *et al.* studied the role of interferon regulatory factor 8 (IRF-8) in cancer progression using a two-dimensional microfluidic co-culture as immunodeficiency model.

Integration of microvalves as well as non-invasive biosensors have proven suitable to increase the through-put and facilitate automation of such two-dimensional microfluidic cell migration systems. For instance, Gao and co-workers introduced an enhanced *in vitro* co-culture platform consisting of pneumatically activated micro-valve system to physically separate different cell types within microfluidic channels (Fig. 2A).[58] In this PDMS-based microdevice, direct cell-to-cell contact was initiated by opening the valves between two cell culture chambers. Cross-migration of murine 4T1 mammary tumour cells and human dermal microvascular endothelial cells (HDVECs) was analysed using live-cell imaging. In the presence of normoxic conditions, tumour and endothelial cells migrated towards each other, while under hypoxic conditions, induced by cobalt chloride ($CoCl_2$), cell migration was predominately hindered for tumour cells. In a similar approach, Zheng and co-workers developed a microfluidic microarray containing integrated central separation barriers to create a parallel cell migration device (See Fig. 2B).[59] Using a 4 x 4 interconnected microchamber array, controlled cell seeding, co-culturing, medium exchange as well as multiplexed migration analysis was simultaneously achieved. In addition to the integration of microvalves and separation barriers into microdevices, another reported technological advancement is the combination of complementary and orthogonal sensing strategies to monitor cell-to-cell interactions.[38] Fig. 2C shows a microfluidic biochip containing embedded interdigitated impedance sensor arrays and organic photodetectors for optical light scattering measurements to assess the interaction of a small number DU-145 prostate cancer cells at an endothelial cell barrier and stromal cell culture.[38] Results of the study showed that DU-145 prostate cancer cells are not able to invade a functional endothelial cell barrier under physiological flow conditions but can freely pass through stromal cells pointing at their low metastatic ability. The authors demonstrate how non-invasive biosensors can be employed for automation of cell migration and tumour cell invasion assays on-chip, being highly sensitive to cell population responses without the need for cell staining or fluorescent transfection.

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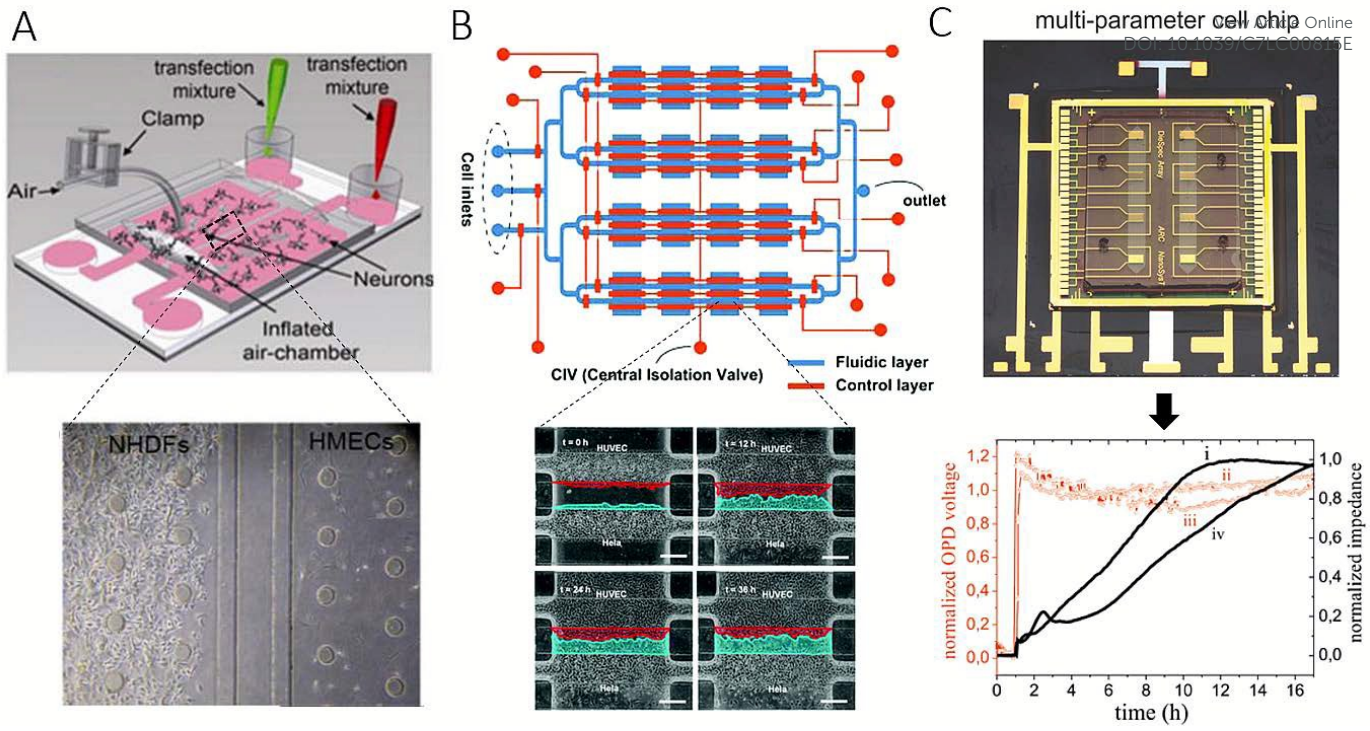

Fig. 2 Examples of complex microfluidic devices for studying of cell-to-cell interactions. (A) Gao *et al* controlled cell-to-cell contact between tumour cells and endothelial cells with integrated pneumatic valves. In (B) Zheng *et al* could monitor cell-migration in 4x4 interconnected microchambers simultaneously by using pneumatic-controlled valves. (C) Charwat *et al* studied the invasive capability of DU-145 cells towards vascular cells in a microfluidic chip with integrated impedance sensor arrays and organic photodetectors.

1.2 On-chip cell migration and cancer in the third dimension

Since human physiology is complex in architecture, the last two decades research efforts have been invested to bioengineer *in vivo* physiology three-dimensional level and some methods were even translated onto microfluidic models. Such three-dimensional systems that are *in vivo*-like, not only with respect to cellular function but also to tissue and organ geometry, can shed more insight into the structural and functional relationship of vasculature, the immune system and cancer. Although most microfluidic cancer studies are conducted using two-dimensional monolayer cell cultures, these planar cancer models are known to have limited practicality in understanding the complex tumour physiology *in vivo*. The main reason is that *in vivo*, both tissue structures and geometry significantly influences growth rates of tumour cells. To address this limitation, a number of microfluidic devices and methods for three-dimensional cell cultures, so-called spheroids, have been established in recent years. [5, 44-47] Microdevices featuring three-dimensional co-culture of cancer spheroids have been developed to mimic the three-dimensional cancer environment for a variety of cancer types including salivary glands and lung cancer, as well as for intra- and extravasation studies. For instance, to increase the relevance of tumour invasion and cell migration assays for breast cancer biology, Sung and co-workers proposed sequential loading of cell types at different time-points using surface-tension driven pumping to establish a three-dimensional breast cancer invasion model (Fig. 3A).[60] Using the microfluidic breast cancer device, the authors confirmed the relevance of their model by monitoring the transition from ductal carcinomas *in situ* (DCIS) to invasive ductal carcinoma (IDC) *in vitro*, which showed good agreement with *in vivo* xenograft models for tumour invasion studies. Also based on spheroids, Liu and co-workers proposed a three-dimensional microfluidic network consisting of intersecting cell culture chambers to determine the influence of carcinoma-associated fibroblasts (CAFs) on malignant cancer progression (Fig. 3B).[61] Invasion of salivary gland adenoid cystic carcinoma (ACC) spheroids was exclusively observed in the presence of CAFs expressing α-SMA, thus pointing at an involved mechanism of invasion. An important aspect in cancer research constitutes cell migration, which can lead to intravasation and extravasation events during metastasis formation. As alternative strategy to spheroid technology, cell-laden hydrogels have also beenintegrated on-chip for cancer research. For instance, a three dimensional microfluidic breast cancer metastasis model introduced by Bersini and co-workers consisting of a vascular and a hydrogel tissue compartment was used to study MDA-MB-231 cancer cells motility in an osteo-cell conditioned microenvironment (Fig. 3C).[62] These extravasated and active cancer cells proliferated and formed micro-metastases of increased sizes up to 132 cells at 5 days of culture. Since precise control of biochemical factors is a key parameter in understanding the tumour-vascular interface, Zervantonakis and co-workers established a three-dimensional microfluidic model to study intravasation of cancer cells that lead to the formation of secondary tumours (Fig. 3D).[63] Using a three-dimensional microfluidic vascular barrier model based on triple co-culture of endothelial cells, macrophages and cancer cells, the authors observed similar percentage of tumour cells in various vascular barriers and found that tumour necrosis factor alpha (TNF-α) secreted by macrophages was the main driving force compromising the tight endothelial barrier and allowing tumour cells to circulate in the vascular channel compartment, thus pointing out the key role of immune cells and soluble factors in progression of cancer via metastasis.

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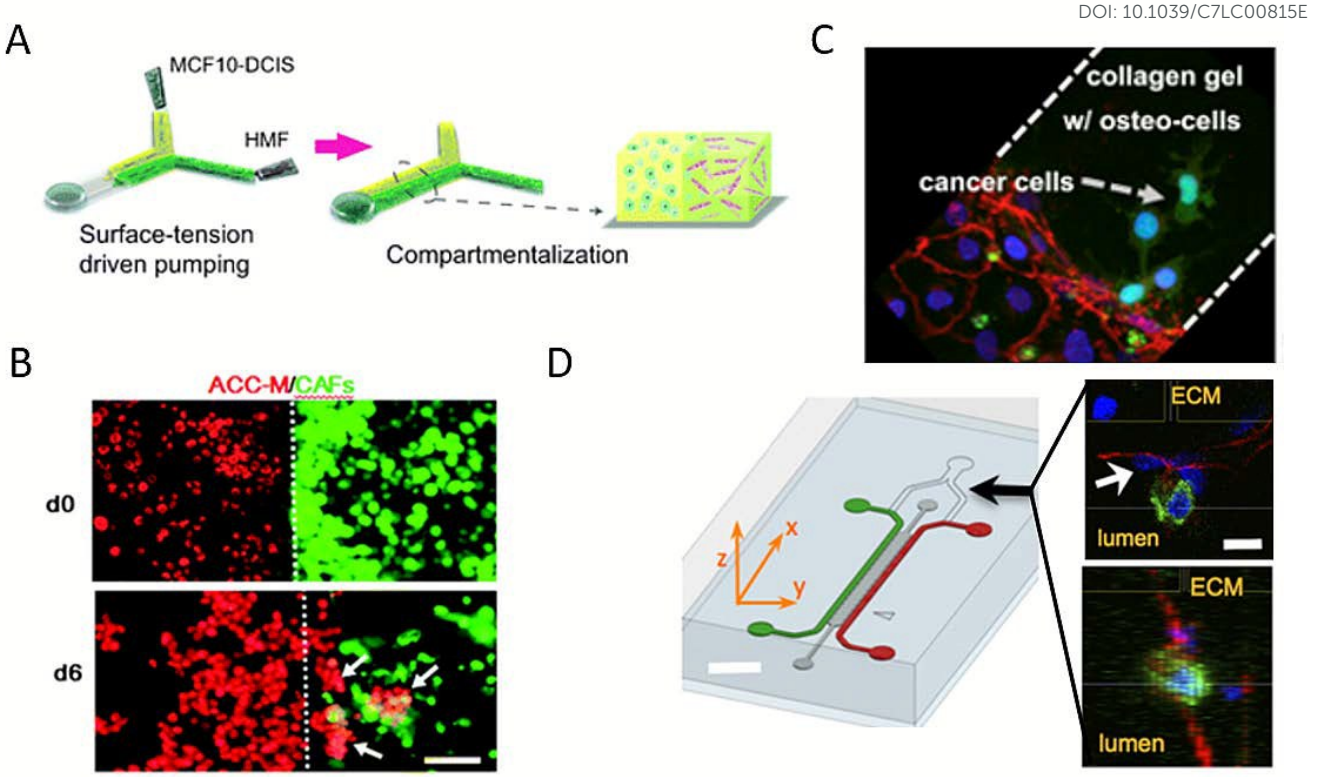

Fig. 3 Three-dimensional strategies for on-chip bioengineering of the cancer microenvironment. (A) Sung *et al.* established a 3D breast cancer invasion model by sequential loading of cells with surface-tension driven pumping. In (B) Liu *et al.* co-cultured carcinoma-associated fibroblasts (CAFs) and salivary gland adenoid cystic carcinoma (ACC) cells in a 3D matrix, demonstrating that CAFs was promoting ACC cell invasion and thereby indicating its critical role in cancer invasion. Scale bar 100 µm. (C) Bersini *et al.* studied extravasation and micrometastasis generation of breast cancer cells within a bone-like microenvironment. (D) Zervantonakis *et al.* demonstrated the impact of macrophages on tumor cell intravasation with a three-dimensional microfluidic barrier model. Scale bar 30 µm.

The importance of vascularization in cancer biology, the connection between invading tumour cells and vascular barrier cells, further led to the development of various microsystems that more closely resembles vessel architecture (round channel morphology like a cellular lumen). For instance, Wong and Searson established a live-cell analysis platform capable of investigating the behaviour of metastatic cancer cells within a functional artificial ECM-based microvessel (Fig. 4A).[64] A refined bioengineering approach has also been established by George and co-workers who exploited the self-organizing nature of human microvessels to perfuse cancer cell spheroids (Fig. 4B).[47, 65] Another demonstration of the usefulness of complex bioengineered systems containing cancer and vascular microenvironments for the establishment of meaningful tumour *in vitro* models is provided by Buchanan and co-workers who showed that all tumour-expressed proangiogenic genes were significantly upregulated during co-culture with endothelial cells and when exposed to shear stress (Fig. 4C).[66] Additionally, angiopoietin 2 (ANG2) and platelet-derived growth factor B (PDGFB), both factors that are involved in breast cancer angiogenesis,[67] upregulate upon cellular stimulation with shear stress.

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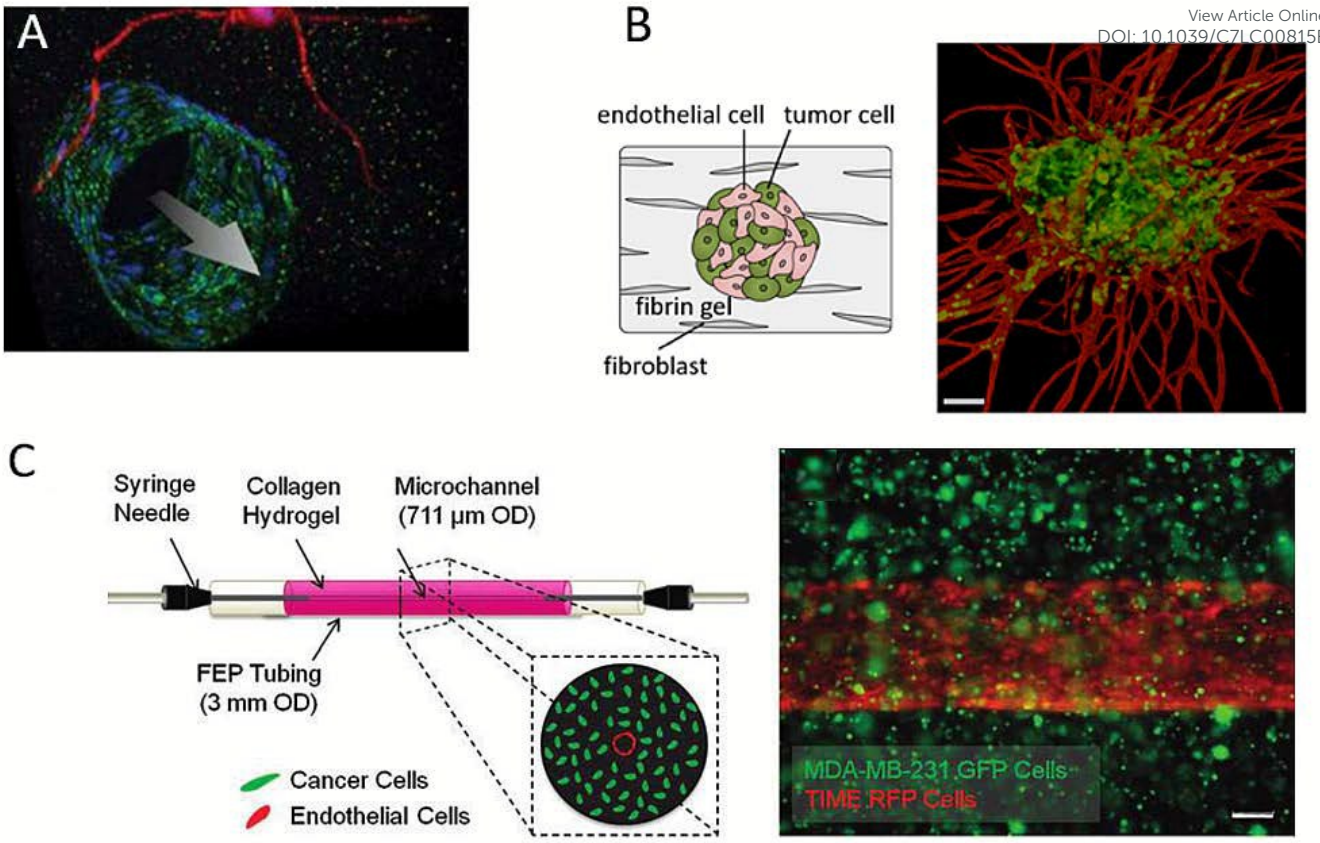

Fig. 4 Microsystems mimicking complex vasculature structures. (A) Wong *et al.* investigated the behaviour of metastatic cancer cells within a functional artificial ECM-based microvessel. (B) Ehsan *et al.* investigated early events of solid tumour progression with a prevascularized tumour (PVT) model composed of spheroids of endothelial and tumour cells, embedded in a fibrin matrix containing fibroblasts. Bar size 100 µm. (C) Buchanan *et al.* developed a tumour vascular model and could show that tumour cells significantly increase expression of proangiogenic genes in response to co-culture with endothelial cells under low flow conditions. Scale bar 200 µm.

1.3 Microfluidic cell co-culture systems for anticancer drug screening

As discussed in the last section, a variety of bioengineering approaches are capable of generation of more relevant *in vitro* cancer models. Within this section, we want to focus on the application of such models and highlight the recent advances in drug screening aiming for better therapeutic outcomes in cancer. The main limitation of most on-chip models for drug screening is that these microdevices in most cases do not have enough through-put to be an effective screening tool. For instance, Choi and co-workers developed a microfluidic drug screening device capable of co-culturing breast tumour spheroids and human mammary ductal epithelial cells as well as mammary fibroblasts using a compartmentalized 3D microfluidic device (Fig. 5A). Using this "breast cancer-on-a-chip" the impact of treatment with paclitaxel, a clinical anticancer drug, on DCIS spheroid size was investigated to demonstrate the drug's efficacy in arresting tumour cell proliferation and thus preventing the growth of DCIS lesions in a microenvironment that closely resembles breast ductal carcinoma. Even though the microsystem shows exceptional bioengineering to create a complex tumour model, each device consists of only one cell culture thus failing to provide enough through-put needed for drug screening. To create a system capable of a higher throughput Xu *et al.* established a system of 4 x 3 hydrogel culture chambers in parallel for anti-cancer drug screening using co-culture of human non-small cell lung cancer cell line (SPCA-1), human lung fibroblast cell line (HFL1) and patient derived lung cancer cells (see Fig. 5B).[68] For chemotherapeutic screening of different anti-cancer drugs the authors implemented on-chip concentration gradient generators (CGG) to apply three different concentrations per functional screening unit automatically. The microdevice enabled accurate screening of different anti-cancer drug sensitivities on eight patient-derived lung cancer samples in parallel, resulting in appropriate dose, single as well as multi-drug chemotherapy schemes. A different three-dimensional microfluidic cell culture system containing a concentration gradient generator, also called SpheroChip system, offers on-chip formation of liver and colon cancer spheroids for drug sensitivity testing.[69] In the work of Bauer *et al.* a platform containing 96 arrayed single microfluidic channels was used for a paracrine signalling analysis of a 3D co-culture of human mammary fibroblasts and T47D breast carcinoma cells (Fig. 5C).[70] With this system, the authors are aiming to replace the conventional microwell-format for three-dimensional cell cultures and enable an automated high-throughput cancer screening for individual patients as well as for drug discovery. Disadvantage of the system may be that heavy automation technology is needed to exchange medium or inject drugs due to the lack of nutrient administration via microchannels and pumps, both major advantages of microfluidic systems. Another very promising pharmacological

approach was reported by Imura and co-workers, who established a bioassay system that can assess intestinal absorption, hepatic metabolism and bioactivity of ingested substances including anti-cancer therapeutics (Fig. 5D).[71, 72] The idea is similar to the micro cell culture analogue (μCCA) concept introduced by Sung and Shuler already in 2009, highlighting the importance not only of cellular but also organ-level function.[73] The authors evaluated the microsystem with the inclusion/exclusion of digestion processes during anti-cancer drug administration. The anti-cancer activity of TGF was lost in all cases, indicating that the drug was degraded by the synthetic gastric juices being in concordance with the known properties of these drugs. These results highlight that the pharmacological aspects especially for oral uptake of anticancer medication is a very important parameter to be observed for new therapeutics since enzymes can degrade bioactive substances rendering them ineffective.

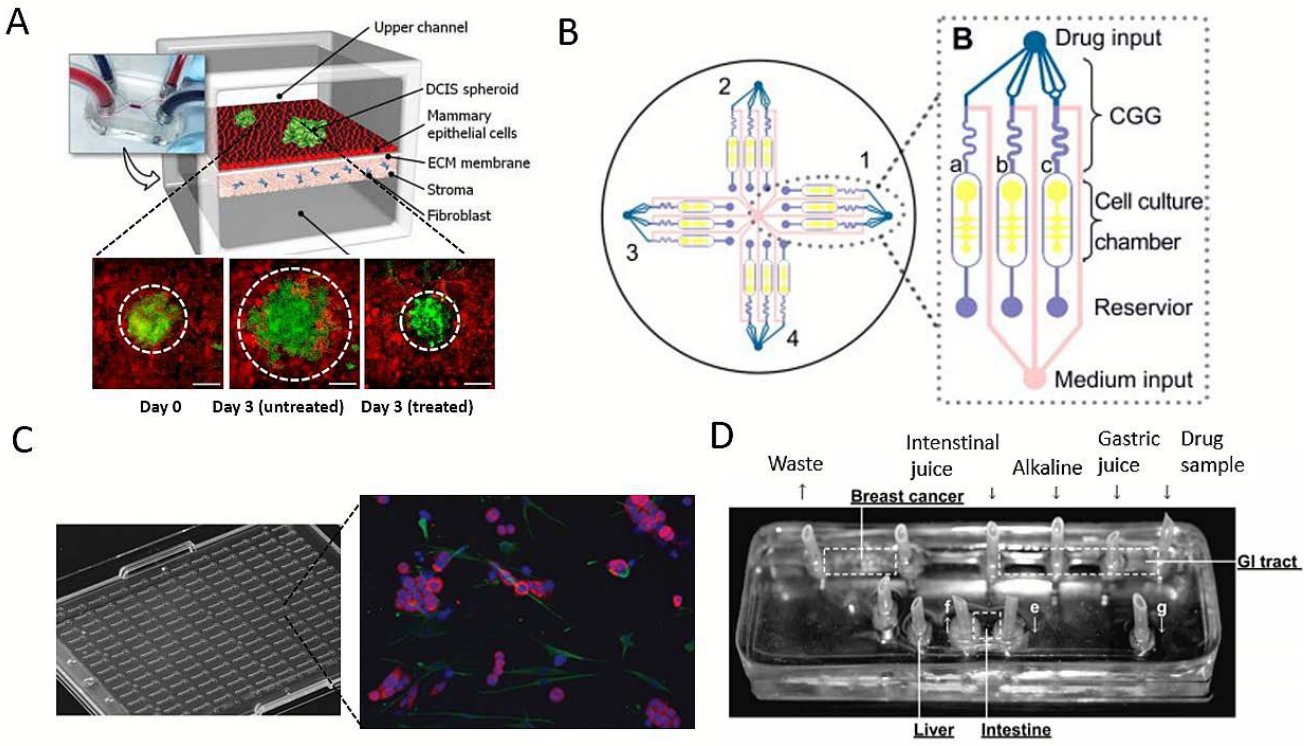

Fig. 5 Microfluidic models for screening of anticancer drugs. (A) Choi *et al.* developed a breast cancer-on-a-chip for drug screening purposes. The efficacy of the anti-cancer drug paclitaxel was demonstrated by treating a DCIS spheroid and monitoring the size reduction. Scale bars 100 μm. (B) Xu *et al.* established a microfluidic three-dimensional co-culture system for drug sensitivity testing of a lung cancer model. (C) Bauer *et al.* performed high-throughput screening on 3D co-culture of mammary fibroblasts and breast carcinoma cells. (D) Imura *et al.* established a micro total bioassay system mimicking physiological processes with the aim of evaluating orally administered cancer drugs.

1.4 Recent advances in immunotherapy-on-chip

Immunotherapy in general is the clinical application therapeutic agents that can enhance immune effector mechanisms. More recently, cell-based therapeutic agents have gained momentum as rationale behind personalized patient-derived anticancer treatment. Among the variety of blood cell populations available, antigen-presenting dendritic cells as well as CD8+ T cells are two promising cell types that can increase the potency of nowadays anticancer therapies. A more comprehensive review about the state-of-the-art in adoptive cell-mediated cancer immunotherapy based on T cells as well as dendritic cell vaccines can be found elsewhere.[74, 75] Dendritic cells can act as an adjuvant due to their role in identifying and presenting antigens to T cells to create an antitumour immune response. Therefore, recently microsystems have emerged that aim to shed light into the complex cellular mechanisms that guide antitumour response to be able to create more effective cell-bases immunotherapies. For instance, Parlato *et al.* presented a microfluidic system (Fig. 6A) to analyse the tumour suppressive capability

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of interferon alpha-conditioned (IFN) dendritic cells.[76] The device enabled the analysis of cell migration as well as infiltration of dendritic cells into the tumour compartment. The authors show that the combinatorial treatment of interferon alpha with romidepsin (RI) results in high cell apoptosis and increased phagocytosis of tumour cells by IFN-dendritic cells. Overall, this microdevice allowed the dissection of dendritic-cancer cell interactions within a three-dimensional tumour microenvironment and the identification of major underlying factors (e.g. CXCR4) thus proving the potential of such microfluidic devices as innovative tools of efficacy testing of novel immunotherapeutic strategies. Our group has reported a lab-on-a-chip system(Fig. 6B) for non-invasive multi-parametric dynamic monitoring of T cells and cancer cells.[38] Initially, we demonstrated how such a system can be used for label-free automation of a conventional T cell proliferation assay, where primary T cells were stimulated using CD3/CD28-labelled Dynabead nanoparticles for T cell expansion and proliferation. Further, dual parametric analysis was employed in the microsystem to identify how T cell priming with Dynabeads leads to tumour-suppressive response in OCM-1 melanoma cells with the formation of two-dimensional, irregular cancer cell aggregates. These results indicate that label-free biosensing methods can prove worth for quality control as well as non-invasive read-out of such delicate bioengineered co-culture systems. In a three-dimensional approach Pavesi *et al.* incorporated human cancer hepatocyte (single cell or as tumour cell aggregate) in a 3D collagen hydrogel of a microfluidic device to screen tumour suppressive capability of engineered, T cell-based anticancer vaccines (Fig. 6C).[77] The authors demonstrated how human T cells engineered to express tumour-specific T cell receptors (TCR–T cells) migrate and kill the tumour target cells. Also, they analysed soluble factors under conditions of varying oxygen levels and in the presence of inflammatory cytokines. Interestingly, cancer aggregates but not conventional cultures were affected by environmental changes. In a final set of experiments the authors demonstrated that the 3D microdevice can be used to analyse the TCR–T cell efficacy in an immune-suppressive cell-cancer co-culture scenario. Overall, the main advantage of applying microfluidic strategies lies in the inherent capability of such systems so geometrically confine cells therefore enabling the analysis of cell-cell interaction studies using microscopy and label-free biosensors.

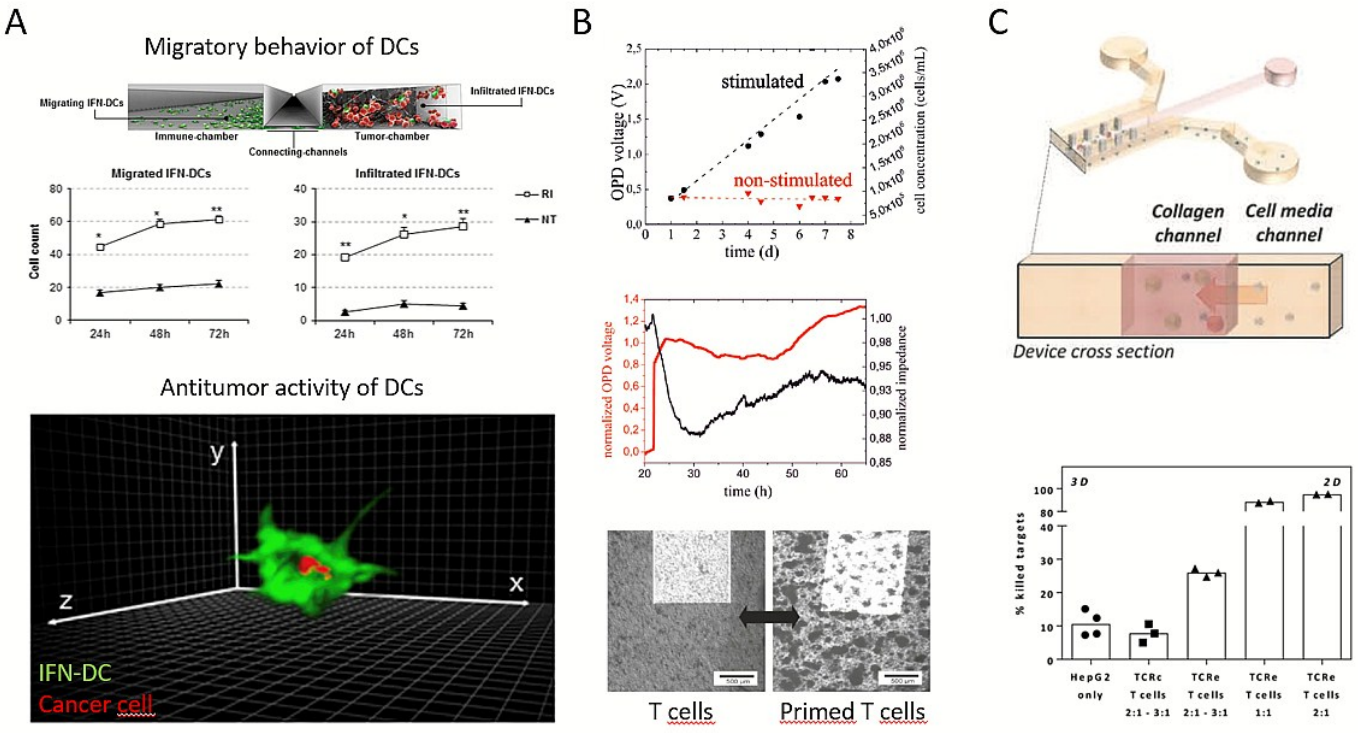

Fig. 6 Microfluidic systems for cell-based immune therapy applications. (A) Parlato et al. demonstrated with their multi-compartment microsystem how IFN alpha preconditioning on dendritic cells (IFN-DC) in combination with romidepsin (RI treatment) results in increased migration, infiltration and cancer engulfment of dendritic cells. (B) Charwat *et al.* studied both tumour invasion and suppression in a microfluidic chip with integrated impedance sensor arrays and organic photodetectors. (C) Pavesi *et al.* showed with their microfluidic strategy how genetically engineered T cells selectively interact and kill cancer target cells.

2. Microfluidics and neuronal biology

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The nervous system of the human body can be structurally divided into the central nervous system (CNS) comprised of brain and spinal cord and the peripheral nervous system (PNS), which includes all other nervous structures throughout the body. Along these lines, established lab-on-a-chip and organ-on-a-chip systems mimic the physiology of these different tissue structure to gain a deeper understanding of various aspects of brain development and dysfunctions including the onset and progression of neurodegenerative diseases. The premise of these advanced microdevices is to overcome existing limitations of conventional cell-based technologies that mainly record neuronal data based on the activity of cellular clusters, thus only providing information on subpopulations of neurons.[78] Consequently the following section microfluidic technology mainly highlight more recent developments that provided deeper insights into neurobiology. A basic overview of on-chip neurobiology and a progress report prior 2010 can be found elsewhere.[79, 80]

One recent technological advance involves the generation and manipulation of synapses on-chip, where rat hippocampal neurons formed synapses in a microgroove array to study the effect of chemical injuries between two spatially resolved neural cell culture compartments.[81] The presented system enables spatial and temporal control over the neuronal microenvironment, which is not possible to achieve in conventional culture systems (Fig. 7A). Similarly, Shin *et al.* developed a microfluidic microgroove system to reliably generate stem cell-derived neurons on-chip as a preplacement for primary neuron cell cultures.[82, 83] Higashimori and Yang used a similar design for application as a microfluidic co-culture platform to study the interaction of neurons with glia cells. They demonstrated how microfabricated systems enable the use of delicate imaging instrumentation and therefore allowing for deeper analysis of cell-to-cell interactions of the central nervous system (Fig. 7B).[84]

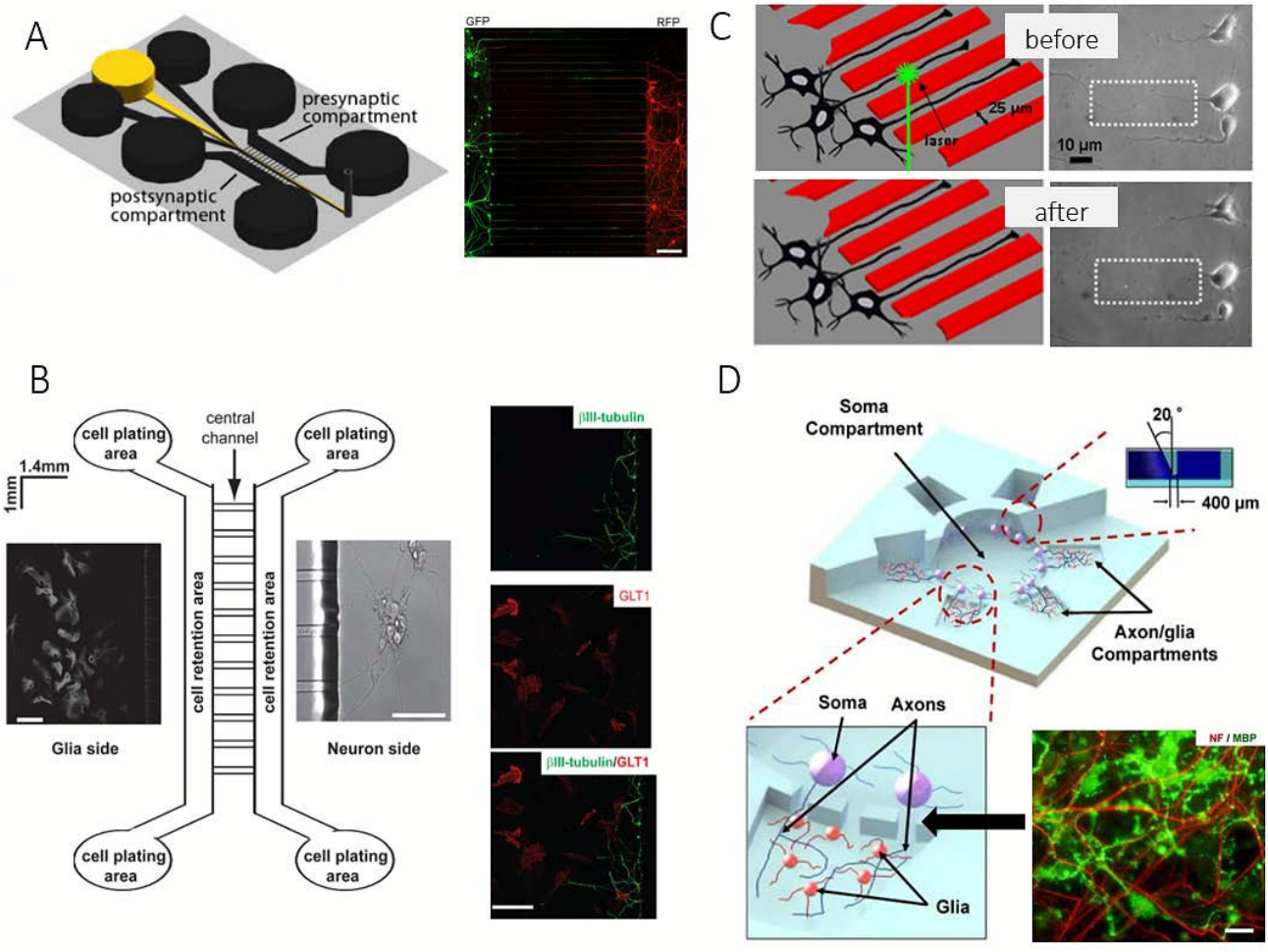

Fig. 7 Microfluidic neuronal models for studying interactions between cells in the central nervous system. (A) Taylor *et al.* studied the effect of chemical injuries between two spatially resolved neural cell culture compartment. Scale bar: 150 µm. (B) Higashimori *et al.* combined a microfluidic co-culture system with delicate imaging instrumentation to examine cell-to-cell interactions between axonal/dendritic and glial cells. Right: Axon bundles (green) and astrocytes (red). Scale bar: 50 µm. (C) Kim *et al.* developed a platform to quantify the regeneration of injured CNS. By generating precise lesions sited in neurons with laser induces axotomy regeneration of single axons could be precisely studied. (D) Park *et al.* established a complex microfluidic 3D platform for studying axon-glia interactions during drug and biomolecule treatment on multiple co-cultures.

For precise control over CNS injury and lesions, Kim *et al.* introduced a microfluidic platform capable of controlling axonal growth by surface modification as well as controlling the soluble factors (Fig. 7C).[85] Additionally the authors demonstrated how an optical setup can be used

for precise generation of lesion sites (laser induced axotomy) as well as analysis of regeneration on a single-axon resolution. A deeper analysis of the central nervous system was further accomplished by introducing on-chip high resolution imaging to study cell-to-cell interactions between neurons and glia cells, also accounting for the complex bidirectional signalling processes taking place in specific neuronal structures such as axons or dendrites. In a related study, Zahavi *et al.* used a similar compartmentalized microfluidic system to establish a neuro-muscular co-culture model to investigate cell-to-cell interaction between motoneurons and muscle at neuromuscular junctions.[86] The authors showed that glial-derived neurotrophic factor (GDNF) modulates growth and muscle innervation at axons in contrast to survival pathways in the soma. In an attempt to increase the number of compartments and to integrate a bigger, central soma compartment, Park *et al.* combined various microfabrication methods including micromilling, hot embossing and soft lithography to fabricate a complex three-dimensional microfluidic culture system (Fig. 7D).[87] This technological advancement allowed for the first time multiple experimental heterotypic culture conditions and different localized biomolecular treatments on a single device to study cell-to-cell interactions, oligodendrocyte progenitor cell (OPC) development, as well as for investigation of axonal response to various chemical stimuli.

Another technological advancement in chip-based neurobiology included the integration of microvalves to open and close connecting conduits between neighbouring cell culture compartments to study interactions between spatially resolved neurons and glia cells.[58, 88] In a follow-up paper the same research group further demonstrated that the valve-integrated microfluidic neuron-glia co-culture platform dramatically increased synaptic stability with elevated levels of soluble factors, thus allowing deeper insight into how synapses may be modulated (Fig. 8A).[89] For instance, on-chip co-culture in vertically-layered orientation with neuron cultures on the microchannel bottom and glia cells cultured on the channel ceiling, led to an increase of dendritic protrusions as well as synaptic contacts indicating that soluble factors alone within conditioned medium is not enough to enhance the stability of synapse contacts. In contrast to microfluidic strategies confining and guiding neuronal cell-to-cell interactions, micro contact printing (µCP) technique has been employed by Marconi *et al.* to control functional properties of neurons based on adjusting surface topography (Fig 8B).[90] The authors concluded that cellular micropatterning, a frequently applied technique in tissue engineering, does not influence the electrophysiological behaviour or the connectivity of neurites. In a similar fashion, nano-volume patterning has been used to establish neuronal live-cell microarrays within biochips.[91] Using such a microfluidic live-cell microarray platform, the authors demonstrated sensitivity and reliability of patterning approaches to screen connectability defects in neuropathophysiology (Fig 8C).[91] Less often used strategies employed in microfluidics constitutes the integration of intact tissue slices, so-called brain slice on-a-chip devices, to study tissue responses within a function in-vivo 3D cellular organization. Since the majority of these systems rely on rodent organ donors, results may not be transferrable to human neuronal (patho-)physiology.[92] It is however important to note that similar as for cancer biology the recent trend in this very promising research field is shifted from two-dimensional co-cultures more and more to complex three-dimensional cultures and artificial microtissues.[93]

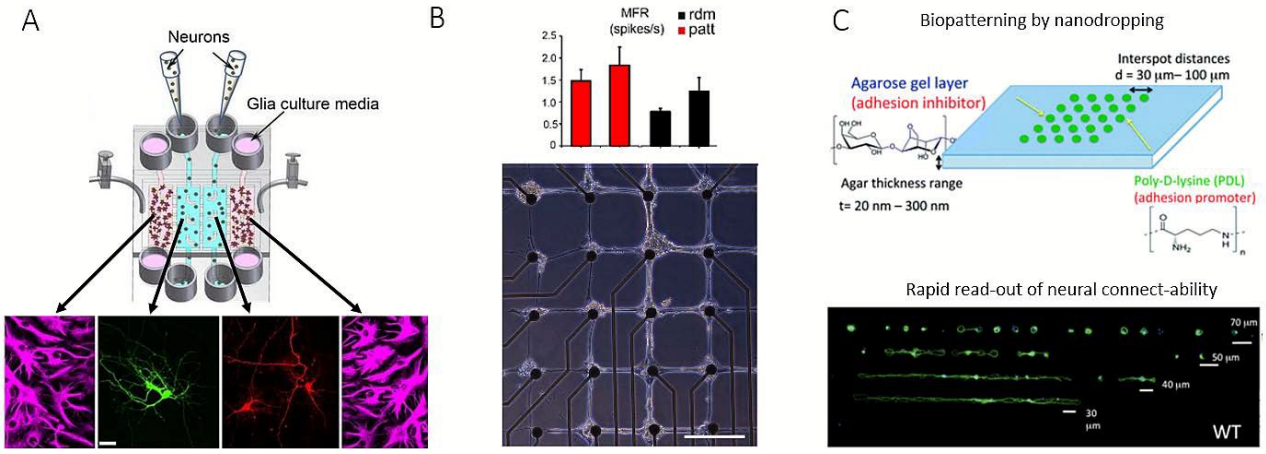

Fig. 8 Further examples of microfluidic systems for investigating cell-to-cell interactions in the central nervous system utilizing different methods for controlling cell growth into complex networks. (A) Shi *et al.* studied synapses with a microfluidic neuron-glia co-culture platform. Scale bar: 25 µm (B) Marconi *et al.* used micropatterning for guiding neurons into forming a neural network with a defined topolgy as well as for investigating the functional properties of the network. Scale bar: 200 µm (C) Petrelli *et al.* established a micro-array for screening of neural connectability by using nano-volume patterning in a biochip.

3. Bioengineering of vascular models on-chip

Blood vessels as a main transporter of oxygen and nutrients play a key role in maintaining organ health and have been incorporated or reengineered into microfluidic devices in the last decade to study effects of increasing shear force conditions, platelet adhesion and nutrient supply into biomimetic tissue structures including the diffusion and uptake of soluble factors and nanomaterials. Since the vascular system and in particular the endothelial wall represents a natural barrier that needs to be breached by migrating tumour cells during intra- and extravasation processes in cancer metastasis, a majority of vascular models have been established to gain deeper insights into cell-to-cell interactions of malignant cells in the circulatory system.[47] Recent effort in advancing microfabrication of artificial vessels using different techniques and biochips are described in the following section.

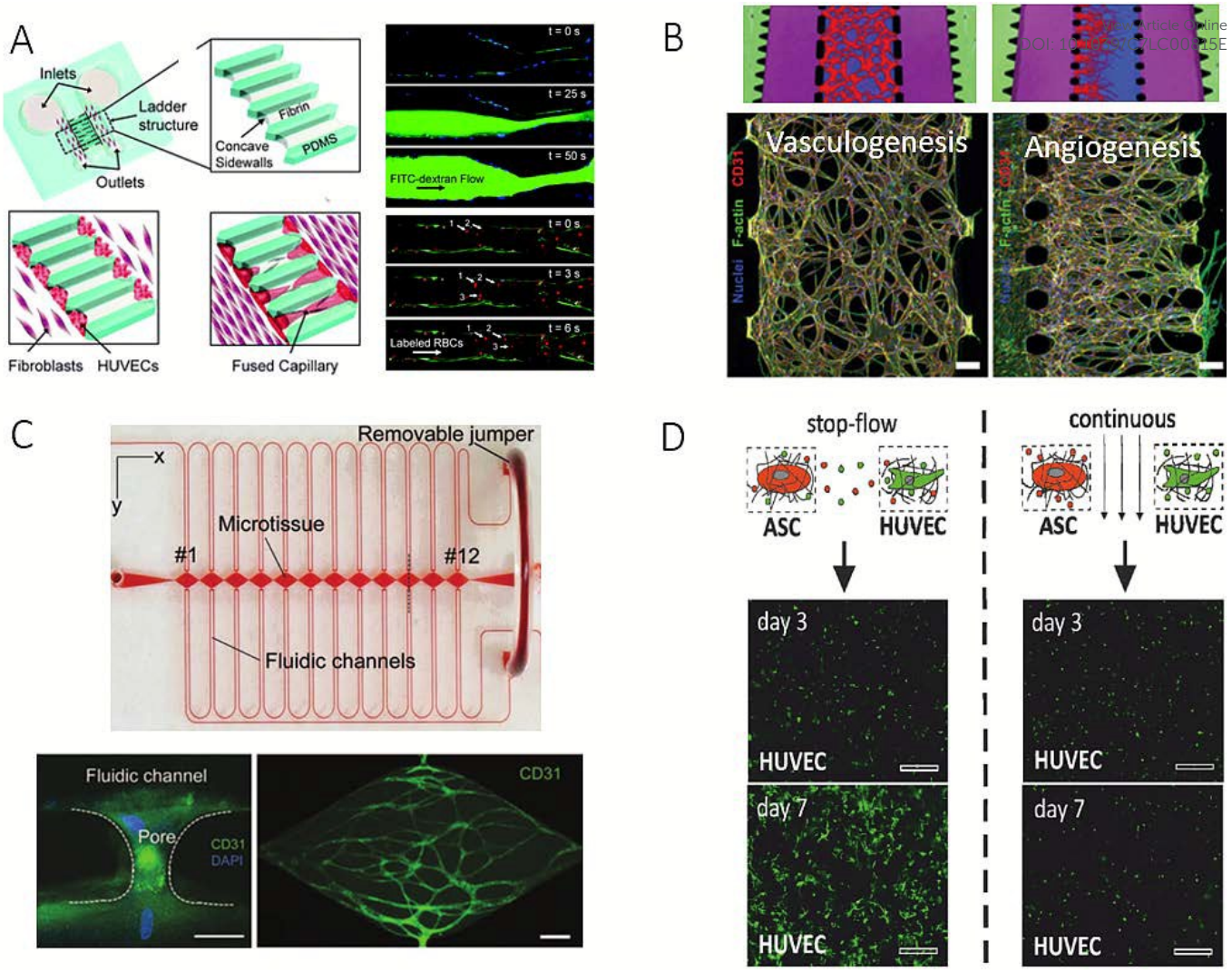

Fig. 9 Examples of microfluidic devices for creation of artificial vascular system. (A) Yeon *et al.* could form a capillary network by growing human umbilical vein cells (HUVECs) in microfabricated channels filled with fibrin gel. (B) Kim *et al.* were able to reproduce vasculogenesis and angiogenesis and could monitor different cell-to-cell interactions. (C) Moya *et al.* created an interconnected capillary network with perfusable lumen inside a high-througput microvessel platform. Scale bar: 50 µm (left) and 200 µm (right) (D) Purtscher *et al.* presented a microfluidic chip capable of tuning secretoma-related aspects of vascular network formation on a mesenchymal stem cell/HUVEC co-culture using stop-flow and perfused culture protocols.

One major improvement over 2D-microfluidic endothelial monolayers [94, 95] constitutes by the creation of perfusable capillaries and lumen-like structures based on either cellular self-organization or as microfabrication techniques employing hydrogels. For instance, a co-culture microdevice based on a versatile polymeric two-component microfabrication system was recently established to spatially separate vein endothelial cells from adipose-derived mesenchymal stem cell cultures that had been embedded in 3D fibrin matrix.[95] Additionally, Yeon *et al.* employed microfluidic technology to form *in vitro* three-dimensional tubular capillary networks on-chip (Fig. 9A) by fostering the invasion of human umbilical vein cells (HUVECs) into fibrin hydrogel-embedded microchannels, which formed perfusable, lumen-like structures over a period of 5 days.[96] Along the same line, Kim *et al.* presented a microdevice containing microstructures to foster on-chip vasculo- and angiogenesis following the addition of pro-angiogenic factors and formation of lumenised microvessels in the presence of endothelial-fibroblast interactions (Fig. 9B).[97] The authors demonstrated that angiogenesis cannot only be fully reproduced, but also enabled the monitoring of diverse types of cell–cell interactions including tumour-induced angiogenesis (endothelial cells and U87MG cells) as well as endothelial–pericyte interactions (endothelial cells, fibroblasts and pericytes). A similar but more complex microfluidic design consists of seven interconnected microfluidic compartments that allow controlled spatial distribution of stromal cells in the vicinity of endothelium to study the impact of multiple soluble factor scenarios on obtained vessel diameter.[98] A higher degree of parallelization was also presented using high-throughput microvessel platform containing 12 interconnected human capillary networks with perfusable lumen (Fig. 9C).[99] The same device was later on used for screening applications of homo- and heterotypic cell-to-cell communication based on multiple stimulation scenarios with different soluble factors. To analyse the pro-angiogenic influence of stem cell proximity and/ or secretoma on vascular network formation, a microfluidic platform containing fibrin-embedded mono- and co-cultures of human umbilical vein endothelial cells and adipose-derived embryonal stem cells were established in a single device (Fig. 9D).[100] Moreover, a fibrin-embedded co-culture system was employed for dynamic tuning of soluble factors within a hydrogel tissue construct, resulting in an inhomogeneous vascular network exhibiting different

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tube dimensions. These and other examples show how microfluidic technology can be used not only as nutrient supply, but also as innovative tool to control vascular network morphology inside a three-dimensional tissue construct including tube size and length.

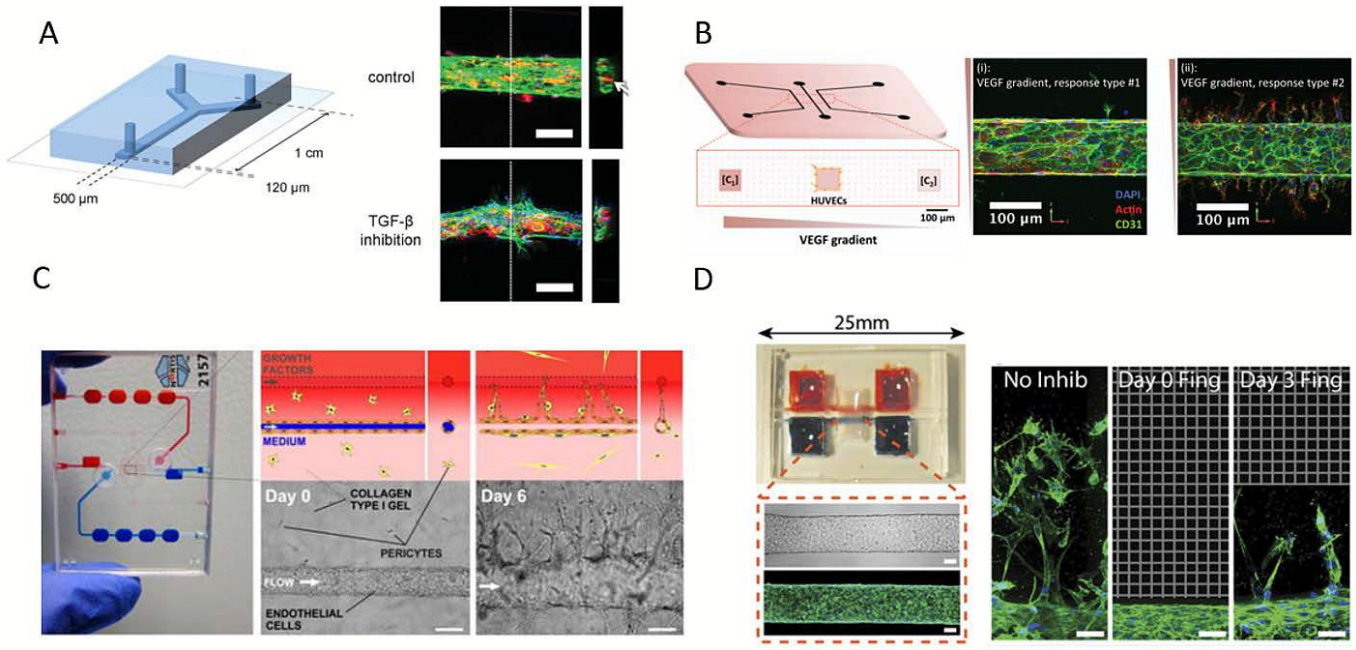

Fig. 10 Microfluidic vascular models based on different 3D matrices. (A) van der Meer *et al.* used rat tail collagen to support growth of an endothelial layer inside a microfluidic channel to study the impact of errors in the transforming growth factor-beta (TGF-β) pathway in vasculo- and angiogenesis. Scale bars: 100 µm (B) Verbridge *et al.* studied endothelial sprouting in response to vascular endothelial growth factor (VEGF) gradients in type I collagen hydrogel-embedded channels. (C) Tourovskaia *et al.* could form a mature microvessel in a collagen I matrix for investigating angiogenesis, barrier permeability and cancer cell migration. Scale bars: 125 µm. (D) Nguyen *et al.* studied multicellular endothelial sprouting as an effect of angiogenic factors in a vascular model formed in collagen. Scale bars: 50 µm.

Although fibrin hydrogel-based microsystems are most commonly used to support the growth and invasion of vascular sprouts over several days, alternative 3D matrices have been used. For instance, van der Meer *et al.* presented novel approach for on-chip formation of a microfabricated vessel that is based on a delicate co-culture of human umbilical veins endothelial cells and human embryonic stem cell-derived pericytes co-injected with a rat tail collagen I (Fig. 10A).[101] The authors showed that a mature endothelial layer with proper cell-to-cell interactions is formed within 12 hours of culture to study defective vasculo- and angiogenesis related to errors in the transforming growth factor-beta (TGF-β) pathway in human disease. Another interesting approach is a PDMS-glass-hydrogel microfluidic device containing an integrated micromolded collagen microchannel (Fig. 10B) to identify distinct endothelial cell invasion types upon VEGF stimulation/depletion.[102] A human vascular microsystem for angiogenesis was developed to study barrier permeability during cancer cell migration using collagen I matrix (Fig. 10C).[103] After formation of a mature microvessel, human umbilical vein endothelial cells induced sprouting through the collagen I matrix towards the established gradient of a growth factor cocktail. In a similar manner, Nguyen established a microdevice comprising of two micromolded collagen microchannels for morphogenic analysis of endothelial sprouting and the impact of angiogenic inhibitors on sprouting morphogenesis (Fig. 10D).[104] Overall, even though different microengineering strategies were used and also hydrogels vary between the proposed microdevices, the common feature is to form a perfusable bioengineered microvessel for future angiogenesis and vascularization research.

4. Cell-cell interaction on single-cell level?

In the first sections of this paper, we reviewed microfluidic systems where *in vivo*-like models were created to study cell-cell interaction between a multitude of cells. The different cell populations in these devices are most commonly seeded individually, initially separated with a barrier, and the cell interactions are observed dynamically due to cell motility and changes in cell proliferation.[105] Cells at physical distance are communicating by releasing signalling molecules which diffuse through the extracellular fluid to the target cell, where they are detected by membrane proteins.[1] Aside from indirect communication cells in direct contact also communicate by sending signalling molecules across gap junctions.[1] To investigate the molecular mechanisms underlying cell-cell interactions between cells in direct contact, cell populations must be decomposed and isolated to single-cell level. In this manner differences in phenotype and genotype within the same cell population, referred to as cellular heterogeneity, can be studied.[106] Since microfluidic technology enables spatial control of the cells, microsystems offer the unique possibility of pairing two cells next to each other.[107] Cell pairing can be realized either horizontal or vertical, and cell trapping in

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microfluidics can been conducted with several different approaches, such as with micro-wells[108], dielectrophoresis[109], surface-acoustic waves[110], encapsulation in microdroplets[111], and hydrodynamic trapping[112]. In the following section, microfluidic platforms for cell-cell interaction studies at single cell level are shortly reviewed to the best of our knowledge.

4.1 On-chip cancer biology on the single cell level

As cancer-stromal cell interaction is a critical process in tumourigenesis and is important to study for the understanding of cancer development and progression. In a work of Yoon et al[113], a platform was developed for cell-trapping and pairing by hydrodynamic capture schemes in a dual stream. Cell-pairing ratio and cell-cell interaction time could be precisely controlled with electrolytic bubble generation. With a cell-interaction assay between prostate cancer cells (PC3) and myoblast cells (C2C12) at different cell-pairing ratios, it was demonstrated how the proliferation rate of the myoblasts cells were enhanced with higher pairing ratio of cancer cells. The enhanced proliferation of C2C12 can be explained by growth factors secreted by PC3 cells, known to increase the reproduction of C2C12 cells, which in turn has an effect on tumour growth and progression.[114] To investigate angiogenic potential of tumour cells in response to cell–cell interactions at single-cell level, Tan et al. developed a peel-off cell-culture array capable of patterning and culturing cells at a high scale. The cell culture array was microfabricated by etching rectangular cavities into parylene and thereafter filling the cavities with fibronectin for cell adhesion. After peeling off the parylene template, cells were seeded onto the fibronectin array, and depending on the fibronectin feature size the tumour cells were cultured in clusters or at single-cell level. With this system, the authors investigated the impact of proliferation of human oral squamous cell carcinoma (OSCC3) and human prostate carcinoma (DU145) cells at individual level compared to cell clusters and the role in regulation of tumour progression and angiogenesis. Secretion of the angiogenic factors VEGF, bFGF and IL-8 was analysed as a measure for cell-cell interactions promotes angiogenesis. In the presented study, it was seen that VEGF secretion was up-regulated for incubated cell-clusters comparing to single-cells, which indicated that cell-cell interactions promoted enhanced proliferation and angiogenetic potential. Overall, single cell technology is an important strategy to identify cell heterogeneity within a single cancer type, however, for cell-cell interaction studies Boyden chamber-like or hydrogel based multi-compartment chips seems physiologically more relevant and *in vivo*-like.

4.2 Neurobiology & microfluidics at single cell level

As evident from the preceding chapter on neuronal on-chip biology, most of the microdevices presented result in data at the single cell resolution due to the availability of high resolution live-cell imaging and high-content biosensing strategies. For the sake of completeness, we mention one intricate approach for cellular alignment at single cell level. Qin *et al.* used a live-cell printing technique, termed “Block-Cell-Printing”, to investigate cell communication in heterotypic cell pairs.[115] The system was applied to analyse the gap junction mediated intercellular communication of individual primary rat cortical neurons. Neurons could be printed with a high precision and efficiency, and were cultured for 14 days with a continuous monitoring of morphology and neurite outgrowth. After 7 days of on-chip culturing, single and paired neurons with highly branched dendrites could be obtained on such arrays submerged in petri dishes. Also, axons were successfully printed to the substrate, which would enable to measuring electrical signals for further analyses in future experiments. Even though appealing on a technological view, the application of three-dimensional hydrogel cultures as well as two-dimensional networks of neurons on MEAAs as mentioned earlier looks more promising with respect to high-content cell analysis on the single cell level. Further, “Block-Cell-Printing” still needs to find its way into perfused microfluidic systems (on-chip integration).

4.3 Single cell microdevices for immunotherapy research

Vaccination with dendritic cells-tumour fusions is an intricate strategy for cancer immunotherapy. A conventional method for fusion of differentiated dendritic cells and tumour cells is by using electrofusion, but this method is challenging due to the high operation voltage and high probability of multiple cell fusions. Lu *et al.* have developed a microfluidic device for a precise and rapid fusion of homogenous or heterogenous cell types. [116] Cells are paired with a combination of hydrodynamic trapping and positive dielectrophoresis (pDEP) and thereafter fused by electrofusion inside the cell-traps. The function of the system was tested by capturing, pairing, and fusing cells of human lung carcinoma cell line A549 with cells from a human peripheral blood acute monocytic leukaemia cell line (THP-1). Cell fusions could be obtained with 64% efficiency. After cell-fusion, the cells could be removed from the microfluidic chip by applying negative dielectrophoresis (nDEP), a feature that that makes this device distinguishable from other microfluidic electrofusion designs. Even though the efficiency may be high, it is questionable how this technology can be used to generate millions of clinic-grade anticancer cell-based vaccines necessary for therapy. To generate a cell immunotherapy using a microdevice with a higher throughput, Han *et al*. developed a single cell high-throughput transfection tool comprising of an array of small microstructures.[117] Based on biophysical deformation, cells are temporarily perforated and take up molecules. The authors proposed this method for high-throughput transfection of hard-to-transfect cell types and demonstrate it for gene editing of immune cells. Overall, both methods present amazing techniques to generate novel and effective anticancer cell-based vaccines, however, these methods don’t seem to be applicable as microdevices for cell co-coculture, maintenance and analysis.

5. Organ-on-a-chip technology – a decade of more relevant on-chip biology?

In this section, we briefly give an overview of organ-on-a-chip technology over the last decade with focus on microdevices that employ heterotypic cell-to-cell interaction schemes (> 2 cell types). In addition, we want to highlight the current state-of-the-art on organ models

and how this interdisciplinary research field evolved. More focussed and comprehensive reviews on organ-on-a-chip systems that do not meet the criteria for cell-to-cell interaction on-chip can be found elsewhere.[118-125] As pointed out in the last sections, it is evident that *in vitro* cell models are increasing in complexity and that a third dimension is a key requirement for generation of functional microtissues and organoids of meaningful physiology *in vivo*. Cell- and organ-on-a-chip systems present a promising and enabling technology for in vitro drug screening and development of novel medicaments. As seen in Table 1, complex organ systems have been integrated in functional microdevices mimicking organ physiology such as brain, heart, intestine, kidney, lung, placenta, vasculature and even teeth. Most of these microdevices employ a combination of primary as well as cancer cell types for mimicking specific organ physiology. Recently, a clear trend is observable for the application of microdevices to interconnect more organ-on-a-chip modules to a human-on-a-chip to gain deeper insights into the complex human physiology on a completely different scale compared to standard *in vitro* assays. Even though the concept of human-on-a-chip was already introduced by Albert Li (integrated discrete multiple organ culture - IdMOC[126]) and Michael Shuler (micro cell culture analogue - µCCA[127]) in 2004, over a decade of interdisciplinary research was necessary to refine the cell biology to enable integration, to create sophisticated microfluidic biochips as technical aid, and finally bring those two components together to recreate more than just a single physiological function at organ-level on a single microdevice.

Initially, the concept of organ culture interplay was introduced by Albert Li and co-workers, referred to as Integrated discrete Multiple Organ Culture (IdMOC) systems and were based on a "wells within wells" approach.[104] The rationale of IdMOC is the dynamic interplay of multiple cell types cultured in a single well while allowing biochemical communication via the supernatant. To allow more control over the cellular microenvironment and scaling down of the cell cultures, µCCAs advanced IdMOC by combining multiple cell culture compartments with a microengineered fluidic channel network that allows for precise control over the cellular microenvironment with respect to pharmacology. After a decade of refinement, the µCCAs nowadays enable multi-organ toxicity testing in a four-organ system under continuous flow conditions in serum-for pump-less long-term cell maintenance.[128] As a further three-dimensional approach, a reconfigurable microfluidic hanging drop system has been established for multiplexed fabrication and analysis of multi-cell organoids.[129] This intricate system comprising of PDMS enables high degree of flexibility with respect to organoid size distributions and organ arrangement and interconnection. Overall, an open microfluidic network is combined with hanging drop microarray allowing for optimal gas exchange during cell culture handling while tuning tissue size and maintaining tissue functionality and integrity. Tissue can be formed as hanging drop cultures in static fashion where important parameters, such as spheroid diameter can be controlled as well as monitored in a time-resolved manner. For on-chip experiments multiple organoid chambers are interconnected and bioassays including drug administration and pharmacology (e.g. bioactivation of prodrugs). Currently, this approach was upgraded by integration of peristaltic micropumps[130] and electrochemical biosensors (amperometry[131] and electric impedance[132]) for automated and inline multi-parametric cell analysis. This device constitutes a promising example of organ-on-a-chip combined with lab-on-a-chip concepts to automate the majority of steps of cell culture procedures, including biomass/tissue generation, culture maintenance as well as time-resolved high-throughput analysis of multiple micro-arrayed tissue samples. In addition, a further combinatorial approach was established containing a multi-layered organ-on-a-chip system with integrated electro-chemical biosensors, both multi-electrode arrays (MEAs) as well as trans epithelial resistance electrodes, on a single chip.[133] As proof-of-principle for this approach, simultaneous measurements of cellular electrical activity and tissue barrier integrity were carried out in a two-organ system combining endothelium with beating heart cultures (human cardiomyocytes and primary human endothelial cells). These studies highlighted that simultaneous detection of dynamic alterations of vascular permeability and cardiac function on the same chip. For instance, when challenging the system with tumour necrosis factor alpha (TNF-α) and cardiac targeting drugs, barrier permeability was increased thus allowing drug-related change in cardiac beating rates. Overall, such microsystems enable to tune the interaction, thus retention times, of chemicals and drugs on a specific cell type by changes in microchannel geometry and can simulate the physiological *in vivo* situation. In contrast, organ-on-a-chip devices have also been engineered to act as a biomimicry simulating the actual mechanical movement/actuation of cell cultures on chip. For instance, the lung-on-a-chip from the Ingber group is a biomimetic microsystem capable of mechanical actuation of lung and vascular cell cultures on-chip with cyclic mechanical strain, which reconstitutes the critical functional alveolar-capillary interface of the human lung. [32, 134] As proof of principle this device has initially been tested as model for organ-level responses to bacteria, inflammatory cytokines (e.g. asthma) as well as nanomaterials introduced into the alveolar space. The most recent development and advancement of this biomimetic device is the establishment of a smoker lung-on-a-chip that can recreate the cellular microenvironment of a lung exposed to cigarette smoke with programmable smoking behaviour thus dosage scenarios. A more recent development is the blinking eye-on-a-chip of the group around Dan Huh.[135] The microdevice looks like an actual eye and simulates blinking movements of the eyelid applying strain at the air-liquid interface. Overall, organs-on-a-chip technology looks a promising strategy for creation of more relevant in vitro models applicable in a broad range of scientific fields. Further, recently more research groups start to not only work on bioengineering but also biosensing strategies on-chip to actually gain more control and insights into these complex models aside from conventional live-cell microscopy. The only downside to date is that organ-on-a-chip systems mostly comprise of a single model per functional chip, which is insufficient and laborious with respect to screening applications.

Table 1 Overview over organ-on-a-chip models including single as well as multi-organ systems.

Organ model	Cells (+/-primary)	Features	On-chip sensing	Reference
Single organ devices				
Blood-Brain Barrier	b. End3 (+) C8D1A astrocytes (-)	Mechanical modulation (shear stress), barrier integrity and permeability	Trans epithelial resistance (TEER)	[136]
	hCMEC/ D3 (+)	Mechanical modulation (shear stress),	Trans epithelial resistance (TEER)	[137]

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		inflammation, barrier integrity		
Intestine	Caco-2 (-) Lactobacillus (+)	Mechanical modulation (pneumatic actuation, shear), co-culture with intestinal microbes, tissue functionality (aminopeptidase assay), barrier integrity	-	138
	HCT-116 (-)	Open microfluidic hanging drop device, on-chip spheroid formation, real-time lactate monitoring, metabolic activity	Lactate, glucose	139, 140
Kidney	Human proximal tubule cells (+)	Mechanical modulation (shear stress), static vs. flow experiments, inflammation, barrier integrity, cellular transport (fluorescent albumin uptake, glucose transport), pharmacology	-	141
	Rat inner medullary collecting duct cells (+)	Mechanical modulation (shear), static vs. flow, Viability, Apical and basolateral markers, cytoskeletal morphology, drug screening	-	142
	Human proximal tubular epithelial cells (+)	Modeling of renal drug clearance and drug-induced nephrotoxicity	-	143
Lung	HPMEC (+) A549 (-)	Mechanical modulation (pneumatic actuation), double barrier model, tissue functionality (aminopeptidase assay), barrier integrity (fluorescent albumin transport), inflammation, nano-toxicology (NPs)	-	
	H441 (-) HPMEC (+)	Mechanical modulation (pneumatic actuation), double barrier model, barrier integrity and permeability, oxygen transport	-	134
Tooth innervation	Embryonic trigeminal ganglia (+) Molars (+) Incisors (+)	Two-compartment microfluidic, neural growth	-	144
Eye	human corneal epithelial cells (HCECs)	Bioengineered 3D tissue, mechanical actuation (blinking)	-	135
Heart	Human iPS-derived cardiac cells (+)	Open microfluidic hanging drop device, impedance biosensor on-chip, beating analysis	Impedance	145
	Human iPS-derived Cardiomyocytes (+) neonatal rat cardiac cells (+)	Array of hanging posts to confine cell-laden gels, pneumatic actuation system to induce homogeneous uniaxial cyclic strains		146
Liver	Rat hepatocytes (+) Sinusoidal endothelial cells (+)	Microfluidic microplate, oxygen transfer, viability, phenotypic analysis	Oxygen	147

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	Rat hepatocytes (+) Hepatic stellate cells (+)	Spheroid live-cell microfluidic microarray, mono vs. co-culture, spheroid morphology, viability, albumin and urea synthesis	-	[148]
	Rat liver cells (+) HCT-116 (-)	Open microfluidic hanging drop device, micropumps, on-chip spheroid formation, substance exposure, on-chip bio-activation loop, pro-drugs	Amperometry (lactate & glucose)[131], Impedance[149]	[129, 130] [129]
	Rat hepatocytes (+), human hepatocytes (+)	Drug screening	-	[150]
Vasculature	HDMEC (+)	No other organ models integrated in this paper	-	
Placenta	Jeg-3 (-) HUVEC (+)	Glucose transport (offline)	-	[151]
	BeWo (-) HUVEC (+)	Vitrified collagen membrane, Mechanical modulation (shear stress), Glucose transport (offline), microvilli morphology, Ca^{2+} ion channels	-	[152, 153]
	BeWo (-) HPVECs (+)	Mechanical modulation (shear stress), microvilli morphology, syncytialization, glucose transport (offline)	-	[154]
	BeWo (-) HUVEC (+) adMSC (+)	Polycarbonate membranes, hydrogel cultures, mutli-layered microchannels	-	
Multi-organ devices				
Organ model	Cells (+/-primary)	Features	On-chip sensing	Reference
Liver/vasculature	HeLa (-) HUVEC (+)	Co-planar model, control of directional medium flow	-	[155]
	Rat hepatocytes (+) RAMEC (+) BAEC (+)	3D Mechanical modulation (flow conditions), hepatocyte differentiation, urea synthesis, phenotypic markers	-	[156]
Liver/intestine/skin/kidney	HepaRG (+) Human primary hepatic stellate cells (+) Human juvenile prepuce cells (+) Human proximal tubule RPTEC/TERT-1 cells (+)	Organ interconnection (human-on-a-chip), integrated micropumps, air-liquid interface (skin), phenotypic analysis, gene expression patterns	-	[157]
Liver/skin	HepaRG (+) Human primary hepatic stellate cells (+) Human juvenile prepuce cells (+)	Organ interconnection, micropumps, fluid flow, metabolic activity, phenotypic analysis, troglitazone screening	-	[158]
Blood/blood brain barrier	MVBEC (+) Erythrocytes (+)	Cell-to-cell adhesion, parasite infection of erythrocytes	-	[159]
Liver/bone marrow/ tumour	HepG2 (-) Kasumi-I (-) HCT-116 (-)	Multi-organ chip, pharmacokinetic-	-	[160]

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		pharmacodynamic model (PK-PD),		
Intestine/liver/tumour	Caco-2 (-) HepG2 (-) U 251 (-)	Pharmacokinetic model, cytotoxicity	-	[161]
Vasculature/heart	HUVEC (+) Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs, +)	Membrane-integration, barrier integrity, cardiac beating, drug screening, barrier alterations	Trans epithelial resistance (TEER), Multi-electode arrays (MEAs)	[133]
	Human umbilical cord vascular endothelial cells (+) Human induced pluripotent stem cell-derived cardiomyocytes (hiPSCCMs, +)	Endothelialized myocardium, real time and simultaneous assessment of cell barrier function and electrical activity	Trans epithelial resistance (TEER), Multi-electode arrays (MEAs)	[162]

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6. Conclusions and future prospects

Microfluidic systems provide the technology to develop *in vivo* like environments for studying of a broad variety of medical models. In this review, we present the latest developments in microfluidic co-culture systems for cell-to-cell interaction studies, with focus on cancer research, vascular models, and neuroscience. Here, we give an overview of both 2-dimensional systems as well as more complex 3-dimensional models and the current technological development of multi-organ-on-a-chip systems. The integration of 3D matrices into microfluidics has enabled a transition from 2D culture models to 3D and multi-organ systems. For cancer biology, decades of bioengineering have resulted in highly complex three-dimensional systems that mimic a variety of different cancer types, such as breast, lung, liver or bone cancer. The main application areas are anti-cancer drug screening and fundamental research on cancer metastasis. Fundamental research aims to understand the biological processes behind cancer and tries to create relevant models by applying 3D cell culture techniques to microfluidic devices, and frequently include drug testing efforts. However, to create clinical relevance, the biggest challenge will be to establish through-put and high-content analysis within the microsystems for testing new drug candidates. An even greater challenge is the standardization of 3D models as well as the adaptation of this technology for a commercial use, thus to hospitals to provide personalized anti-cancer drug screening platforms applied for individual patients. The biggest opportunity for microfluidic technology stems from novel emerging therapeutic strategies that are still hard to analyse with conventional cell analysis techniques such as immunotherapy, cell based vaccines and cellular gene editing. In neurobiology, most applied microdevices are based on a similar design principle, but the combination of multi-disciplinary research stems lab-on-a-chip systems with cell separation microstructures, surface micropatterns that guide cell adhesion and migration, cell manipulators for trauma and regeneration studies as well as biosensors for non-invasive monitoring of cell-to-cell interactions. However, neurobiology-on-a-chip still needs to adapt from well-established two-dimensional models to more complex and relevant three-dimensional models. The challenge will be to transfer the existing knowledge to organ-on-a-chip like systems with three-dimensional architecture as well as organ-specific functions on a cellular level. The aim of vascular biochip models is fundamental research on angiogenesis as well as vasculogenesis. On the one hand, screening of biomolecules that promote the formation of vascular systems as well as regeneration thereof are interesting for research in developmental biology. On the other hand, screening of inhibitory compounds and drugs may prove value for studies in cancer biology as well as anti-cancer drug screening. Also, cell motility and movement across vascular barriers is of high interest especially for research on metastasis. Overall a variety of different hydrogels and matrices have been applied to successfully create 3-dimensional vessels, most frequently fibrin based gels. Interestingly, none of the presented systems employ on-chip analysis strategies other than standard optical microscopy. The main challenge for on-chip application lies in the formation of microvasculatures that allow for continuous perfusion. Potentially, these microstructures enable the integration of engineered anastomoses between different organ-on-a-chip models. The state-of-the-art is still flexible endothelialized PDMS channels that are easy to fabricate rather than complex 3-dimensional bioengineered microvasculatures. The emergence of organ-on-a-chip technology has recently reinvented studies on cell-to-cell interaction on a microdevice. Single organs-on-a-chip comprise of up to four different cell types (primary as well as cancer cell lines) making up the organ function within the microsystems. Some create organ function by the chip itself using mechanical stimuli by integrating actuated flexible membranes. Others rely on classical bioengineering, including self-assembly and organization of different cell types, thus cell-to-cell interaction to create actual organ function on a chip. Most recent developments interconnect multiple organs within a single chip to even recapitulate human physiology, so-called human- or body-on-a-chip systems. Overall, only a few of the presented systems can be categorized as lab-on-a-chip systems, which can only be fulfilled by the combination of complex biology with integrated biosensors, such as electric activity, TEER, impedance, oxygen, lactate, and glucose.

To conclude, microfluidics is a powerful tool and set of techniques to control and analyse cell-to-cell interactions on different levels of complexity such as single cells as well as microtissues. However, most of these efforts are still proof-of-principle rather than a fully developed and broadly applicable alternatives to existing models. To create relevant microfluidic systems for cell-to-cell interaction studies, the integration of primary cell models in combination with standardized microdevices is desirable. Both, primary cell models as well as plastic microchips are readily available for research purposes with a variety of companies are readily commercializing biochips, organ-on-a-chips, in vitro cell models as well as a magnitude of biosensors. Future efforts may lie more in the selection and combination of standardized modules and model and the application thereof within microfluidic chips to generate more human-like biology.

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Notes and references

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‡ Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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