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A guide to Organs-on-Chips (OoC) technology

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I. What are “Organs-on-chips” (OoC)?

1. Definition

For decades, live cell experiments have been performed using cells cultured under static conditions on 2D substrates coated with either serum or extracellular matrix (ECM) molecules. Although these conditions provide ideal conditions for cell proliferation, they often fail to promote tissue-specific functions. The different functions among human organs rely on intricate interactions between various specialized cell types at well-defined interfaces, arranged in complex geometries and interacting with specific microenvironments.

These limitations combined with recent advances in microfabrication, bioengineering and stem cell biology have led to the development of a novel 3D cell culture model. The so-called Organs-on-chips (OoC). The term “on-chip” comes from the design principles based on microchip technology and the term “organs” stem from engineered microenvironment mimicking organ-level functions. However, they are not miniature organs on a silicon chip soldered to a circuit board. OoC, also known as microphysiological systems, are bioengineered microdevices that recapitulate key functional aspects of an organ or a tissue. These microfluidic 3D culture devices can promote structural complexity and incorporate dynamic fluid flow as well as mechanical stimulations. This has led to the demonstration that dynamic culture conditions significantly influence the physiological maturation and function of *in vitro* systems. A wide variety of OoC designs exist and all of them attempt to recreate key aspects of human physiology such as the cell-cell interface between an epithelium and the endothelium of organs, the tissue-level organization of an organ, and the systematic interaction of multiple organs. OoC technology therefore presents the potential to reproduce tissue barriers, parenchymal tissues as well as inter-organ interactions.

An organ-on-chip system constitutes the following elements: 1) a microfluidic chip, a credit card size device containing microchannels for media flow and microchambers for cell culturing; 2) one or several compartments to culture cells (commercial cell line, primary cells, stem cell-derived cells or organoid) or tissue slices; 3) the presence of fluid flow through one or multiple microchannels/tubing providing culture medium 4) optionally: a porous membrane to create barrier models or a 3D gel onto which cells are grown to simulate physiological structures; 5) optionally: biosensors to measure endpoints such as barrier integrity, metabolism, or bio-actuators that stimulate relevant physical stimuli.

2. Applications

Although animal models have greatly contributed to our understanding of physiology and disease and to the development of new medicines, scientists have long been aware of the frequent discrepancies between animal and human studies. There is a crucial need for modelling and testing platforms that would be more predictive of human responses.

Drug candidates may be terminated for lack of efficacy in animals, or discovery of hazards or toxicity in animals that might not be relevant to humans. Despite significant developments in computational and *in vitro* biology and toxicology in the past two decades, more than 80% of investigational drugs still fail in clinical testing, with 60% of those failures due to lack of efficacy and another 30% due to toxicity[1] . Drug-induced cardiac, liver or kidney toxicity may not be predicted by classical pre-clinical models. In addition, assessing the teratogenic effects of drugs and environmental chemicals on fetal development is currently not possible in humans. The increase of the cost, timing and ethics of data obtained from animal testing that frequently fail to predict human response during clinical trials, has increased the focus on finding alternative models. OoC has attracted great interest and attention from the pharmaceutical industry as it emerged as a successful *in vitro* human microphysiological systems that can mimic organ-level and even organism-level functions. OoC represents a significant advance in drug discovery and development as it offers novel tools for disease modelling and characterization, as well as providing potentially more predictive methods for assessment of the toxicity and efficacy of new compounds and therapeutics.

II. Why use OoC models?

1. "Breaking the *in vitro* impasse"

Understanding human physiology and pathogenesis requires *in vitro* models that mimic tissue biology. However, current models consist of cells forming a single cell layer that do not reflect the complexity of human tissues and do not reproduce key biological functions (2D cell culture) (Figure 1). One of the major challenges is to find experimental physiological relevant models. Commonly used models include cell aggregates and organoids (3D cell culture), Organ-on-chip systems and animal models (such as rodent, zebrafish, drosophila...). These models all require compromises between relevance to human diseases; complexity, experimental reproducibility and cost.

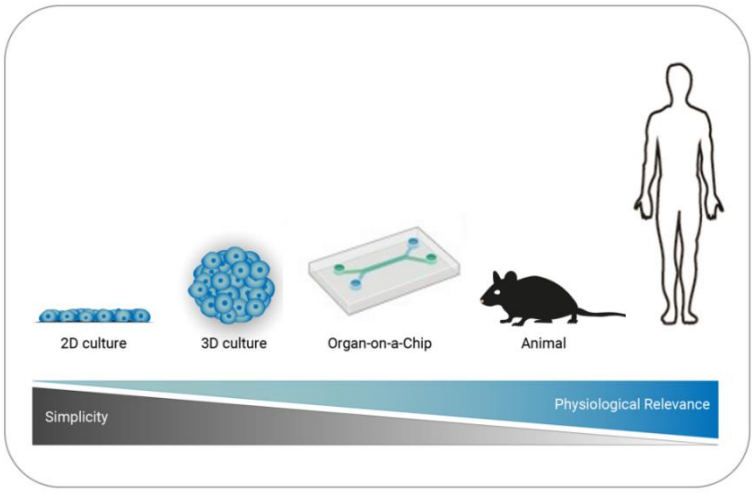


Figure 1: Biological model systems used in biomedical research ranging from 2D cell culture to model organisms in terms of simplicity versus physiological relevance (adapted from[2] & scienceinschool.org website)

Organs-on-chips which are microengineered *in vitro* tissue models have the potential to break this "*in vitro* impasse"[3]. They can provide a physiologically relevant level of tissue organization, geometry, soluble gradients and mechanical stimulation in a well-defined and controlled manner. The possibility to combine these microfluidic chips with integrated sensors opens the way to high throughput analyzes.

2. An alternative to animal models - 3R principle

The concept of rationalizing animal testing was originally formulated by Russell and Burch in 1959 in their book "The Principles of Humane Experimental Technique". They proposed three principles now commonly referred to as the "3 R's":

- Reduction: to minimize the number of animals included in research protocols
- Refinement: to favor the use of technical approaches that decrease the incidence and the level of pain inflicted to animals
- Replacement: to find an alternative animal model that may be less sensitive to pain and distress or to find a complete non-animal substitution model

Simple cell culture systems cannot reflect the complexity of an entire tissue, organ or organism. However, the combination of mathematical models, computer simulation, and complex *in vitro* biological systems should be considered. Complex *in vitro* systems include organoids (3D aggregates of one or more cell types) and organs-on-chips. Microfluidic devices can provide high physiological relevance while offering a cost-effective alternative. OoC technology is a promising tool to reduce the reliance on animal models.

3. Advantages of OoC models

Organ-on-chip technology lies at the interface of biological microelectromechanical systems - bioMEMS (microengineered systems in a biological context), microfluidics (the control of fluid delivery) and biomimetics (that implements relevant biological functions in bioengineered systems). OoC offer the possibility to culture one or more cell types in a small bioreactor of a chosen design (cf section III.2) that mimic, in a miniaturized but efficient and controlled manner *in vivo* physiological conditions. Micro-engineered systems also enable parallelization and increased throughput. The presence of tissue–tissue interfaces, dynamic fluid flow (cf section III.3), and physiological mechanical stimuli (cf section III.4) enhances the fidelity of cell differentiation and increases expression of tissue-specific function. Tissues cultured in OoC can be analyzed using classical methods such as high resolution microscopy, Liquid chromatography/Mass spectrometry (LC/MS), flow cytometry, transcriptomics, proteomics, metabolomics, and histological analysis (cf section III.5). OoC can also be combined with in-line electrochemical, mechanical, and optical sensors to incorporate real-time readouts and enable probing of tissue barrier integrity, neuron electrical activity, oxygen utilization, pH, molecular transport, and other parameters (cf section III.5). These measurements can be carried out under dynamic flow conditions as occur *in vivo*, and many would be difficult, if not impossible, to carry out in animal studies or human trials. Human cells from different individuals can be tested to reflect the natural variation between populations and could enable the development of personalized treatments (see section VII). The micron-scale dimensions of OoC allows for consumption of small amounts of cells, culture media or drugs to perform experiments. All these advantages make OoC the ideal platform to build innovative and realistic *in vitro* models.

4. Technical challenges

OoC has great potential to change the landscape of *in vitro* testing for fundamental and applied research in biology, drug development and toxicology. To gain broad implementation for tissue culture replacement, 3 main challenges have to be considered when developing OoC models: 1) To choose the right design, 2) To master the cell culture and biological parameters, 3) To propose integrated systems easy to handle by the end-users.

Key parameters such as vascularization and interaction with other cellular compartments may be difficult to integrate. In addition, most tissues are submitted to mechanical forces *in vivo* such as the heart that beats to pump blood, lungs that stretch out during inhalation or bones that encounter mechanical loads during movement. Trying to recreate or simulate these physical aspects using appropriate devices and/or sensors represents a technical challenge for the OoC field.

III. What are the current OoC technologies available?

The main requirement for developing a relevant OoC model is to first understand and identify key aspects of the geometric, mechanical and biochemical microenvironment of the tissue of interest. Once they have been identified, these are integrated in the microengineered system to obtain complete control over the defined parameters. Cells can then be introduced inside the microfluidic chip and subjected to the engineered stimuli. Ultimately, cells will respond to the stimuli and self-organize until reaching a functional organization more realistic than when they are cultured in conventional conditions.

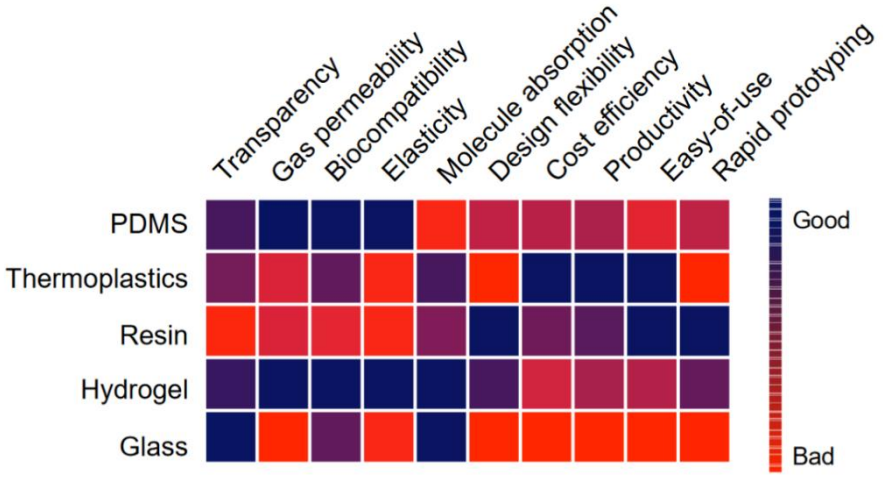
Biomimicry is crucial for understanding highly complex human physiology. This involves the creation of an environment that resembles the native environment, in a compartmentalized and structured manner. Although more physiologically relevant than conventional *in vitro* culture models, OoC are still a simplification of the actual microenvironment of the tissue of interest. The design philosophy of an OoC arises from the balance between complexity, ease of use, and reliability. There are several key parameters that can be included and controlled in the system such as fluid flow, mechanical stimuli, 3D spatial organization of cells and crosstalk between cell types. The biological question to be addressed or the physiological process to be modeled drive the design. For instance, to implement vascular systems, endothelial cells must be cultured in a relevant microenvironment connected to a perfusion system offering external control of the flow rate and flow pattern[4] . In other applications, the use of a porous membrane enables the simulation of tissue barrier functions necessary to recapitulate skin, gut, placenta or blood-brain barrier (BBB) physiology and pathology. It is important to not oversimplify the model if one wants to obtain meaningful results.

OoC devices vary with the materials used, layouts, fluidic perfusion systems and mechanical stimulations. Many measurement classically used in molecular biology, cell biology and immunology can be performed to study the functional responses of tissues cultured in OoC. The addition of multiple sensors within the devices permit one to follow the production/consumption of metabolites and other physiological parameters.

1. Materials used for OoC devices

Materials used should be evaluated based on their biocompatibility, the possibility to be sterilized, and their physiochemical properties. Essential physiochemical properties to consider are optical transparency for observation, gas permeability for cells requiring oxygen, lowest possible absorption of molecules, chemical and thermal resistance, as well as stiffness. Common materials used for creating OoC devices are silicone substrates and polymers such as Polydimethylsiloxane (PDMS). While physical properties are useful for cell culture applications (such as

transparency and gas permeability), the ability to absorb small hydrophobic molecules can significantly change solution concentrations and alter experimental outcomes[5]. Other transparent materials which do not absorb small hydrophobic molecules such as inert thermoplastic polymers (Cyclic olefin copolymer (COP), cyclic olefin copolymer (COC) or glass[6] can be an alternative to PDMS. These have a very low permeability to oxygen and water vapor, allowing precise control of those gases by controlling their concentration in the culture medium. Of note, these materials allow one to perform hypoxia experiments[7]. The material and fabrication method should thus be chosen depending on the experimental purpose. A comparison of most common materials used for OoC fabrication is depicted in Figure 2 (from [8]). In addition, a table presents their advantages and drawbacks as well as their main applications (from [8]).



Materials	Advantages	Drawbacks	Experimental model
PDMS	Gas-permeability Optical transparency Elasticity Biocompatibility	Absorption of small molecules Difficulty in mass production	Disease modelling Mechanical and chemical stimuli Electrode patterning
Thermoplastics	Optical transparency Mass production Cost-effective Low absorption	Rigidity Difficulty in producing complex structures Low permeability	Drug screening Large-scale experimentations
3D printing resins	High mechanical and thermal properties Low cost Complexity and design freedom	Autofluorescence Opacity Toxicity Low permeability Surface roughness	3D design modelling Rapid prototyping
Glass	Optical transparency Inert Biocompatibility Low autofluorescence	Laborious fabrication Fragile Expensive	Electrode patterning
Silicon	Low absorption Generation of high-resolution channels on the nanoscale	Laborious fabrication due to need for clean-room facilities Expensive	On-chip sensors Formation of diffusive barriers

OoC, organ-on-a-chip; PDMS, poly(dimethylsiloxane).

Figure 2: Comparison of various materials used in OoC fabrication (figure from [8])

2. OoC layouts classically used to recreate organ functions

The design of an OoC device strongly depends on the biological function that need to be recreated *in vitro*. For instance, tissue barrier function of organs such as the skin, liver, gut or BBB is usually reproduced using compartmentalized devices in which different cell types are cultured in two parallel microfluidic channels separated by a porous membrane (Figure 3a). The porous membrane is often made of flexible synthetic material coated with extracellular matrix (ECM) proteins such as collagen or fibronectin to promote cell adhesion. An epithelial cell layer is cultured on one side of the membrane (in the upper chamber) and an endothelial cell layer is cultured of the other side of the membrane (in lower chamber). Measurements such as trans-epithelial electrical resistance (TEER) or diffusion of fluorescent molecules make it possible to assess the tightness of the epithelium.

OoC design was first developed to recreate a major functional unit of the human body, the lung alveolus[9]. Human lung alveolar epithelial cells were seeded on one side and human vascular endothelial cells on the other side, thereby recreating the alveolar-capillary interface. This device allows to mimic the air-liquid interface (ALI) of the lung (essential for lung differentiation and function) by perfusing the endothelium with culture media and introducing air into the epithelial channel. In addition, the device comprises two empty side channels in which suction is applied (vacuum channels) to create cyclic stretches of the flexible central part of the device to recreate breathing motions. This chip design has been used by multiple groups to develop various OoC models and have been reviewed by Donald E. Ingber in a recent publication[10]. It has recently been modified to gain access to the upper channel allowing the incorporation of a thick slab of natural hydrogel (tissue-specific ECM) in which cells can be embedded. The hydrogel can be exposed to cyclic mechanical strain throughout its thickness[11] . This Open-Top Chip is capable of imposing mechanical forces, while enabling the inclusion of a relatively large volume of natural ECM hydrogel with embedded mesenchymal cells, overlaid with epithelial cells and supported by a vascular component. It reproduces both the complexity of the 3D tissue architecture and its mechanical environment and offer the possibility to extract the gel for tissue staining and analysis. Using this design, the authors have developed a skin-on-chip and an alveolar-on-chip model[11] .

The presence of a natural 3D scaffold enables the organization of complex cellular structures modeling native tissues. In some OoC designs the porous membrane is replaced by a hydrogel (usually made of ECM) to promote cellular ingrowth and formation of 3D structures. In these cases, the design of the chip is often planar and can contain more than two microfluidic channels (Figure 3b,c). The central channel is loaded with a hydrogel in which cells can be embedded. The hydrogel channel does not have solid sidewalls to constrain the gel. Instead, it is the presence of either phase guides (Figure 3b, MIMETAS) or spaced micropillars/microposts that restrain the gel in the central channel[12–14] (Figure 3c). Within the hydrogel, encapsulated cells will organize based on self-assembly[12] . These designs are preferentially adopted to promote microvascular network formation[14] or to support neurite outgrowth[15] and neuronal network formation to understand how neurons process information[16].

Alternatively, hollow channels can be shaped within the hydrogel and lined with cells. In the end, cells will either cover the inner surface of the channel or fill the whole channel section. This can be achieved using glass mandrels (Figure 3d, Nortis) or microfilaments[17] that are removed after hydrogel gelation as occurred. Similarly, sacrificial networks made of hydrolysable material such as gelatin can be included in the hydrogel and removed by adding aqueous solution once the hydrogel has reticulated[18]. Sacrificial networks can also be created using 3D printing to deposit bioinks [19] (Figure 3e). In addition, hydrogel can be patterned *in situ* using laser ablation to create scaffolds with a well-defined and complex geometry that will then be cellularized[20]. These carved structures will guide the formation of the cellular structures. Cylindrical channels carved within collagen hydrogels and seeded with endothelial cells can lead to the formation of blood vessel analogs[20]. Recently, the group led by Matthias P. Lutolf has used laser ablation of hydrogel loaded in microfluidic chip to extrinsically guide the self-organization of stem cells into functional mini-intestines[21] (Figure 3f).

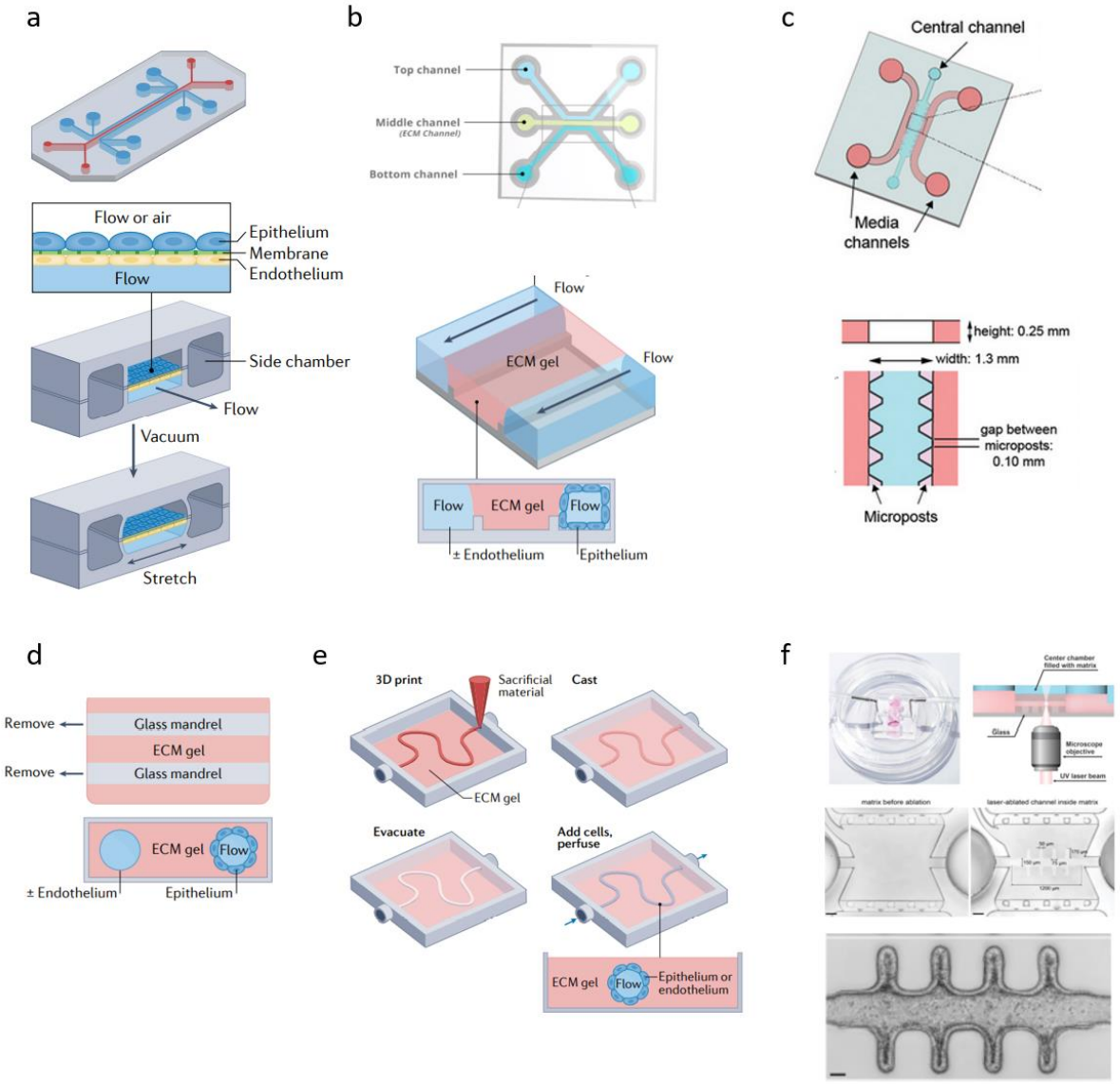


Figure 3: Overview of OoC layouts and features. (a) Mechanically actuated OoC made of PDMS comprising two parallel channels separated by a flexible porous membrane and two distal hollow channels connected to a vacuum generator. Application of cyclic suction to the hollow channels induced rhythmic stretching of the porous membrane and attached tissue layer to introduce mechanical forces in the system[9] *(b) Planar OoC design made of three adjacent channels separated by phase guides. Loading of 3D hydrogel in the central channels is constrained by the presence of these phase guides. Outer perfusion channels enable the development of complex co-cultures configuration. This platform enables perfusion, high resolution imaging but cannot be stretched to impose mechanical strains (Mimetas) (c) Planar OoC design made of three adjacent channels separated by micropillars that will restrain the hydrogel in the central channel based on surface tension phenomenon*[14] *(d) Hollow channels are created inside the bulk of hydrogel by placing glass mandrels and removing them once gelation of the hydrogel has occured (Nortis) or by using microneedles or microfilaments further removed*[17] *(e) OoC created using 3D printing to deposit sacrificial bioinks of defined geometry within a bulk of hydrogel that will eventually be removed and result in the formation of empty structures within 3D matrix*[19] *(f) Laser ablation of hydrogel loaded inside a microfluidic chip to create scaffold that extrinsically guide the self-organization of stem cells into functional mini-intestines*[21]*. This figure has been adapted from* [10]*.*

Other design have transformed the membrane-based microfluidic devices to an open multi-well design. A pneumatic deflection of a membrane suspended in an open well is used to apply mechanical strain on the tissue (Alveolix).

3. Perfusion systems to deliver physiological flow

Fluidic perfusion is essential in OoC devices to ensure continuous supply of nutrients and oxygen but also to remove waste products, CO_2, and dead cells. Perfusion also generates mechanical stimuli such as laminar, pulsatile and interstitial shear stress. The shear stress applied on cells exposed to fluid flow is known to affect their phenotype, morphology and their maturation. Flow control is essential to emulate key environmental factors (biochemical gradients) and to promote proper cell signaling (mechanotransduction).

While the microfluidic chips in which cells are cultured are compact and easy to handle, a certain amount of tubing and external pumps must be plugged to the microfluidic chip. To avoid complexity, passive fluid handling methods can be adopted which eliminates the need for external pumps. The simplest method is gravity-driven flow using a rocking platform to induce passive flow. Here, flow results from the difference in liquid height between the inlet and the outlet (due to hydrostatic pressure) and the resistance of the system[22]. It is bidirectional as it is derived from the oscillatory motion of the rocking plate. This approach is transient in nature and prone to performance variation. A major drawback is the absence of

control over the applied flow rate, shear stress, and flow pattern. Conversely, active perfusion using pressure-based flow controllers or peristaltic pumps can provide such fluidic control. Flow parameters can be tuned and several microfluidic channels can be perfused in parallel using microvalves connected to the pressure controller. Tuning flow enables control and circulation of media by perfusing the entire system with common medium which simplifies the engineering of complex human physiology on chip. Peristaltic pumps do not deliver robust steady-state flows for long periods of time as they rely on pulsatile flows and are more susceptible to contamination and air-bubbles[7]. The choice of perfusion method will greatly depend on whether the culture runs on a single-pass type of perfusion flow or a recirculatory flow configuration. Conventional syringe pumps and pump-free gravity-driven flows typically support single-pass perfusion flow. Other pump variants such as pressure controllers and peristaltic pumps are amenable to driving recirculatory flow. Building an integrated system can offer a way to control several key culture parameters crucial to stimulate physiologically relevant crosstalk between cellular components, such as supply of nutrients and oxygen, removal of cellular waste and toxins and diffusion of metabolic or angiogenic factors. This can be achieved by maintaining continuous media recirculation in the culture of microtissues using a small amount of media. One way is to integrate built-in micropumps that eliminate the need for tubing connections and the use of large media reservoirs to enable media recirculation. These micropumps are often based on pneumatically controlled membrane deflection to drive fluid flow.

Unidirectional flow ensures a stable supply of fresh nutrients, but inter-tissue communication is only possible in a sequential, downstream manner. Recirculating flow has the advantage of recirculating important molecules, which can accumulate overtime within a single chip or allows chemical communication between the different tissues of a multi-chip device. Recirculation does not provide continuous replenishment of nutrients, and can lead to an accumulation of waste products. Although OoC systems can be maintained for several days without medium exchange, periodic replenishment with fresh medium or removal of a portion of medium may be necessary for longer term studies. This removal can be useful to sample material for offline analysis[8].

4. Mechanical stimulation

OoC devices are well suited for applying a range of physical environmental stimuli on cells. As fluid flow is inherent in many OoC systems, these systems are used to mimic physiological shear stresses exerted by blood and interstitial fluids (cf review about how to control shear stress in microfluidic devices).

OoC devices can also be designed to apply mechanical, tensile or compressive forces to cells. Mechanical stimulation plays an important role in the development, function and maintenance of specific tissues. This is often achieved by growing cells on thin flexible PDMS membranes that are incorporated into the OoC device such that they can be flexed cyclically by vacuum or pressure pneumatic actuators. Mechanically active OoC devices have been implemented to mimic breathing-

induced stretching of the airway[9], or peristalsis motions of the gut[23]. Actuated hydrogel (natural or synthetic) can also be used to generate mechanical stimulation on cells embedded within the hydrogel. This has been done to mimic the mechanical forces exerted onto the articular cartilage during motion[24]. Contractility of cardiac tissues has been emulated by coupling 3D cell-laden hydrogel embedded with cardiac cells and a pressure-actuated chamber[25].

5. Readouts and sensors of physiological responses

- Experimental readouts

Tissue cultured in chips can be analyzed using classical methods such high resolution microscopy, LC/MS, flow cytometry, transcriptomics, proteomics, metabolomics, and histological analysis, and as recently demonstrated, automated high content confocal imaging with fluorescent reporters[26]. The authors created an end-to-end, automated workflow to capture and analyze confocal images of multicellular OoC. They were able to characterize detailed cellular phenotypes across large batches of chips. By automating this process, data acquisition time is reduced and process variability and user bias are minimized.

- Electrical sensors

The most common electrical sensing system in OoC is trans-epithelial/endothelial electrical resistance (TEER). It relies on the resistance obtained between electrodes on opposite side of a semipermeable membrane on which a epithelial and endothelial tissue are cultured. High TEER is indicative of tight junction formation, a readout for tissue barrier function. Cell impedance can also arise from the attachment of a cell to a substrate containing patterned electrodes. This is called electrical cell-substrate impedance sensing (ECIS) and is applied to assess cell attachment, growth, morphology and motility. Measurements are restricted to local area as opposed to TEER[27].

Henry et al. have developed an OoC design comprising embedded electrodes to assess formation and disruption of tissue barrier function in a human airway-on-chip and in a human gut-on-chip model[28]. They used a layered approach to assemble their microfluidic chip and integrated semi-transparent, sensing electrodes to measure TEER as well as cell morphology using 4-points impedance measurements at varying frequencies. They were able to analyze the barrier function of human primary small airway epithelial cells cultured under ALI (Air-Liquid Interface) conditions as well as human intestinal cells under flow. Addition of the chelating agent EGTA resulted in impaired barrier function, confirming the proper functioning of their assay[28]. These real-time, non-invasive TEER measurements can be applied to any type of cultured cells. A simple and cleanroom-free way to integrate four platinum electrode wires horizontally in a microfluidic device has been reported[29]. Their specialized chip design containing two crossed channels makes it difficult to be apply to a typical OoC device often

containing two parallel channels. A recent study reported another cleanroom-free fabrication method for the integration of platinum electrode wires within a two layer OoC device[30]. They performed TEER measurement in a gut-on-chip and a BBB-on-chip model.

- Metabolic and Oxygen Sensors

A critical aspect of OoC systems is the ability to monitor both the cell culture conditions and biological responses of the cultured cells (such as proliferation and differentiation rates) but also the release of signaling molecules and metabolic activity. It is often done using microscopy techniques and off-chip analyses and assays. Integrated microphysiometers within the chip enable on-line measurement of metabolites allowing continuous measurements and faster readouts. Electrochemical sensors are typically composed of gold (Au) or platinum (Pt) working and counter electrodes, and silver/silver chloride (Ag/AgCl) reference electrodes. A multianalyte screen-printed electrode was developed for the detection of analytes such as glucose, lactate, oxygen, and pH[31]. Their sensor was enclosed within a microfluidic channel loaded with macrophages to demonstrate its capabilities to achieve real-time microphysiometry measurements. Amperometric glucose and lactate sensors using platinum electrodes was also developed to measure current under a polarization of 450 mV. Here, current is produced by the oxidation of glucose or lactate to H_2O_2 by membrane-embedded glucose or lactate oxidase[32]. Electrochemical sensors have demonstrated the existence of an oxygen gradient in a liver-on-chip device using human hepatocytes. The authors have integrated multiple sensors in a thin, porous membrane inside the OoC using inkjet-printing along the microfluidic channel[33]. An enzyme-based multi-analyte biosensors was integrated into a multi-tissue culture platform for 'body-on-a-chip' applications. The sensor modules were designed as small glass plug-ins featuring four platinum working electrodes. The electrodes were functionalized with oxidase enzymes to enable continuous monitoring of lactate and glucose through amperometry[34] .

Although a wide variety of human organ-on-a-chip models have been created, the integration of multiple sensors to assess the dynamic responses of the organs to pharmaceutical compounds over extended periods of time is challenging. A fully integrated multi–organ-on-a-chip system was developed to achieve continuous and automated sensing of biophysical and biochemical parameters. This modular sensing platform was interfaced through a fluidics-routing breadboard.

IV. What kind of cells are used to create OoC?

An important consideration in the field of OoC is to decide what types of cells should be used to design the microphysiological systems of interest.

1. Cell lines

Cell lines represent the easiest and more reliable cell source as they usually undergo extensive validation tests for purity and viability. They generally quickly assume their proliferative state, are easy to culture and to transfect thanks to standard protocols provided by suppliers. Their stable and overall predictable behavior contribute to high reproducibility. A major drawback of cell lines is their capacity to proliferate indefinitely acquired either through genetic or artificial modifications. This may cause a drift in their phenotype that may not reflect their primary cell type origin. In addition, they are very systematic and uniform in nature and do not capture the true diversity of a living tissue and are not patient-specific.

2. Primary cells from human donors

Primary cells most closely represent the tissue of origin as they are directly taken from human donors. They have a limited lifespan but offer more advantages compared to continuously cultured cell lines. Several factors such as age, sex, health status can thus be considered, accurately reflecting the heterogeneity of the human population. When sourced from donors with specific diseases, primary cells can allow to model pathologies. With the growing trend towards personalized medicine, donor variability and tissue complexity can be achieved with such cells. Variability among donors or batches can occur leading to higher standard deviation within replicate experiments and thus less reproducibility. Some primary cell types can be difficult to obtain or can even be not available at all (such as neural cells). Primary cells require more complex and specific culture media that can be an issue when several cell types are seeded onto the same chip or when multiple chips are sequentially connected. In these cases, finding a common culture media could be problematic.

3. Human iPS (induced pluripotent stem cells)

Another potential cell source is induced pluripotent stem cells (iPS) from either healthy or diseased populations. They are adult somatic cells genetically reprogrammed back into an embryonic-like pluripotent state. They can divide

indefinitely and produce different types of cells as part of their progeny. iPS possess the potential to create an OoC platform dedicated to each individual in order to model their tissues and disease phenotypes. They are particularly useful when trying to generate patient-matched tissues in a multi-OoC system[35]. A certain amount of time and resources are needed for creation, passaging and differentiation of these cells. In addition, steering stem cells towards a well-differentiated state and maintaining this differentiated phenotype can be difficult. Their immature or fetal phenotype can also limit their utility.

4. Fragmented Organoids

Organoids are self-organized 3D tissue cultures derived from human iPS. Such cultures can be crafted to partially replicate the complexity of an organ, or to express selected aspects of it. They can be seeded onto OoC platform either keeping their 3D architecture by surface treatment with passivating agent or by promoting the formation of a cell layer by coating the device with specific proteins or ECM. In this scenario, organoids must be first fragmented to be opened. This coating is required for the formation of attached, confluent monolayers of cells to create intestinal epithelium in the gut-on-chip model (ref). Finally, cells grown as organoids represent only the epithelial component of the tissue, not the stroma or vasculature, limiting their application.

5. Human biopsies

OoC can be developed using biopsy-derived organoids. Human primary cells isolated from biopsies or tissue resections of living human organ are expanded as 3D organoids, dissociated and cultured in specific chips. This strategy has been implemented to develop a relevant Intestine-on-chip model[36]. Cancer-on-chip models could also benefit from this approach since using cell lines can result in inconsistencies between the *in vitro* model and the patient tumor cells[37].

V. What are the current single OoC models available?

Several types of tissues have been successfully modeled to reproduce corresponding functional subunits, including, the lung[9,38], intestine[23,36,39], liver[40,41], kidney[42], the BBB[43,44], heart[45], blood vessels[12,13,44].

A complete and updated list of all single OoC available can be found in recent reviews[10,46].

Importantly, these OOC devices can reproduce organ level response to exogenous agents such as inflammatory responses of the lung to silica nanoparticles[23] intestinal epithelial-microbiome crosstalk[47], early liver fibrotic activation in response to anti metabolites chemotherapy drug[48] as well as flow dependent recruitment of circulating immune cells[49], and organ specific inflammatory reaction *in vitro*. Moreover, they can also effectively mimic many types of organ specific disease states, including pulmonary edema and thrombosis, asthma, inflammatory bowel disease[37].

1. Example 1: Lung-on-a-chip – The first OoC

The human lung alveolus chip with two parallel microchannels lined with human lung epithelial cells on one side and human endothelial cells on the other side separated by a microporous membrane was the first OoC to replicate complex integrated organ-level physiological and pathophysiological responses, rather than to simply demonstrate retention of cell- or tissue-level differentiated functions [9] (Figure 4). Once the cells have reached confluence, air can be introduced into the epithelial compartment to create an air-liquid interface, to mimic the lining of the alveolar air space. The compartmentalized channel configuration of the microdevice makes it possible to manipulate fluid flow, as well as delivery of cells and nutrients, to the epithelium and endothelium independently. These chips were challenged with either living bacteria to mimic infection or nanoparticles to simulate inhalation of airborne smog nanoparticles. Both stimulations resulted in the stimulation of circulating immune cells and secretion of inflammatory cytokines at it occurs *in vivo* (Figure 4). Pulmonary edema toxicity caused by Interleukin-2 (IL-2) treatment in some patients was successfully demonstrated in another study using the same lung-on-chip device[23].

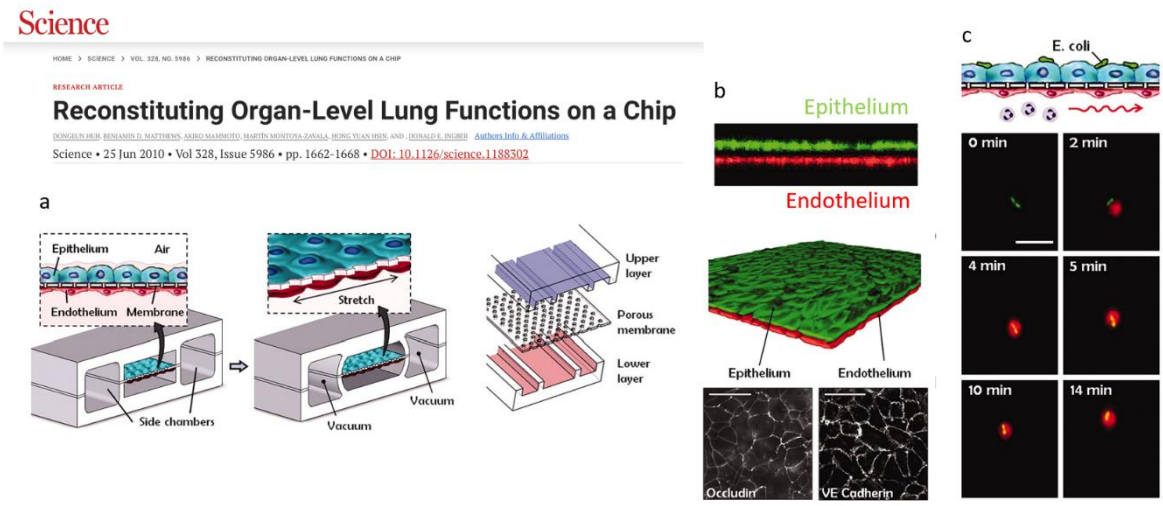


Figure 4: Reconstituting Organ-Level Lung Functions on a Chip (Science 2010)

(a) Microfabricated lung-on-chip device is made of compartmentalized PDMS microchannels to form an alveolar-capillary barrier separated by a thin porous flexible PDMS membrane coated with ECM. The device recreates physiological breathing movements by applying vacuum to the empty side chambers, causing mechanical stretching of the PDMS membrane. Three PDMS layers are aligned and irreversibly bonded to form two sets of three parallel microchannels separated by a 10 μm-thick PDMS membrane containing an array of through-holes with an effective diameter of 10 μm (b) Long-term microfluidic co-culture produces a tissue-tissue interface consisting of a single layer of the alveolar epithelium (Epithelium; stained with CellTracker Green) closely apposed to a monolayer of the microvascular endothelium (Endothelium; stained with CellTracker Red), both of which express intercellular junctional structures stained with antibodies to Occludin or VE-cadherin, separated by a flexible ECM-coated PDMS membrane. (c) Time-lapse images showing phagocytosis of GFP-expressing Escherichia. coli (green) bacteria on the epithelial surface by a neutrophil (red) that transmigrated from the vascular microchannel to the alveolar compartment. (figure adapted from[9])

The possibility to apply mechanical stimulation in the chip revealed that breathing-like motion enhance inflammatory reactions and enhanced nanoparticles absorption, features not observed in static culture systems[9]. Another study later demonstrated that applying dynamic flow in the lung-on-chip system could lower nanoparticle toxicity as compared to static conditions[50]. Several other studies using lung-on-chip models have been performed to successfully model healthy and diseased human lung airways such as inflammation-induced thrombosis[51], chronic obstructive pulmonary disease[52], asthma, cystic fibrosis[53] and more recently SARS-CoV-2 infection to test anti-viral molecules[10].

2. Example 2: Gut-on-a-chip

Several OoC models of the small and large intestine seeded with intestinal epithelial cells with or without underlying endothelium have been created to model various diseases as well as to study drug metabolism and toxicities[10]. The presence of dynamic fluid flow has been found to be necessary and sufficient to promote villi formation in small intestine-on-chip as well as production of high numbers of goblet cells and accumulation of a thick mucus layer in colon chips. The application of peristalsic-like mechanical motions is required for optimal tissue differentiation[54]. Cyclic mechanical stretches and the presence of dynamic fluid flow in colon-on-chip enhanced bacterial growth of the bacteria Shigella[55].

A challenge in gut-on-chip models is to recreate the anaerobic environment of the intestinal lumen, while keeping the endothelium oxygenated. It is particularly true when one wants to recreate human microbiome on chip. This was accomplished through the creation of a hypoxic gradient across the epithelial-endothelial interface and confirmed by integrated on-chip oxygen sensors[56]. This system allowed the culture of a complex gut microbiome in direct contact with human intestinal epithelium and naturally secreted mucus. Another human intestine-microorganism interface was developed in which an oxygen gradient was recreated to support the growth of a commensal anaerobic bacteria[57].

3. Example 3: Tumor-on-a-chip

OoC are rapidly emerging as powerful tools for oncology research. Indeed, Tumor-on-chip can reproduce certain key aspects of the tumor microenvironment (TME), such as biochemical gradients and niche factors, dynamic cell–cell and cell–matrix interactions, and complex tissue structures composed of tumor and stromal cells[37]. Several models have been developed to manipulate the TME and assess tumor cell behavior such as a microfluidic model to study cell response to metabolic gradients[58]. In addition, Tumor-on-chip models have been used to characterize how Cancer-associated fibroblasts (CAF) modify the TME and how TME favor CAF motility and associated tumor dissemination. OoC designs for recreating tissue–tissue interfaces, crucial for reconstituting in a physiological context the interaction occurring between tumor and its environment during cancer invasion and metastasis have been nicely reviewed[37]. Several other interesting reviews about Tumor-on-chip can be found with a focus on tumor–endothelial–immune cells interactions[59–61].

The metabolism and drug resistance of tumor cells *in vivo* are often induced by hypoxia[62]. However, no therapeutic targeting hypoxic cells in tumor exists, probably due to the absence of available *in vitro* model that accurately reproduces hypoxia as found in the tumor microenvironment. Microfluidic devices fabricated with PDMS permit oxygen diffusion. This can be blocked by an embedded sheet of polymethyl methacrylate (PMMA)[63]. In a recent study, a 3D engineered oxygen model (3D-O) that supports the growth of breast cancer cells and generates physio-

and pathophysiological oxygen levels was developed to understand the role of oxygen availability in tumor-immune interactions[49]. Low oxygen level induced within 3D-O scaffolds validated known tumor hypoxia characteristics such as reduced cancer cell proliferation, increased extracellular matrix protein expression, increased extracellular vesicle secretion and enhanced immunosuppressive marker expression. It constitutes a promising platform for the evaluation of immunological events and as a drug-screening platform tool to overcome hypoxia-driven immune evasion.

VI. How to combine several OoC to create a "body-on-chip"?

The ultimate goal behind the development of single microphysiological systems is to couple and combine multi-organ systems in a single device. It is possible to fluidically link multiple organ models to simulate organ interactions. This would yield a device with complex, interacting organ systems to create a full "body-on-chip". Such advanced *in vitro* approaches incorporating a systemic dimension and multiple organs must be developed to faithfully emulate human health and pathophysiology.

1. Multiplexing of single OoC

The principle here is to model each organ in separate chips and connect them with tubing to each other to create a modular and independent multiorgan model. This modular approach allows reconfiguration of the multi-OoC platform and supports the use of individual vascularized organs by using organ-specific microvasculature endothelial cells. Furthermore, the single OoC modules can first be established and matured using specific medium before they are connected to each other.

For instance, two human BBB-on-chip spaced by a Brain-on-chip were fluidically coupled to model drug influx across the human BBB, permeation into the brain parenchymal compartment, and efflux across the barrier[64]. The authors used this system to mimic the effect of intravascular administration of a psychoactive drug (methamphetamine). They also uncovered a previously unknown metabolic coupling between the BBB and neurons. In another study, a pancreas-on-chip and a liver-on chip were fluidically coupled to study organism-level, hormonal cross-talk between these two compartment to look at of insulin production[65]. The authors found a functional hormonal feedback loop between the liver and insulin-secreting pancreatic tissue suggesting that this system may be useful for studying diabetes in humans.

Some limitations to this approach are the requirement for a common medium, difficulty to reduce metabolic wastes that go to the next organ, organ-specific flow

rates and adequate level of oxygenation of the different organ models[66]. An alternative to direct coupling is functional coupling, defined as the transfer of media from one organ module to the next in a physiological sequence. Advantages of functional coupling include the ability to adjust flow rates and media composition for each module. Vernetti *et al* [67] achieved functional coupling of four human OoC models (intestine, liver, kidney, BBB) and assessed three compounds for their organ-specific processing. Their results were consistent with clinical data and led to the discovery that one compound was crossing the BBB.

2. Multiple organs into a single plate (multi-OoC plates)

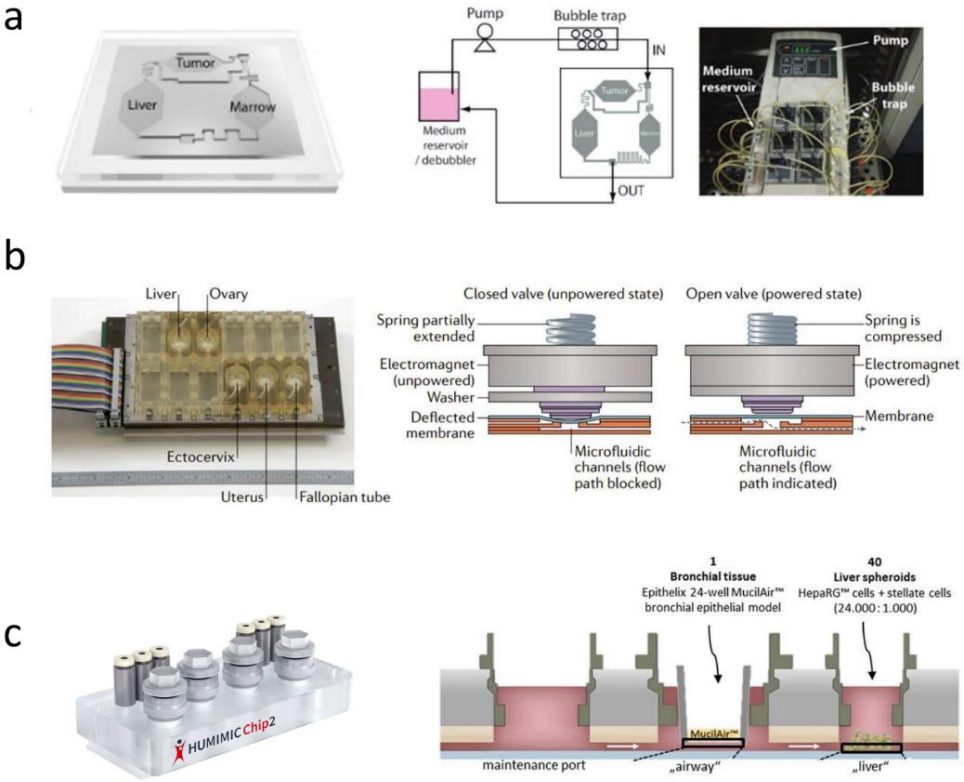
Multi-OoC plates are more compact and user-friendly as they do not require manual interconnection and heavy use of tubing. This limits the risks for leakage and minimizes the total recirculation volume. These microfluidic devices are designed to mimic important aspects of the human metabolism by interconnecting fluid flows from multiple *in vitro* tissue cultures on the chip in a physiologically relevant manner so that metabolites are consumed, produced, and exchanged (via recirculation) between all tissues at physiologically relevant concentrations.

However, organ-specific vascularization and specific organ modeling appears more challenging. In addition, common media must be used to maintain all the different organ models. Tedious optimization is necessary to include all the required supplements while ensuring that they are not detrimental for any other organ.

One of the first experiments to show how multiple-organ systems can mimic human responses to drugs was performed by Sung et al. using a device containing 3 types of organoids (liver, bone marrow, and colon tumor) on a single chip with closed circulation (Figure 5a). They were able to reproduce how the liver metabolizes Tegafur (an anticancer drug) and demonstrated that by itself, this compound did not harm the colon cancer organoid, but became active and fatal for cancer cells only after going through the liver[68]. Another example of complex multi-OoC is the simulation of the female reproductive system[69]. Xiao et al. developed a microfluidic system which supports murine ovarian follicles to produce the human 28-day menstrual cycle hormone profile. Their microfluidic platform EVATAR simulates the female reproductive tract and peripheral organs by integrating up to five tissues in a single system[69]. Actuated micropumps were embedded within the platform to enable precise flow control over a wide dynamic range. Modules included flow ports to allow recirculation within each module as well as within the whole-system (Figure 5b). It represents a powerful *in vitro* tool to simulate fundamental mechanisms of the menstrual cycle and reproductive function and could be used in drug discovery and toxicology studies. Recently, Schimek et al. have successfully combined a bronchial OoC with a liver-on-chip model to investigate the potential toxicity of inhaled substances on a systemic level[70]. Their so-called HUMIMIC Chip comprises optimized medium supply and oxygenation of the organ cultures in separate culture compartments within a closed circulatory perfusion system (Figure 5c). This robust microfluidic chip-based

co-culture of human bronchial tissue and liver spheroids enable 14 to 28 days of culture with effective crosstalk between the two compartments. In order to model preclinical drug toxicities and metabolic conversion, a multi-OoC containing microelectromechanical systems has been setup[71] (Figure 5d). Each tissue module is cultured on a plate with specific coating to promote cell adhesion and act as extracellular matrix. A common medium is delivered to the system by gravity-induced flow using a rocking platform. Liver, neurons, cardiac and skeletal muscle modules were used and combine with multi-electrode arrays to stimulate and record their activity. Another outstanding example of human Body-on-chip model is a robotic system comprising an automated liquid handler, a custom software and an integrated mobile microscope that allows for the continuous perfusion, linking and imaging of several OoC models. The so-called 'Interrogator' can maintain the viability and organ-specific functions of vascularized, two-channel OoC (intestine, liver, kidney, heart, lung, skin, blood-brain barrier and brain) for 3 weeks in culture when intermittently fluidically coupled via a common blood substitute through their reservoirs of medium and endothelium-lined vascular channels. It could appear as a relevant device to perform pharmacokinetic/pharmacodynamic and physiologically based pharmacokinetic modelling[72] (Figure 5e).

Together, these studies show how linked multi-organ systems can help understand systemic or off-target drug effects and create Body-on-chips systems



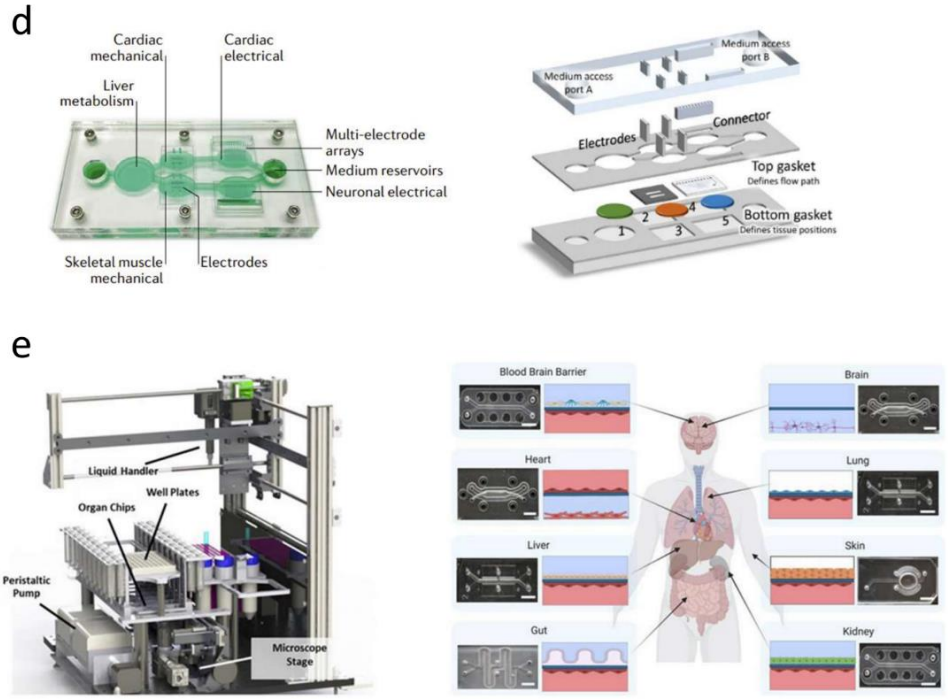


Figure 5: Example of multi-OoC systems. (a) The micro cell culture analog consists of liver, tumor and marrow chambers, interconnected with channels mimicking the blood flow pattern in the human body. Medium recirculates from the reservoir, goes through a bubble trap before reaching the organ chambers and goes back to the reservoir for recirculation. A picture of the micro cell culture analog system containing multiple chips is depicted[68] *(b) Female reproductive system containing five tissue modules (ovary, cervix, uterus, fallopian tube and liver) linked by pneumatic and electromagnetic pumps*[69] *(c) HUMIMIC chip containing three thick pump membrane acting as valve to promote medium flow in the system. The medium passes through the medium reservoir and then through the airway model (MucilAir culture from Epithelix) and liver model.*[70] *(d) Multi-organ system in a single microfluidic chip. Schematic representation of the platform assembly and design of the multi-chamber system comprising microelectrode arrays*[71] *(e) Robotic system comprising an automated liquid handler, an integrated microscope and a custom software for the continuous perfusion, linking and imaging of several OoC models (named the "Interrogator"). It can host up to 10 OoC*[72].

3. Challenges of the multi-OoC field

A challenge is the combination of parenchymal tissues (kidney, heart; liver, spleen, pancreas) and a physiological barrier model (skin, lung, gut, BBB) since their design rely on completely different bioengineering and fluid perfusion strategies.

Human Body-on-Chips platforms need to be designed to model the organism-level properties of interest. Recent studies suggest that meaningful results can be obtained using this approach that may be even more useful than those obtained from animal studies, as demonstrated by the ability to quantitative predict human drug PK parameters using a first pass intestine–liver–kidney model[73] which could potentially accelerate clinical trials. This inability of a single Organ Chip to model all facets of that organ's function, and the need to modify both single and multiple Organ Chip systems based on the questions being posed, are challenges that animal models do not have.

VII. Applications of OoC in academia and pharmaceutical industry

Building a truly comprehensive *in vitro* system can potentially partially replace animal testing. Because of their small size, OoC can be parallelized and used in early stages of drug development; in which the pharmacokinetics and pharmacodynamics of multiple compounds need to be tested. In addition, these compounds can be so specific for human that they would not show any activity in non-human models or may exhibit a different effect.

1. Towards an OoC rather than an *in vivo* validation experiment?

Donald E. Ingber recently raised the question of whether continuing to ask for data resulting from animal testing still makes scientific or ethical sense[74]. Instead, more physiologically relevant human OoC models might better serve this purpose? It is common for researchers that submit a grant application or a publication to be asked for additional animal experiments to validate their *in vitro* work. However, with the current progress in the development of complex, relevant 3D cell culture such as organoid or engineered OoC, performing animal testing may become irrelevant. In addition, when researchers directly compare the predictive value of murine models of various human disorders, the results can be biased or artifactual. Indeed, genetically engineered mice can mimic the phenotype of some human disease, the underlying molecular mechanisms can be completely different[75,76].

Before any animal model can be replaced, the OoC must be designed so that it contains the key features of the relevant organ unit that is the key site of this activity, while minimizing system complexity. For example, while a Lung Alveolus Chip containing only alveolar epithelium, endothelium, and immune cells might be used to replace an animal model of pulmonary edema that is focused on preventing pulmonary vascular leakage, it would need to be modified by adding additional connective tissue cells to replace an *in vivo* pulmonary fibrosis model.

2. Drug development (Efficacy and Safety)

The failure of pre-clinical studies to uniformly predict drug safety and efficacy in humans results in billions of wasted dollars each year and slows the development of new treatments. For some diseases, treatment options are scarce, insufficient, or do not exist. The current drug development process is slow and extremely expensive. It takes around 12 years to bring a drug to the market with costs estimated to amount to at least hundreds of millions of dollars up to $ 2.6 billion per drug that reaches the market[77]. The low predictive value of animal models for effects in humans poses a major problem. An important factor driving development of more complex systems is that drug failures mostly arise from lack of efficacy, not drug toxicity. Many therapies were not advanced because they failed to produce positive results in animals, yet they could have potentially saved human lives. This is especially true for cancer and chronic diseases without clear single gene defects, such as diabetes, Alzheimer's, and arthritis, where multiple organs and tissues may be involved in the pathology. There is an obvious need for novel approaches to capture *in vitro* the complexity of human physiology. Disease modeling, with the aim of evaluating targets and efficacy, is emerging as a prominent frontier for the field, driving development of complex OoC with perfusable microvascular networks[13].

3. Pre-clinical ADME-Tox assay

The characterization of how the body affects the fate of the drug are usually known in pharmacology as pharmacokinetics (PK). When a drug is administered to a human, it goes through at least four processes that include absorption, distribution, metabolism and excretion (ADME)[78]. Conversely, the characterization of how the drug affects the body is called pharmacodynamics (PD). It is important to address how a drug may circulate through an entire body to specifically target a diseased organ (efficacy) and discriminate between its intended target and other parts of the body (safety). PK and PD are usually studied together and consists of several readouts that influence dosing, benefit, and adverse effects[79]. To characterize the PK/PD or ADME of a drug candidate in the conventional way, animal testing is

broadly used. Unfortunately, differences in the underlying biological mechanisms can result in inaccurate prediction of human drug responses. In addition, animal models require high financial investment. Therefore, alternative model are being developed starting with well-characterized and validated individual organ systems (such as liver, kidney, gut). The ultimate aim is to develop a human-body-on-chip system where multiple organ models are fluidically linked to allow the study of interdependent PK, PK/PD and ADME relationships that can benefit safety and toxicity assessments.

A human OoC model of the kidney proximal tubule combined with quantitative computational modelling has been used to perform *in vitro*-to-*in vivo* translation (IVIVT) of experimental results. It was applied to the biomarker kidney injury molecule-1 (KIM-1), predicted human drug-induced renal toxicities and helped to identify favorable dosing regimens[80]. The same approach was used to reveal a unique mechanism by which an FDA-approved antibiotic drug (polymyxin) induces kidney injury, and to identify related chemical analogues that do not induce this toxicity[81]. Importantly, human OoC can replicate pathophysiological responses observed in humans but not in preclinical animal studies. One example is a human Kidney-on-chip model that can recreate toxicities induced by cisplatin through a human-specific protein that is not expressed in animals[82].

While most studies have focused on creating OoC containing human cells, other species-specific OoC also have been made. Rat, dog, and human Liver-on-chip have been created and that confirm species-specific hepatotoxicities induced by multiple drugs[83]. This is important because preclinical rat and dog liver toxicity studies are key requirements for regulatory approval, and they can often produce conflicting results, leading to confusion or removal of good drugs from the pipeline. These results suggest that human OoC offer a potential way to cross-validate these results between animals and humans early in the drug development process.

4. Personalized medicine

The need for personalized preclinical models will grow as the healthcare industry transitions to precision medicine. It has become obvious that there is a great variability of responses to therapy across individuals (among different genetic or ethnic populations for example)[1]. As such, it is debatable to model human genetic disorders using unrelated animal models. Preclinical human *in vitro* models seeded with organ-specific cells isolated from surgical biopsies, iPS or organoids generated from patients could become a more reliable alternative. The promises of using OoC in personalized medicine are huge. It could lead to faster and cheaper drug development, faster identification of drug targets, better outcome of drugs in clinical trials, fewer drug failures, more effective treatments, fewer negative side effects, better prediction of disease onset in individuals with inherited disease, better understanding of the mechanisms underlying complex diseases and altogether, a better quality of life for patients.

VIII. Conclusion

As stated, OoC do not mimic the function of a whole organ but rather that of a major functional subunit of an organ. The goal of OoC is not to replace animal models in a generic way but to become a human-specific experimental platform for preclinical research and drug testing. This could ultimately reduce the reliance on animal testing of experimental compounds in the pharmaceutical industry. OoC technology is still full of engineering and biological challenges.

The successful adaptation of OoC technology depends on the robustness of the microfluidic devices and the organ tissue models used. It is a long process as all OoC models must be experimentally validated in terms of their ability to reproduce the key physiological or pathophysiological properties of the particular organ, disease state, or multi-organ coupling in a consistent and robust manner. In addition, there is a clear consensus in the OoC community, industry and regulatory bodies on the need for standardization to advance the field[84]. Advances in standardized device manufacturing, organ tissue culture protocols and development of high throughput systems is required for OoC to outperform traditional models.

According to the 2018 Gartner report, OoC (referred to as 'biochips' in that report) are now in the 'peak of inflated expectations' phase. A stall in progress often occurs after this phase because the technology fails to live up to the preliminary expectations. The aim for emerging technologies is to reach this productive plateau as quickly as possible, when 20–30% of the potential audience has adopted the innovation. This is estimated to be in 5–10 years for OoC[85]. The coordinated global efforts of the OoC community will hopefully help this technology to transform science, medicine and patients' lives.

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