Lab on a Chip

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Powering Ex Vivo Tissue Models in Microfluidic Systems

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This Frontiers review analyzes the rapidly growing microfluidic strategies that have been employed in attempts to create physiologically relevant 'organ-on-chip' models using primary tissue removed from a body (human or animal). Tissue harvested immediately from an organism, and cultured under artificial conditions is referred to as ex vivo tissue. The use of primary (organotypic) tissue offers unique benefits over traditional cell culture experiments, and microfluidic technology can be used to further exploit these advantages. Defining the utility of particular models, determining necessary constituents for acceptable modeling of in vivo physiology, and describing the role of microfluidic systems in tissue modeling processes is paramount to the future of organotypic models ex vivo. Virtually all tissues within the body are characterized by a large diversity of cellular composition, morphology, and blood supply (e.g., nutrient needs including oxygen). Microfluidic technology can provide a means to help maintain tissue in more physiologically relevant environments, for tissue relevant time-frames (e.g., matching the natural rates of cell turnover), and at in vivo oxygen tensions that can be controlled within modern microfluidic culture systems. Models for ex vivo tissues continue to emerge and grow in efficacy as mimics of in vivo physiology. This review addresses developments in microfluidic devices for the study of tissues ex vivo that can serve as an important bridge to translational value.

Introduction

Much biomedical research relies upon individualized cells in petri dishes (in vitro) or whole animals (in vivo) to address complex questions of health and disease.[1] In between these two extremes is a need to represent complex organs in vitro to enable more accurate understanding of the biological basis of disease as well as improving accuracy of drug screening. The varied needs of different cells in an organ complicate the development of a system to accurately reflect the function of that organ outside of the animal (ex vivo). The pursuit of more physiologically relevant ex vivo models drives the development of organotypic tissue approaches.[2] Blood flow is fundamental to all organs in vivo and microfluidics can provide an ex vivo source of fluid flow. Organotypic tissue models present difficulties, principally modulating appropriate local oxygen tensions, and using culture media compositions that are efficacious for a chosen organ system. While some of the challenges inherent in tissue culture are similar in nature to the challenges associated with dissociated cells in culture, they have added challenges related to the thickness and cellular heterogeneity of the specimens. Microfluidic technology offers a promising avenue for addressing many of the challenges related to numerous types of ex vivo approaches, from dissociated cells to more complicated organotypic systems.

Microfluidic approaches to media circulation address a number of issues with static organotypic tissue culture systems, and have been used to maintain numerous types of tissues, including, liver, intestine, retina, artery, lymphoid, tumor xenografts, and testis.[3-10] Current technologies are evolving to address the heterogeneous, complex nature of mammalian tissues and provide more consistent and useful results. Nonetheless, microfluidic organ-on-chip systems must be assessed for tissue health using multiple endpoint measurements and validated for ex vivo function in the context of in vivo physiological functions.

Although tissue models cannot replace the high throughput potential of cell line studies, nor the importance of tests in live animals and people, organotypic models derived from organs in vivo can provide a critical bridge between the two levels. Tissue-based models have a strong potential to recapitulate complex physiological mechanisms that are missing from models built from one cell type at a time. This quality makes tissue models, and their developing microfluidic components, an important asset for research and development in the biomedical research enterprise. It is particularly notable in the final steps of therapeutic drug design, discovery, and safety analyses on the preclinical side to personalized medicine on the clinical side. Microfluidic technologies are a critical addition for tissue models to unlock this potential.

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Cell and tissue models used with microfluidics

Standard culture systems of the 20th century required that media be changed manually and regularly every 1-7 days, depending on the density and cell types being cultured.[11] The advent of microfluidic technology in the late 20th century, and explosion in popularity in the early 21st century removes this need.[12] What gets placed in vitro can vary greatly, ranging from dissociated cells (primary or immortalized), dissociated cells reconstructed in a device to form biological units (e.g., monolayers), organoids derived from stem cells, or tissue/organotypic explants or slices from explants. All of these in vitro model systems offer advantages and limitations. When comparing models comprised of cells to organotypic tissues, one principal advantage that sets tissue cultures apart is the diversity of cell types (depending on the target tissue) and biological structures that hold the tissue together (e.g., extracellular matrix) that are often not accounted for in dissociated cell cultures (e.g., glial cells when studying neurons). These factors enable organotypic tissue models to provide potential access to the cell-cell communication and mechanical signalling experienced in vivo.[13]

The drive towards the developing "organs-on-a-chip" has typically involved the culture of cell lines or stem cells, but the improved cellular diversity of organotypic tissue makes it attractive for certain applications and/or for bridging the in vitro/in vivo gap. This relevance is exemplified by examining intestine on-chip models. The simplest intestine on-chip systems, regardless of the microfluidic flow paradigms, involve culturing of Caco-2 epithelial progenitor cells, and allowing them to proliferate to mimic intestinal epithelium. These systems are sufficient to recapitulate the intestinal mucosa, a dense network of epithelial cell types constantly proliferating, dying off, and producing secretory factors.[14-16] These models do not recreate the full cellular diversity of the in vivo intestinal mucosa. Intestinal organoids on a chip add a layer of diversity, better recapitulation of epithelial structure, and can include the formation of intestinal villi and crypts, something that Caco-2 systems only partially recapitulate.[15] They still lack the diversity of cell types of the intestinal wall in vivo. Organoids lack representation of the enteric immune system (e.g. lymphocytes, dendritic cells and macrophages) and the vast enteric nervous system (e.g. neurons and glia). Communication between these neural-immune components is known to impact gut function in healthy and diseased states.[17] Models that maintain cellular diversity have been achieved in static culture with varied temporal control, ranging from 24hr to 6 days ex vivo.[18, 19] When incorporation of organotypic tissues into microfluidic systems has been accomplished, tissue health analyses have been limited and validation of epithelial cell turnover incomplete.[4, 20]

Advantages and applications of microfluidic technology to ex vivo tissue culture

Improve Tissue Viability and Longevity

The use of microfluidic technology to maintain mammalian tissue ex vivo has been shown to improve tissue viability when compared to traditional static systems that do not incorporate continuous media exchange, particularly when long-term culture is necessary.[21, 22] Maintaining viable tissue is particularly challenging when using organotypic tissues with a thickness greater than 400μm.[19] In static cultures, tissue thicker than approximately 400μm prevents adequate diffusion of oxygen and nutrients throughout the entirety of the tissue, and cytotoxic cellular waste products can rapidly build up within the media.[19] This results in a direct correlation between tissue thickness and overall health in static culture.[23] Inadequate nutrient diffusion into the core of the tissue establishes limitations on the variety of tissue that can be effectively cultured and the length of time the tissue will remain healthy and viable ex vivo. Numerous non-microfluidic based techniques have been established to improve nutrient exchange to cells and tissues.[24] Culture dishes can be continuously shaken, rotated, or stirred to refresh the media within the diffusive boundary layer at the biological surface.[24] These techniques are useful for prolonging the viability of cultured tissue; however, these techniques result in microenvironments that are relatively under characterized and uncontrolled.[25-27] A further complication is that the rates of diffusion and uptake of essential solutes vary based upon the preparation and the type of tissue being cultured.[28, 29]

A simplified mathematical model was developed to estimate the limits of oxygen diffusion through spherical tumor tissue explants in static culture.[3] The transport of oxygen within tissue can generally be modeled using Fick's second law:

$$\frac{\partial C}{\partial t} = D\nabla^2 C - q \quad (1)$$

where C represents the concentration of oxygen at time t and distance x; D is the diffusivity coefficient of oxygen; and the term q represents the volumetric consumption rate of oxygen by the tissue.[3] Using this equation, two partial differential equations were derived to model the concentration of oxygen within the tissue and the surrounding culture media. By assuming a constant rate of oxygen consumption, infinite media surrounding a spherical explant, as well as a steady state system; the paired equations were solved, and a conservative estimate of the maximum depth of oxygen penetration (R_c) and critical spherical tissue diameter ($2R_c$) were determined as follows:[3]

$$2R_C = 2\sqrt{\frac{6D_T D_M C_{max}}{\rho Q(2D_T + D_M)}} \quad (2)$$

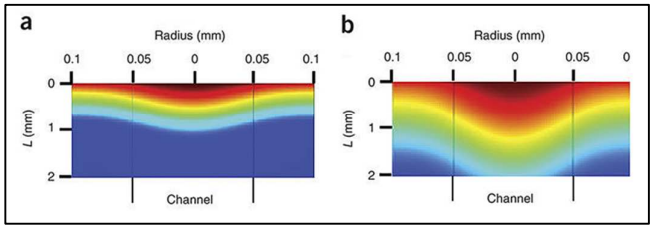
Here, D_T and D_M are the diffusion coefficients of oxygen within tissue and culture medium, respectively; C_{max} is the maximum possible concentration of oxygen in the media; and ρQ (cell density multiplied by average cellular oxygen consumption) is an estimate of oxygen consumption by the tissue. For the

parameters associated with tumorous tissue in static culture, the critical diameter was calculated to be 424μm.[3] The theoretical critical diffusive diameter will vary depending on the shape and type of tissue being cultured.

Based on experimental evidence, diffusive limitations are mitigated in tissue that is maintained no thicker than 250μm with a vibrating microtome.[18, 30] These organotypic slice models maintain their respective tissues in physiologically relevant environments for limited time frames (e.g., up to 6 days for murine intestine) and for many tissues, the 250μm limit works reasonably for maintaining sufficient three-dimensional physical microenvironment.[18] However, due to the limited thickness of the slices, diffusion of natural chemicals and mechanical cues in differential physiological compartments that exist within tissues in vivo can be disrupted ex vivo. Gradients formed by oriented diffusion of critical signaling molecules guide cellular movement or growth and differentiation; and disruption of these gradients can alter cell interactions and tissue physiology.[27, 31, 32] If the tissue of interest contains spatial boundaries, limited thicknesses may make it difficult to obtain slices with a full representation of cellular diversity. For example, mature ovarian follicles (from mice) are difficult to fit within 250μm thick slices.[33] Microfluidic approaches automatically offer a potential method for combatting waste buildup, and nutrient deficiencies that might otherwise be problematic for experimental needs at longer time points.[34]

The continuous perfusion of media across the surface(s) of an explant increases the efficiency of nutrient and waste transport to and from the tissue, respectively.[15, 35-38] This improvement in efficiency is equivalent to an increase in the media diffusion coefficient term (D_M) in the mathematical expression shown above in equation 2.[3, 39] A larger media diffusion coefficient results in an increase in the maximum steady state depth of oxygen penetration (R_c) into the tissue. Finite element analysis was performed using FEMLAB 2.2 software to simulate oxygenated media perfusion through 100μm diameter circular channels at low (490 μms^{-1}) and high (1.35mms^{-1}) velocity.[40] The resulting concentration profiles of oxygen diffusing into the surrounding tissue compartment are shown in Fig 1.

Fig. 1 Simulations of the steady state oxygen concentration (μM) profiles within a tissue compartment surrounding a 100μm diameter channel under different flow conditions. The flow velocities of media are 490 μms^{-1} (panel a) and 1.35mms^{-1} (panel b). The depth of oxygen penetration (mm) is increased at higher flow velocities. Adapted with permissions from Macmillan Publishers Ltd: Nature Protocols (ref. 40), 2008.



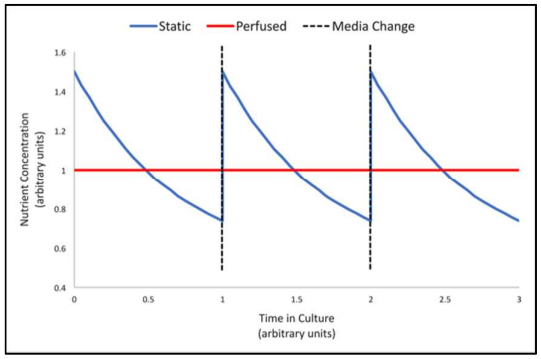


Fig. 2 Stylized representation of the varying nutrient concentration available to tissue or cells in static culture, compared to the constant media composition achievable through perfusion.

Controlled Nutrient Delivery

Static culture systems use relatively large volumes of nutrient-rich media to keep the tissue alive. The culture media must be removed and replenished on a periodic basis to avoid the deleterious effects of insufficient nutrients and the buildup of cytotoxic metabolic waste products in the culture dish. This may result in inconsistent delivery of nutrients and removal of waste to the tissue.[34] The rate of delivery in vivo of nutrients, oxygen, and other soluble factors to cells is variable, and dependent upon a variety of environmental and physiological factors, such as stress, exercise, and diet.[41, 42], The rate of nutrient delivery fluctuates following the ingestion of meals.[43] Microfluidic perfusion offers the ability to control the rate of nutrient delivery and create a defined microenvironment.[25] The desired rate of delivery of soluble factors may be periodic, as in the case of modeling reproductive hormonal regulation, or constant, such as delivery of nutrients to cells.[44] For the latter cases, microfluidic technologies provide continuous media perfusion, ideally leading to a more consistent media composition, and thereby a more consistent delivery of nutrients and removal of waste from the tissue (Fig 2).[22, 34]

A recent example of the potential of microfluidic systems to enable analyses of a complex tissue construct was the demonstration of a model of human reproductive function.[21] A combination of murine and human reproductive tissues were maintained ex vivo for 28 days, and effectively modeled the human menstrual cycle. The system consists of five culture chambers connected by microfluidic channels and electromagnetically actuated micro-pumps to drive media flow and physiological hormones throughout connected chambers (Fig. 3). The microfluidic platform enabled the co-culture of ovary, fallopian tube, uterus, cervix and liver explant tissue in series, and allowed for hormonal and cellular communication to occur between the tissue chambers. Explant tissue from each representative organ was also cultured in parallel using conventional static culture techniques, with 50% media replacement every 24hours. The authors noted a significant improvement in a number of assays linked to tissue health and in vivo physiological function when compared to the conventional static culture of tissue. These include increased ovarian follicle steroid hormone secretion, an extended period of ciliary beating in fallopian tube explants, and an indicator of proliferative competent cells (higher Ki67 expression) in uterine endometrium. This model of human reproductive function ex vivo provides an example of the strong potential of

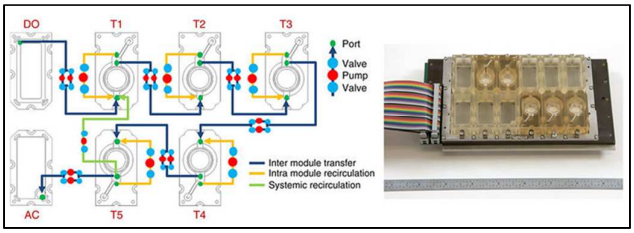


Fig. 3 Schematic representation and image of the five-tissue microfluidic culturing platform. The tissue modules (T1-T5) are fed fresh media from the donor module (DO) via electromagnetic micro-pumps. The tissue modules are connected fluidically to enable tissue-to-tissue communication. Adapted with permission from ref. 21, licensed under CC BY.

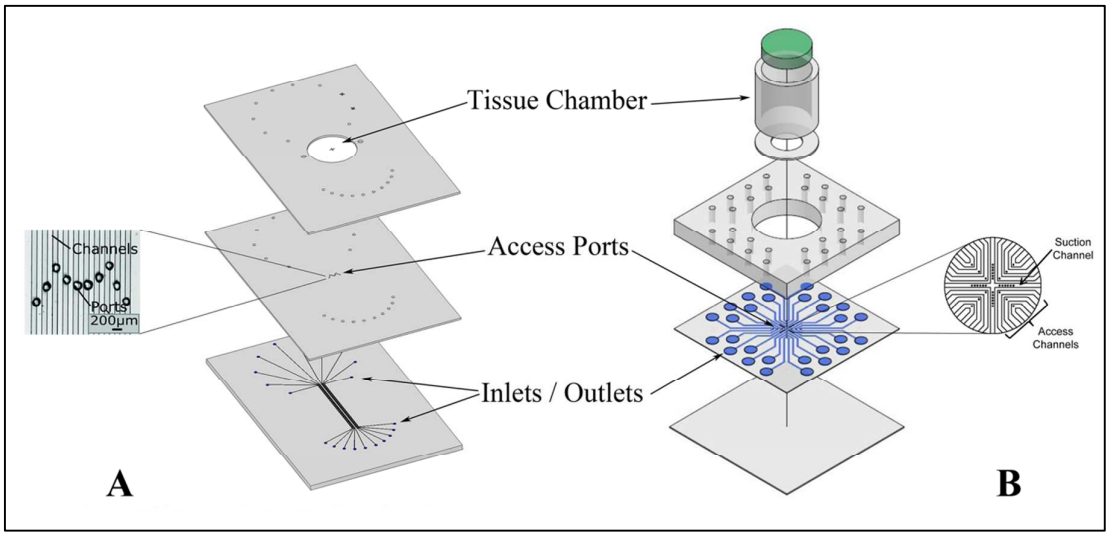
microfluidics to be used to increase culture times and viability of complex tissue and organ components.

Spatially-Controlled Reagent Delivery

Microfluidic techniques have been used to study local cellular signaling within ex vivo tissue cultures. In a traditional static culture, drugs and reagents can be introduced in a global media change or perhaps with the spatial precision of a local pipette application or even a slow release source place in the dish. Microfluidic flow has the potential to provide spatially-controlled reagent administration and spatially-resolved sampling of tissue responses, making it easier to study local cellular mechanisms and responses. Development of these techniques can be particularly useful in understanding the physiology of tissues that are structured into discrete cellular units, defined by spatial boundaries. For example, lymph nodes are organized into distinct regions, with small B-cell zones in the periphery, surrounding larger, centralized T-cell zones; and infections, vaccinations and drug delivery are believed to elicit a differential immune response based on regional stimulation.[8, 45] To investigate response differences in lymph nodes with regional specificity, a microfluidic platform was recently developed to stimulate and monitor discrete zones of live lymphoid tissue.[8] Murine lymph nodes were removed and sliced on a vibrating microtome to a thickness of 300µm. Each slice was cultured in a multi-layered PDMS device with 80µm ports spaced underneath the tissue slice (Fig 4A). A fluorescently-labelled mock therapeutic glucose conjugated to bovine serum albumin was driven through microfluidic channels aligned to the ports, and glucose-facilitated uptake was monitored in real time. The small ports restricted the uptake of the fluorescent molecule to a 200-300µm region of tissue, providing sufficient resolution to specifically target lymph node regions in future studies.

Microfluidic spatial control over reagent delivery has been used to observe microglia migration in live murine retinas.[5] Excised retinas cultured in a tissue chamber were gently suctioned onto a thin PDMS layer containing molded microfluidic channels and regularly spaced 100 µm access ports (Fig 4B). Small amounts of lipopolysaccharide, an inflammatory component of bacterial cell walls, delivered selectively through 100µm ports was shown to induce microglia that were in the retinal slices to migrate to the microfluidic access points. The local nature of the stimulation and the response demonstrates the ability to create microenvironments in discrete regions of ex vivo tissue, while simultaneously allowing for on-chip microscope detection of cell migration. The use of microfluidic ports could allow research teams to vary conditions (i.e. drug dosage) over spatially defined regions of a single explant, reducing the amount of tissue needed for experimental analysis.[46, 47] Development of this technique would be particularly useful for situations in which tissue samples are scarce, for example testing drugs on human biopsies. The utility of such techniques would depend on the rapidness of the tissue response and the immediacy of detection of the analyte of interest. For example, it would be reasonable to stimulate norepinephrine release across adrenal tissue in a spatially defined manner, and measure the biochemical concentration in real-time using an electrode array.[47] However, it might not be reasonable to compare endpoint cellular viability using this experimental design.

Fig. 4 Device designs for spatially resolved reagent delivery to lymph node (Panel A) and retinal tissue (B). Panel A adapted from ref. 8 with permission from the Royal Society of Chemistry. Panel B adapted from ref. 5 with permission of Springer.



High Throughput Assays

Static cell cultures win the day for high throughput. Robotic pipetting systems and uniformity of cells that can be handled in large batches provide expediency. The independence of sample handling provides shelter from cross contamination and the power of endless replicates. The win, however, comes at the price of cellular complexity and model validity. This limits the translation of cell culture into an accurate use for drug screening in the pharmaceutical and diagnostic industries. For the single cell based approaches, microfluidics may not provide a meaningful advantage because for each of 1 million wells with cells you would need 2 lines of fluid flow (in and out) and they might not provide appreciably more information. Additional value is provided when you consider cell-based systems that are multi-cellular.

Ex vivo organotypic tissue will not replace cell culture for mass screening of toxicity and drug interactions. The smaller number of matching tissue samples that can be processed comes with the opportunity to gather a richer pipeline of data provided by more complex cellular interactions. Microfluidic systems augment the value of organotypic tissue because it provides for temporal resolution of physiological changes. In this respect, the capability to culture multiple tissue samples in parallel is still important. Implicit in this complexity is a large number of biological variables; many that vary from tissue source to tissue source. With a sufficient number of replicates, otherwise subtle biological differences can be uncovered.

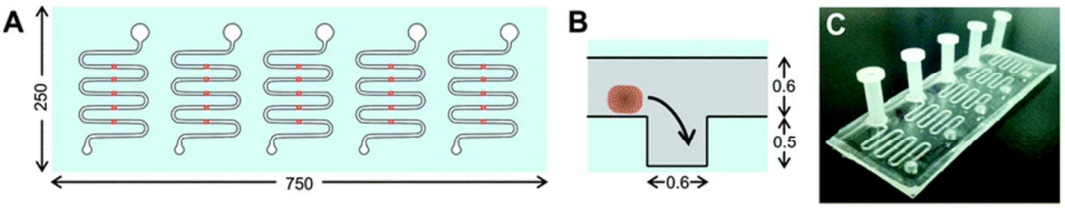
The supply of healthy (or diseased) tissue is a limiting factor for the number of replicate samples that can be cultured for any given experiment. Acquiring viable excised human tissue often requires proximity and access to major healthcare centers and for tissue only from 1 patient at a time. For animal tissues the labor-intensive process required to culture may limit the number of animals in any one preparation period. To maximize data efficiency microfluidic techniques should be applied to multiple simultaneous experiments on separate small tissue sections, thereby multiplexing the assay, maximizing the number of replicates given a finite tissue supply, and minimizing the labor required.[48]

Microfluidic multiplexing strategies have been developed for cultures involving human tumor tissue. A recently developed culture system allowed for the parallel culture and chemosensitivity testing of multiple microdissected tumor biopsies (approximately 400μm diameter).[3] The microfluidic platform included 5 separate fluidic channels. Rectangular wells on the bottom of each channel were used to capture and culture individual biopsy tissue sections (Fig 5). The authors estimated that 300 microdissected tissue sections could be prepared and loaded onto the microfluidic platform in less than 6 hours. Validation of the viability of the cells in tumor explants was achieved by labeling dead cells with the DNA binding dye propidium iodide, and labeling apoptotic cells with annexin V and 7AAD dyes (PE Annexin V Apoptosis Detection Kit I, BD Biosciences). On-chip microscopy allowed for real-time tracking of cell death within the tissue. The multiplexed capabilities of the platform make it appealing for personalized medicine and for clinical screening of chemotherapeutics.

The standardization of loading tissues into microfluidic devices can also improve the throughput of ex vivo tissue culture. A multi organ chip (MOC) was developed to simultaneously culture, in series, human juvenile prepuce skin and single hair follicular units obtained from human skin samples.[22] The MOC contains two tissue compartments linked by microfluidic channels, and connected to an on-chip micropump that simulates peristaltic blood flow. A key advantage of the MOC design is that standard Transwell® inserts were loaded with tissue samples, and then rapidly inserted into the microfluidic chip. The ease of use and flexibility associated with the operation of this culture model system expedites the culturing of multiple tissue replicates simultaneously, and may facilitate wider utility. Standardization of inserts for microfluidic culture systems could also make automated readout systems more accessible. Plate readers for conventional 96-wellplate cultures allow for convenient multiplexed detection of a variety of assays that could readily mesh “lab on a chip” with microfluidic culture systems. Due to the decreased diffusional distances associated with microfluidic culture, the results of chemical assays can be obtained more rapidly than in a conventional culture.[49] For instance, a biomarker detection device for human cancerous tissue slices decreased immunohistochemical development from 120 minutes in conventional system to 3.5 minutes in a microfluidic device.[49]

Present Considerations and Future Challenges

Fig. 5 High-throughput microfluidic platform for the analysis of microdissected tumor biopsy tissue. The platform consists of five microfluidic channels (panel a), each lined with five rectangular biopsy traps (panel b). This enables simultaneous experimentation on 25 primary tumor sections under 5 different conditions. Reproduced from Ref. 3 with permission from The Royal Society of Chemistry.



Choosing the Right Material

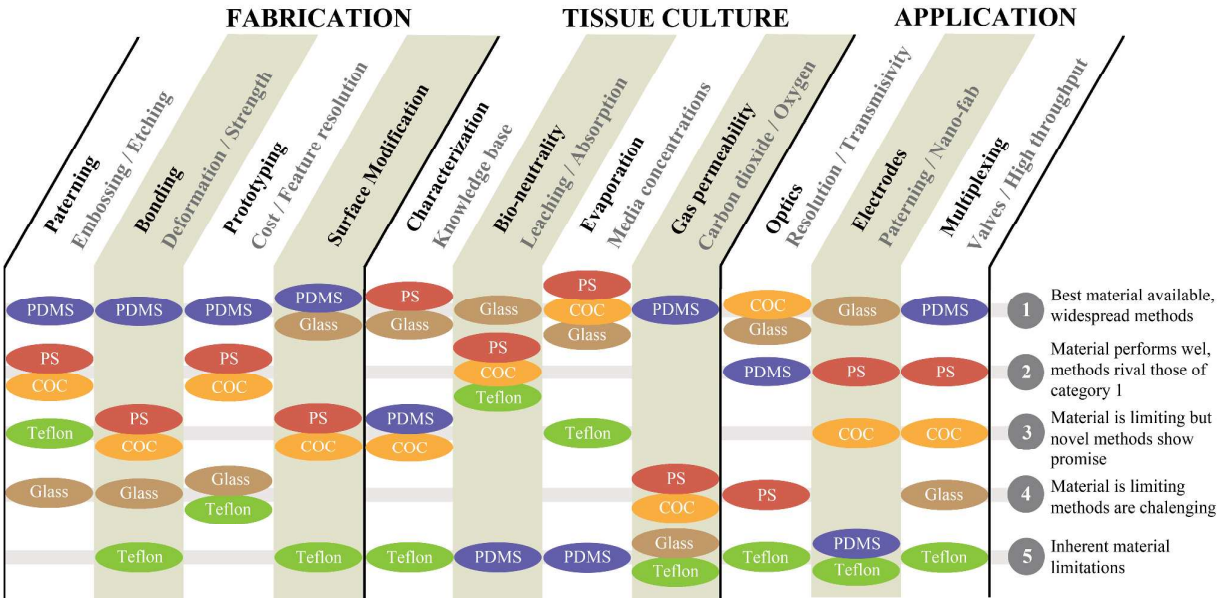
Choosing a proper material for microfluidic device fabrication is critical when designing for live tissue. Ideally, fabrication should be easy and cost effective, and the chip material should be biocompatible with the chosen tissue and support the most physiologically relevant environment possible. The contributions of material properties upon live tissue physiology and possibly even media composition are hard to define and quantify, but understanding these properties is crucial to obtain repeatable and translatable results.

Novel fabrication techniques, from 3D printing to laser ablation, have resulted in a significant increase in the material options available for microfluidic systems.[50] This includes devices fabricated out of thermoplastics such as cyclic olefin copolymer, polycarbonate, and polystyrene, collagen based three dimensional-models, and glass devices, among others.[16, 51-53] The most commonly used material to fabricate microfluidic devices in academic laboratories is polydimethylsiloxane (PDMS).[54] PDMS is an attractive material due to the low overhead cost involved, the ease of fabrication, and the elastomeric properties of PDMS that simplify chip bonding and "world-to-chip" interfaces. The emergence of soft lithography techniques in conjunction with PDMS molding is considered one of the most important developments to help launch the microfluidic revolution.[12] However, there is evidence that PDMS may not be suitable for certain in vitro culture applications.[55, 56]

PDMS is widely considered a biocompatible plastic; however, biocompatibility can mean a wide range of things related to cell health, functionality, and absorption of media components. Past studies have shown that there are no differences in cell death rates for immortalized cell lines cultured on PDMS versus polystyrene (a common laboratory plastic). Other studies, however, have observed changes in gene expression and differentiation of cells cultured on PDMS.[56-58] When attempting to model complex biological processes, physiological measures other than cell death may be needed. A recent study investigated cellular adhesion and migratory properties of cells on PDMS compared to a glass and conventional lab plastic substrate (Thermanox®).[59] Chick embryonic brain and liver explants cultured on the PDMS substrate exhibited less cell migration and increased cell adhesion compared to the other substrates.[59] Such considerations may significantly impact design characteristics of cell or tissue culture experiments to investigate cell migration or membrane dynamics.

Another important consideration in chip design is the diffusivity of the material to molecules within the culture solution. One significant advantage of using PDMS for cell and tissue culture systems is that the material readily allows for diffusion of gas, thus simplifying the process of oxygenating and buffering the culture media.[34] On the other hand, the permeability of PDMS to water vapor can lead to evaporation of small amounts of media out of the system, potentially leading to altered concentrations of media solutes.[55] In some cases where regulation of oxygen tensions is desired the porosity of PDMS may create problems in the other direction. PDMS is a porous hydrophobic material that has been shown to rapidly absorb small molecules and adsorb proteins from contacting fluids.[60, 61] This can pose a major issue when culturing tissue on a microfluidic platform as alterations in biomolecule concentrations in media/tissue due to adherence to the devices material could alter the tissue physiology or response to perturbations such as therapeutic screenings. Plasma and UV treatment of PDMS has been shown to decrease the hydrophobicity of the material, but these techniques may not be appropriate for long-term tissue culture, as the plastic may return to the original hydrophobic state one week after treatment.[34]

Fig. 6 Qualitative comparison of the strengths and weaknesses of common materials used to fabricate microfluidic culture systems. Each color denotes a particular material. Reproduced from Ref. 63 with permission from The Royal Society of Chemistry.



Cyclic olefin copolymer (COC) has emerged as a promising alternative to PDMS for the fabrication of microfluidic culture systems. COC has a high resistance to chemicals, low permeability to air, and minimal adsorption of small hydrophobic molecules.[58] The optical properties of COC give it low autofluorescence and high transparency.[62] Difficulties and expenses associated with COC chip fabrication have discouraged widespread use of COC in the academic laboratory.[62] However, novel and inexpensive techniques to prepare master molds for COC chips have recently been reported.[62] A combination of micromilling and hot embossing was used to create a COC device for the culture of oocytes. A mid-level micromilling machine, Protomat S63 (LPKF Laser & Electronics, Garbsen, Germany), was capable of producing 50μm features. Nonetheless, it is important to note that PDMS is advantageous for many applications. The ease of fabrication, the ability to incorporate on-chip valves and micropumps, and the precision and resolution of PDMS chips are currently unmatched in the academic laboratory.[63] Figure 6 provides a qualitative assessment of the advantages and disadvantages of a selection of materials that can be used to fabricate tissue culture microfluidic systems.[63]

As the complexity of the biological systems being modeled increases, so does the importance of material choice in microfluidic design. For instance, a microfluidic model of hormonal regulation may be problematic for physiological relevance if a relevant proportion of steroids are being absorbed out of solution and into the microfluidic device. Recent requests for applications from the National Institutes of Health (RFA's) have expressed reservation about the use of PDMS in microphysiological systems (e.g., RFA-TR-16-017). The material used for microfluidic chip fabrication should be chosen with care based upon the type of tissues cultured and the intended experimental results. A full analysis of the material's impact on the physiological function of the cultured tissue and cells should be taken into account.

Design Considerations

The principles developed for the design of microfluidic cell-line culture platforms can be translated to tissue culture applications. Similar methods can be used for channel design, chip-to-world connections, and the fabrication of tissue culture microfluidic platforms. Quality reviews are available that detail these techniques.[25, 64] However, the size and three dimensionality of tissue samples creates unique design challenges associated with loading samples. In cell-line derived cultures, cells can be loaded into a microfluidic device by injecting a cell suspension through a perfusion inlet.[25] This method allows the fluidic system to be primed prior to loading the cells. A similar technique has been used to load tissue samples, but only when the tissue sections are smaller than the perfusion channel.[3] For larger tissue slices and explants, this is not a viable strategy. One alternative is to load tissue sections onto an insert lined with a semi-porous membrane (for example a modified Transwell® insert).[22] The insert can then be integrated into the microfluidic chip via a threaded port to form a fluid-tight seal between the insert and the microfluidic chip.[65]

Special considerations are required when attempting to model the function of barrier tissues. To expose two sides of the tissue sample to two different environments, the tissue must form a seal between two distinct fluids. This has been accomplished through uncomplicated techniques, such as using petroleum jelly to form a seal around the tissue, or suturing intestinal tissue to a fluidic port.[4, 20] Alternatively, sub-atmospheric pressure has been applied through microchannels to conform outer surfaces of resected arteries to perfusion channel, and thereby selectively perfuse the lumen of the vessel.[9] In certain applications, for example when targeting spatially-defined regions of a tissue, it is particularly important that tissue samples remain stationary during culture. Negative pressure, applied by a weak vacuum through micro-suction channels, has been used to hold excised retinas in place without damaging the tissue.[5] Alternatively, collagen overlays work to hold tissue in place in other systems.[18, 30]

There are considerations that are selectively important when designing the geometry of a tissue perfusion chamber. Cell-lines are often cultured in straight microfluidic channels; in which each cell positioned transverse to the channel experiences the same laminar flow and mass transport.[64] For tissues in culture this is often not possible, and the perfusion chamber must be designed to fit the three-dimensional shape of the tissue. A small microfluidic channel entering a large perfusion chamber may result in unequal flow. The mass transport at the outer edges of a large circular chamber can be significantly less than the transport near the center of the chamber. This could affect the health and function of the tissue. Computational flow dynamics can be used to predict the extent of mass transport deviations.

Choosing the Right Media

From the advent of tissue culture techniques to the present, determining appropriate media for mammalian tissue culture always has been a challenge. It is important to use culture media that is optimized for amino acids, fatty acids, sugars, ions, cofactors, and vitamins to keep tissues viable.[66] Different cell types can have different nutritional requirements, and that makes finding a universal media more difficult.[67] It particularly complicates the choice of media for the culture of heterogeneous cell populations. For this reason, animal serum is often added to basic tissue culture media. Serum can be derived from a variety of animal sources, but most often is taken from bovine fetuses (FBS).[67] FBS contains a host of undefined hormones, growth factors, and proteins that can help many cell and tissue types thrive in vitro.[68]

The use of serum in cell and tissue culture systems is controversial.[68] The properties of commercial serum are inconsistent from animal to animal, and from lot to lot, and can influence the phenotype of cells maintained in the media.[68] Furthermore, the exact composition of serum is typically undefined or undisclosed, which runs contrary to the desire to maintain control over experimental reagents.[69] Due to these shortcomings, much effort has been expended on

developing defined serum-free medias for the culture of a variety of different cell and tissue types.[67] Ideally, each medium would deliver the same nutrients and small molecules that the particular tissue experiences in vivo. This is difficult to achieve when culturing multiple tissue types in series. There is potential for the development of a relatively "universal" serum free medium that can be used to culture a wide range of tissue types. To this point, neurobasal media (Gibco, Invitrogen) with B27 supplement has been used to maintain tissue viability by one group without serum for murine brain, pituitary, ovary, adrenal and gastrointestinal tissues.[18, 30, 33, 47, 70, 71] It has also been used with human intestinal tissue.[72]

Tissue Response to Shear Stress

At increasing perfusion velocities, a greater depth of oxygen diffusion can be achieved; although care must be taken to avoid high flow rates in which cell-damaging shear stress can develop.[25, 73] Within the healthy human circulatory system in vivo, endothelial cells that line the blood vessels experience shear stresses ranging from approximately 1 to 6 dyne / cm^2 in the venous system to 10-70 dyne / cm^2 in the arterial system.[74] Laminar shear stress within this physiological range has been found to promote certain cytoprotective remodeling processes in vascular endothelial cells cultured in vitro.[75] However, with the exception of certain epithelial-lined organ systems, such as the gastrointestinal tract, lung, and kidneys, non-endothelial lined tissues in vivo are only subjected to extremely low interstitial shear forces (<0.1 dyne / cm^2).[76] For tissues that do not normally experience significant shear stresses, fluidic shear stress greater than 2.5 dyne / cm^2 can influence cell permeability.[77] Therefore, tissues cultured ex vivo should be perfused at a flow rate that maximizes the efficiency of nutrient delivery while minimizing the detrimental effects of shear stress. The introduction of flow at an appropriate velocity can help prevent nutrient and oxygen deprivation within the core of cultured tissue, and enable explants with thicknesses greater than 250µm to remain viable for longer periods of time in culture. Greater detail on determining the proper perfusion rate are available.[64]

Oxygen Control

Oxygen availability in vivo varies significantly depending on the tissue of interest, and is a concern for generating physiologically relevant in vitro models. In addition, oxygen tensions can vary within single organs, such as in the intestines.[78] Many static culture systems of the past 50+ years used either ~20% (150 mm Hg) or 95% oxygen (720 mm Hg) concentration media.[79] Similar dissolved oxygen concentrations in media are observed within microfluidic culture systems, and while these systems often maintain cells alive and functional, they may provide an incomplete view of cell functions in vivo.[15] Mimicking oxygen tensions seen in vivo is important as tissue immune responses depend on them; as do cellular signaling mechanisms, cell proliferation, and health of the tissue.[80-82]

Where oxygen gradients exist across tissues such as for barrier tissues (e.g. intestine, uterus), the recreation of oxygen gradients in vitro can be challenging. In the intestines, as you move from the luminal to serosal aspects of the gut wall, the pO_2 varies between ~10 (Physiological hypoxia) and ~35 mm Hg (~5% O2) respectively.[83] A microfluidic platform developed to investigate microbial-intestinal interactions modeled this oxygen gradient across a Caco-2 cell monolayer.[16] Oxygen was precisely measured in real-time on both sides of a cell layer via optical spot sensors (PreSens). A near anoxic environment of less than 6 mm Hg pO_2 was maintained on the apical side of the cell layer by continuously bubbling N_2 gas through the circulating media, and a near physiological pO_2 of 41 mmHg was achieved on the basolateral side by the simultaneous perfusion of oxygenated media.[16] The relevance of establishing in vivo-like oxygen environments is highlighted when trying to incorporate bacteria into tissue models. Non-physiological concentrations of oxygen may kill some bacteria depending on their metabolic demands and alter the function and metabolism of facultative and obligate anaerobes alike, possibly leading to confounding results.[84] It is important to note that while near-physiological oxygen conditions were established on the two sides of the cell layer, the oxygen tension experienced by the cells would still have differed from physiological conditions.[16] In vivo, the oxygen gradient from lumen to serosa would occur over the full thickness of the tissue comprised of many cell layers thick, rather than a single cell layer.

Vascularization of Organotypic Tissue

One solution to mimic in vivo oxygen tensions would be to perfuse native vasculature within tissue, ex vivo. This has not been accomplished using primary tissues, but there is emerging research in the field of tissue engineering that demonstrates that microfluidic technology may make vascular perfusion ex vivo possible in the future.[85, 86] In a recent study immortalized fibroblasts and human dermal microvascular endothelial cells (HDMECs) were seeded into channels located underneath a media reservoir to culture cancerous spheroids.[86] The media reservoir was connected to the fibroblast and HDMEC channels by micropores 200µm in diameter. After 5 days of culture, the HDMECs sprouted and formed a microvessel network. The network of microvessels formed tight walls, and could be perfused with media selectively, potentially allowing for a more physiologically accurate perfusion of the spheroid.

It may be possible to connect an engineered microvessel network to existing vasculature within primary tissue. A microfluidic chip, similar in design and concept to the one mentioned above, was used to engineer a network of perfuseable microvessels adjacent to a culturing spheroid composed of human lung fibroblasts (HLF) and human umbilical vein endothelial cells (HUVECs).[85] After 9 days in culture, the group observed angiogenic sprouts from the engineered microvessels with connections to vessel-like structures within spheroids (Fig 7). Chip-based perfuseable networks may one day allow for more physiological perfusion of tissue ex vivo. However, further development must be done to determine how connections can be made from an engineered microvascular network to mature vasculature within tissues. Functionality of native vasculature in tissues ex vivo needs to be determined. Capillary-like structures have been maintained in brain slices for up to 14 days in culture, but

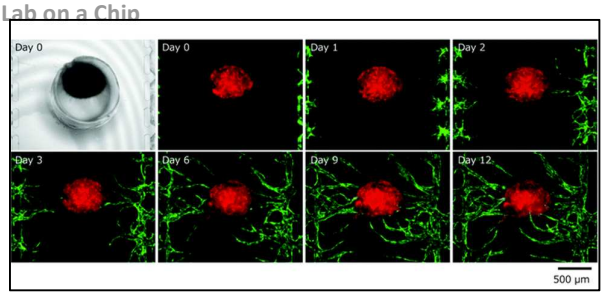


Fig. 7 Time series of micrographs demonstrating the sprouting of HUVECs cultured in adjacent microfluidic channels (green fluorescence). At day 9, engineered microvessels had anastomosed with HUVEC derived vessel-like structures within the organoid (red fluorescence). Reproduced from Ref. 85 with permission from The Royal Society of Chemistry.

the results have not indicated whether the capillary-like structures remain viable.[87]

In mammals, oxygen is carried through the body attached to hemoglobin. Hemoglobin effectively increases the solubility of oxygen within blood and improves the efficiency of oxygen exchange from the lungs to other tissues.[82] Oxygen carrier molecules, however, are absent from the media in microfluidic and conventional tissue model systems.[82] Oxygen delivery to cells is instead accomplished through simple diffusion from the media through the tissue, which is not physiologically accurate. More work is needed to develop artificial oxygen carriers that mimic the function of hemoglobin in vitro. This would be particularly useful in thicker tissue culture systems when oxygen transport is needed over multiple cell layers. Realization of such technology would allow microfluidic culture systems to better mimic in vivo oxygen transport. Other means of measuring and controlling oxygen have been developed. A recent in-depth review offers a detailed look at the strategies used and challenges in implementing oxygen control in microfluidic devices.[82]

On-Chip Tissue Validation

As microfluidic models become more common in the laboratory, developing proper standardized on-chip validation of tissue health and functionality are important for characterizing and comparing biological results. This is particularly true for microfluidic models involving the culture of human tissue ex vivo, in which tissue injury and/or suboptimal timing during processing may occur. In one study, less than 1% of total gene expression had changed in excised prostate tissue one hour after surgery.[88] However, more pronounced physiological changes have been noted post-surgery in excised human tissue, and validation strategies should account for this possibility.[89] There is no single validation method that fits all tissue types or microfluidic designs, making standardization of validation methodologies difficult. Validation strategies are needed based upon the applicable parameters for each tissue of choice. For instance, quantifying epithelial cell proliferation rates is an accepted and commonly used methodology for measuring intestinal tissue health.[18, 90, 91] This same measure may be less sensitive when looking at brain-on-a-chip models, because cell turnover is spatially and temporally more complex compared to intestinal tissue. Quantifying cell death on the other hand, in brain-on-chip models provides a more universal indicator for assessing tissue health.[92] If a system maintains the 3D structure of the tissue in question, the anatomical context may help determine if the observed chemical markers and rates are consistent with in vivo physiology.[8, 18]

Measures of tissue function need to be validated, depending on the physiological process that is being modeled. For example, if a system is designed to model and quantify transport across barrier tissues, it becomes important to verify the integrity of tight junctions that comprise the barrier. There are several ways to approach the question. One approach could be assessing tight junction viability through the quantification of tight-junction protein expression.[16] Measuring the transepithelial resistance (TEER) across a tissue barrier is another acceptable option for evaluation in real-time.[93] Another might be monitoring the passage of a labeled compound (e.g., by fluorescence or a radiolabeled tracer such as mannitol). These measures would be less useful in non-barrier tissues illustrating the need to validate tissue functions that are relevant to the experimental model.

Tissue validation should include comparisons to an appropriate baseline as well as appropriate controls. The answer to the question of 'what constitutes functional and viable tissue?' may not be simple, and depends on the tissue being studied and the context of the experiment. Simple measures of cell health that only quantify cell death and proliferation may be insufficient. These measures, by themselves, are not robust indicators of biological validity or physiological relevance, particularly when the aim of the model is to represent a complex organ system. Additionally, markers of tissue integrity are often compared to past in vitro studies. While this may have some value, in vitro markers of viability ideally should be compared to rates derived from in vivo observation. Creating controls may be as simple as including parallel microfluidic devices during studies when sufficient tissue is available but may also require more creative methods with tissue supplies are limited. The value of all ex vivo systems will be in their predictive validity for events that occur in vivo.

Conclusions

Collaboration between engineers and biologists will be key moving forward to design microfluidic devices of the future. Such devices will maintain physiologically relevant environments, while still offering cost effective and time-efficient device generation. The accuracy of models for in vivo physiology is important for designing organ-on-chip microfluidic devices with heuristic value. For commercialization, it would helpful for models to be scalable and offer high throughput results. Scalability for organotypic systems presents a challenge, but one that can be overcome with advances in device technologies, and further understanding of the physiological demands for tissue-on-chip.

Further utilization of microfluidic technologies and optimization of organ/tissue physiology ex vivo will no doubt yield more relevant tissue models than static culture systems. Controlling for the myriad variables in tissue viability, cell-cell interactions, and extracellular matrix and signaling molecules will be an important challenge. The microfluidic designs of the future will likely improve the efficacy of microfluidic models for applications such as drug development and personalized medicine.

A recent survey shows that approximately 90% of all drugs entering clinical trials are not approved.[94] Estimates for the cost of bringing a drug to market have risen to $2.6 billion dollars.[94] A significant portion of this cost is attributed to the high rate of rejection for drugs that were identified as viable candidates through high-throughput cell culture screening and in vivo animal research. Rejection is often due to unforeseen toxicity, unintended interactions, or lack of efficacy. It has become evident that the current methodologies for pre-clinical research is insufficient and do not accurately capture the complexity of heterogeneous cellular interactions that occur within the human body. Cell-line based assays and in vivo animal testing will continue to serve an essential role in drug development. Ex vivo tissue culture systems can provide supplementary techniques for intermediate test-beds between preclinical and clinical testing. Adapting microfluidic technology to tissue culture systems will make them more accurate, useful, and attractive for the testing of mechanisms and translation to the pharmaceutical industry and clinical settings.

Conflicts of interest

There are no conflicts to declare.

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References

1. F. Pampaloni, E. G. Reynaud and E. H. K. Stelzer, *Nature Reviews Molecular Cell Biology*, 2007, **8**, 839.
2. E. R. Shamir and A. J. Ewald, *Nature Reviews Molecular Cell Biology*, 2014, **15**, 647.
3. M. Astolfi, B. Péant, M. A. Lateef, N. Rousset, J. Kendall-Dupont, E. Carmona, F. Monet, F. Saad, D. Provencher, A.-M. Mes-Masson and T. Gervais, *Lab Chip*, 2015, **16**, 312-325.
4. A. Dawson, C. Dyer, J. Macfie, J. Davies, L. Karsai, J. Greenman and M. Jacobsen, *Biomicrofluidics*, 2016, **10**.
5. K. H. Dodson, F. D. Echevarria, D. Li, R. M. Sappington and J. F. Edd, *Biomedical microdevices*, 2015, **17**, 114.
6. M. Komeya, H. Kimura, H. Nakamura, T. Yokonishi, T. Sato, K. Kojima, K. Hayashi, K. Katagiri, H. Yamanaka, H. Sanjo, M. Yao, S. Kamimura, K. Inoue, N. Ogonuki, A. Ogura, T. Fujii and T. Ogawa, *Scientific reports*, 2016, **6**, 21472.
7. L. Prodanov, R. Jindal, S. S. Bale, M. Hegde, W. J. McCarty, I. Golberg, A. Bhushan, M. L. Yarmush and O. B. Usta, *Biotechnol Bioeng*, 2016, **113**, 241-246.
8. A. E. Ross, M. C. Belanger, J. F. Woodroof and R. R. Pompano, *Analyst*, 2016, DOI: 10.1039/c6an02042a.
9. S. Yasotharan, S. Pinto, J. G. Sled, S.-S. Bolz and A. Günther, *Lab on a Chip*, 2015, **15**, 2660-2669.
10. P. M. van Midwoud, G. M. M. Groothuis, M. T. Merema and E. Verpoorte, *Biotechnology and Bioengineering*, 2010, **105**, 184-194.
11. J. R. Masters and G. N. Stacey, *Nat Protoc*, 2007, **2**, 2276-2284.
12. G. M. Whitesides, *Nature*, 2006, **442**, 368-373.
13. V. van Duinen, S. J. Trietsch, J. Joore, P. Vulto and T. Hankemeier, *Current Opinion in Biotechnology*, 2015, **35**, 118-126.
14. Y. Chen, Y. Lin, K. M. Davis, Q. Wang, J. Rnjak-Kovacina, C. Li, R. R. Isberg, C. a. Kumamoto, J. Mecsas and D. L. Kaplan, *Scientific Reports*, 2015, **5**, 13708.
15. H. J. Kim, H. Li, J. J. Collins, D. E. Ingber, D. J. Beebe and R. F. Ismagilov, *Pnas*, 2015, DOI: 10.1073/pnas.1522193112, E7-E15.
16. P. Shah, J. V. Fritz, E. Glaab, M. S. Desai, K. Greenhalgh, A. Frachet, M. Niegowska, M. Estes, C. Jager, C. Seguin-Devaux, F. Zenhausern and P. Wilmes, *Nat Commun*, 2016, **7**, 11535.
17. D. O'Malley, *Am J Physiol Gastrointest Liver Physiol*, 2016, **311**, G934-G941.
18. L. A. Schwerdtfeger, E. P. Ryan and S. A. Tobet, *American journal of physiology. Gastrointestinal and liver physiology*, 2016, **310**, G240.
19. M. Li, I. A. de Graaf and G. M. Groothuis, *Expert Opin Drug Metab Toxicol*, 2016, **12**, 175-190.
20. N. Yissachar, Y. Zhou, L. Ung, N. Y. Lai, J. F. Mohan, A. Ehrlicher, D. A. Weitz, D. L. Kasper, I. M. Chiu, D. Mathis and C. Benoist, *Cell*, 2017, **168**, 1135-1148 e1112.
21. S. Xiao, J. R. Coppeta, H. B. Rogers, B. C. Isenberg, J. Zhu, S. A. Olalekan, K. E. McKinnon, D. Dokic, A. S. Rashedi, D. J. Haisenleder, S. S. Malpani, C. A. Arnold-Murray, K. Chen, M. Jiang, L. Bai, C. T. Nguyen, J. Zhang, M. M. Laronda, T. J. Hope, K. P. Maniar, M. E. Pavone, M. J. Avram, E. C. Sefton, S. Getsios, J. E. Burdette, J. J. Kim, J. T. Borenstein and T. K. Woodruff, *Nature Communications*, 2017, **8**, 14584.
22. B. Atac, I. Wagner, R. Horland, R. Lauster, U. Marx, A. G. Tonevitsky, R. P. Azar and G. Lindner, *Lab Chip*, 2013, **13**, 3555-3561.

23. Y. Guy, A. E. Rupert, M. Sandberg and S. G. Weber, *J Neurosci Methods*, 2011, **199**, 78-81.
24. H. E. Swim, *Annual Review of Microbiology*, 1959, **13**, 141-176.
25. L. Kim, Y.-C. Toh, J. Voldman and H. Yu, *Lab on a Chip*, 2007, **7**, 681-694.
26. E. Azuaje-Hualde, M. García-Hernando, J. Etxebarria-Elezgarai, M. M. De Pancorbo, F. Benito-Lopez and L. Basabe-Desmonts, *Micromachines*, 2017, **8**.
27. T. M. Keenan and A. Folch, *Lab Chip*, 2008, **8**, 34-57.
28. I. S. Longmuir and A. Bourke, *Biochemical Journal*, 1960, **76**, 225-229.
29. R. De Kanter, M. Monshouwer, A. L. Draaisma, M. H. De Jager, I. A. de Graaf, J. H. Proost, D. K. Meijer and G. M. Groothuis, *Xenobiotica*, 2004, **34**, 229-241.
30. S. a. Tobet, H. J. Walker, M. L. Seney and K. W. Yu, *Integrative and comparative biology*, 2003, **43**, 794-801.
31. J. Wu, Z. Mao, H. Tan, L. Han, T. Ren and C. Gao, *Interface Focus*, 2012, **2**, 337-355.
32. A. A. Khan, Suits, J. M. T., Kandel, R.A., Waldman, S.D., *Biotechnol Progress*, 2009, **25**, 508-515.
33. K. A. Frahm, C. M. Clay and S. A. Tobet, *Biology of Reproduction*, 2010, **83**, 66-66.
34. S. Halldorsson, E. Lucumi, R. Gómez-Sjöberg and R. M. T. Fleming, *Biosensors and Bioelectronic*, 2014, **63**, 218-231.
35. C. M. Finelli, B. S. T. Helmuth, N. D. Pentcheff and D. S. Wethey, *Coral Reefs*, 2005, **25**, 47-57.
36. D. De Beer, P. Stoodley and Z. Lewandowski, *Water Research*, 1996, **30**, 2761-2765.
37. I. J. Hidalgo, Hillgren, K.M., Grass, G.M. et al. , *Pharm Res*, 1991, **8**, 222-227.
38. M. Fröhlich, W. L. Grayson, D. Marolt, J. M. Gimble, N. Kregar-Velikonja and G. Vunjak-Novakovic, *Tissue Engineering. Part A*, 2010, **16**, 179-189.
39. C. Dusny and A. Schmid, *Environmental Microbiology*, 2015, **17**, 1839-1856.
40. M. Radisic, A. Marsano, R. Maidhof, Y. Wang and G. Vunjak-Novakovic, *Nature Protocols*, 2008, **3**, 719.
41. M. C. Moore, K. C. Coate, J. J. Winnick, Z. An and A. D. Cherrington, *Advances in Nutrition*, 2012, **3**, 286-294.
42. M. A. Lomax and G. D. Baird, *British Journal of Nutrition*, 2007, **49**, 481-496.
43. G. Freckmann, S. Hagenlocher, A. Baumstark, N. Jendrike, R. C. Gillen, K. Rössner and C. Haug, *Journal of diabetes science and technology (Online)*, 2007, **1**, 695-703.
44. D. Antoni, H. Burckel, E. Josset and G. Noel, *International Journal of Molecular Sciences*, 2015, **16**, 5517-5527.
45. C. L. Willard-Mack, *Toxicologic pathology*, 2006, **34**, 409-424.
46. K. E. Sung, N. Yang, C. Pehlke, P. J. Keely, K. W. Eliceiri, A. Friedl and D. J. Beebe, *Integrative Biology*, 2011, **3**, 439-450.
47. J. B. Wydallis, R. M. Feeny, W. Wilson, T. Kern, T. Chen, S. Tobet, M. M. Reynolds and C. S. Henry, *Lab on a Chip*, 2015, **15**, 4075-4082.
48. N. R. Wevers, R. Van Vught, K. J. Wilschut, A. Nicolas, C. Chiang, H. L. Lanz, S. J. Trietsch, J. Joore and P. Vulto, *Nature Publishing Group*, 2016, DOI: 10.1038/srep38856.
49. A. T. Ciftlik, H.-A. Lehr and M. A. M. Gijs, *Proceedings of the National Academy of Sciences of the United States of America*, 2013, **110**, 5363-5368.
50. X. Hou, Y. S. Zhang, G. T.-d. Santiago, M. M. Alvarez, J. Ribas, S. J. Jonas, P. S. Weiss, A. M. Andrews, J. Aizenberg and A. Khademhosseini, 2017, **2**, 17016.
51. J. A. Kim, H. N. Kim, S. K. Im, S. Chung, J. Y. Kang and N. Choi, *Biomicrofluidics*, 2015, **9**, 1-15.
52. T. Satoh, G. Narazaki, R. Sugita, H. Kobayashi, S. Sugiura and T. Kanamori, *Lab Chip*, 2016, **16**, 2339-2348.
53. X. Wang, L. Yi and M. G. Roper, *Anal Chem*, 2016, **88**, 3369-3375.
54. V. S. Shirure and S. C. George, *Lab Chip*, 2017, **17**, 681-690.
55. T. Chen, B. Gomez-escoda, J. Munoz-garcia, J. Babic, L. Griscom, P.-y. J. Wu, D. Coudreuse and P.-y. J. Wu, *Open Biology*, 2016, **6**, 160156.
56. K. Futrega, J. Yu, J. W. Jones, M. A. Kane, W. B. Lott, K. Atkinson and M. R. Doran, *Lab Chip*, 2016, **16**, 1473-1483.
57. J. M. Lopacinska, J. Emneus and M. Dufva, *PLoS One*, 2013, **8**, e53107.
58. P. M. van Midwoud, A. Janse, M. T. Merema, G. M. Groothuis and E. Verpoorte, *Anal Chem*, 2012, **84**, 3938-3944.
59. E. Leclerc, J. L. Duval, C. Egles, S. Ihida, H. Toshiyoshi and A. s. Tixier-Mita, *Journal of Materials Science: Materials in Medicine*, 2017, **28**, 0-1.
60. M. W. Toepke and D. J. Beebe, *Lab on a Chip*, 2006, **6**, 1484-1486.
61. B. J. J. van Meer, H. de Vries, K. S. A. S. A. Firth, J. van Weerd, L. G. J. G. J. Tertoolen, H. B. J. B. J. Karperien, P. Jonkheijm, C. Denning, A. P. P. IJzerman and C. L. L. Mummery, *Biochemical and Biophysical Research Communications*, 2017, **482**, 323-328.
62. B.-A. Miguel, S.-A. Maria, M. Roser, Y. Oriol, R.-V. Laura, T.-P. Oriol, A.-C. Julián, P. Mar and L.-B. Manel, *SLAS TECHNOLOGY: Translating Life Sciences Innovation*, 2017, DOI: 10.1177/2472630316684625, 2472630316684625.
63. E. Berthier, E. W. Young and D. Beebe, *Lab Chip*, 2012, **12**, 1224-1237.
64. E. W. K. Young and D. J. Beebe, *Chemical Society reviews*, 2010, **39**, 1036-1048.
65. I. Wagner, E.-M. Materne, S. Brincker, Su, C. Fradrich, M. Busek, F. Sonntag, D. A. Sakharov, E. V. Trushkin, A. G. Tonevitsky, R. Lauster and U. Marx, *Lab on a Chip*, 2013, **13**, 3538-3547.
66. J. P. Mather, Roberts, Penelope E., *Introduction to Cell and Tissue Culture*, Springer US, 1998.
67. J. van der Valk, D. Brunner, K. De Smet, Å. Fex Svenningsen, P. Honegger, L. E. Knudsen, T. Lindl, J. Noraberg, A. Price, M. L. Scarino and G. Gstraunthaler, *Toxicology in Vitro*, 2010, **24**, 1053-1063.
68. B. M. Martin, *Tissue Culture Techniques*, Birkhäuser Basel, 1 edn., 1994.
69. K. Drakou and P. Georgiades, *Biochemical and Biophysical Research Communications*, 2015, **468**, 813-819.
70. A. M. Navratil, J. G. Knoll, J. D. Whitesell, S. A. Tobet and C. M. Clay, *Endocrinology*, 2007, **148**, 1736-1744.
71. G. J. Brewer, J. R. Torricelli, E. K. Evege and P. J. Price, *Journal of Neuroscience Research*, 1993, **35**, 567-576.
72. L. B. Schwerdtfeger, EC; Ryan, EP; Tobet, SA., presented in part at the 2017 Biomedical Engineering Society Annual Meeting, Phoenix Az, 2017.
73. H. Rashidi, S. Alhaque, D. Szkolnicka, O. Flint and D. C. Hay, *Archives of Toxicology*, 2016, **90**, 1757-1761.

74. J.-J. Chiu and S. Chien, *Physiological reviews*, 2011, **91**, 10.1152/physrev.00047.02009.
75. J. Liu, X. Bi, T. Chen, Q. Zhang, S. X. Wang, J. J. Chiu, G. S. Liu, Y. Zhang, P. Bu and F. Jiang, *Cell Death &Amp; Disease*, 2015, **6**, e1827.
76. K. M. Kim, Y. J. Choi, J.-H. Hwang, A. R. Kim, H. J. Cho, E. S. Hwang, J. Y. Park, S.-H. Lee and J.-H. Hong, *PLoS ONE*, 2014, **9**, e92427.
77. Y.-S. Sun, *Molecules*, 2016, **21**.
78. C. J. Kelly and S. P. Colgan, *Cell Host Microbe*, 2016, **19**, 427-428.
79. A. Carreau, B. E. Hafny-Rahbi, A. Matejuk, C. Grillon and C. Kieda, *J. Cell. Mol. Med*, 2011, **15**, 1239-1253.
80. M. Sitkovsky and D. Lukashev, *Nature Reviews Immunology*, 2005, **5**, 712-721.
81. C. Rios, G. D'Ippolito, K. M. Curtis, G. J.-R. Delcroix, L. A. Gomez, J. El Hokayem, M. Rieger, R. Parrondo, A. de Las Pozas, C. Perez-Stable, G. A. Howard and P. C. Schiller, *Stem cells and development*, 2016, **25**, 848-860.
82. P. E. Oomen, M. Skolimowski and S. Verpoorte, *Lab Chip*, 2016, **16**, 3394-3414.
83. L. Zheng, C. J. Kelly and S. P. Colgan, *American journal of physiology. Cell physiology*, 2015, **309**, C350-360.
84. L. Albenberg, T. V. Esipova, C. P. Judge, K. Bittinger, J. Chen, A. Laughlin, S. Grunberg, R. N. Baldassano, J. D. Lewis, H. Li, S. R. Thom, F. D. Bushman, S. A. Vinogradov and G. D. Wu, *Gastroenterology*, 2014, **147**, 1055-1063.e1058.
85. Y. Nashimoto, T. Hayashi, I. Kunita, A. Nakamasu, Y.-s. Torisawa, M. Nakayama, H. Takigawa-Imamura, H. Kotera, K. Nishiyama, T. Miura and R. Yokokawa, *Integrative Biology*, 2017, **9**, 506-518.
86. S. Oh, H. Ryu, D. Tahk, J. Ko, Y. Chung, H. K. Lee, T. R. Lee and N. L. Jeon, *Lab on a Chip*, 2017, **17**, 3405-3414.
87. K. V. Moser, R. Schmidt-Kastner, H. Hinterhuber and C. Humpel, *European Journal of Neuroscience*, 2003, **18**, 85-94.
88. A. Dash, I. P. Maine, S. Varambally, R. Shen, A. M. Chinnaiyan and M. A. Rubin, *The American Journal of Pathology*, 2002, **161**, 1743-1748.
89. W. E. Grizzle, W. C. Bell and K. C. Sexton, *Cancer biomarkers : section A of Disease markers*, 2010, **9**, 531-549.
90. P. V. Senior, C. J. Pritchett, J. P. Sunter, D. R. Appleton and a. J. Watson, *Journal of anatomy*, 1982, **134**, 459-469.
91. M. Asano, T. Yamamoto, T. Tsuruta, N. Nishimura and K. Sonoyama, *Dev Growth Differ*, 2015, **57**, 68-73.
92. H. Ahlgren, K. Henjum, O. P. Ottersen and E. Rundén-Pran, *Cell and Tissue Research*, 2011, **345**, 329-341.
93. B. Srinivasan, A. R. Kolli, M. B. Esch, H. E. Abaci, M. L. Shuler and J. J. Hickman, *J Lab Autom*, 2015, **20**, 107-126.
94. G. R. Langley, I. M. Adcock, F. Busquet, K. M. Crofton, E. Csernok, C. Giese, T. Heinonen, K. Herrmann, M. Hofmann-Apitius, B. Landesmann, L. J. Marshall, E. McIvor, A. R. Muotri, F. Noor, K. Schutte, T. Seidle, A. van de Stolpe, H. Van Esch, C. Willett and G. Woszczek, *Drug Discovery Today*, 2017, **22**, 327-339.