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Review

Multiorgan-on-a-Chip: A Systemic Approach To Model and Decipher Inter-Organ Communication

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Multiorgan-on-a-chip (multi-OoC) platforms have great potential to redefine the way in which human health research is conducted. After briefly reviewing the need for comprehensive multiorgan models with a systemic dimension, we highlight scenarios in which multiorgan models are advantageous. We next overview existing multi-OoC platforms, including integrated body-on-a-chip devices and modular approaches involving interconnected organ-specific modules. We highlight how multi-OoC models can provide unique information that is not accessible using single-OoC models. Finally, we discuss remaining challenges for the realization of multi-OoC platforms and their worldwide adoption. We anticipate that multi-OoC technology will metamorphose research in biology and medicine by providing holistic and personalized models for understanding and treating multisystem diseases.

Why Go Systemic?

Interactions between multiple organs are essential to ensure proper physiological functioning of the human body. Although organs are physically separated *in vivo*, their communication is mediated via the blood and lymph circulation by various signals (soluble factors, exosomes, cells, etc.) to maintain overall viability and homeostasis. For example, the journey of orally ingested substances (nutrients, chemicals, drugs, etc.) is well orchestrated and involves different organs through a specific sequence in which each organ has a specific function: the small intestine absorbs the (digested) substances, the liver metabolizes them, they are then delivered to target organs via the blood circulation, and the kidney excretes corresponding waste products. This complex process of **absorption/distribution/metabolism/excretion/toxicity** (**ADMET**; see Glossary) affects the fate, distribution, efficacy (if applicable), and possible toxicity of exogenous substances (e.g., food, drugs, additives, environmental pollutants) [1] through unwanted side-effects in secondary tissues.

In addition, many functions and processes in the body depend on regulatory pathways and hormonal feedback loops that involve organs of the endocrine system. The reproductive system, which comprises multiple tissues, relies on endocrine loops that control peripheral tissues. Similarly, Langerhans islets in the pancreas secrete insulin that promotes glucose uptake by the liver. Together, this systemic and cross-organ communication is key to deciphering and emulating the temporal processes involved in physiological functions.

As a direct consequence, many diseases such as sepsis, osteoarthritis, gout, infertility, and neurodegenerative diseases involve multiple organs, and systemic approaches must therefore be pursued to accurately model them. Similarly, deciphering this cross-organ communication is essential for identifying biomarkers in body fluids for diagnostic purposes. For instance, tumor

Highlights

Multiorgan-on-a-chip (multi-OoC) devices, by supporting cross-organ communication, allow the study of multiorgan processes and modeling of systemic diseases.

Multi-OoC approaches provide new insights that would be lost using single-OoC models.

Various coupling configurations have been proposed for building multi-OoC platforms, and these present different levels of user-friendliness.

Multi-OoC platforms have the potential to transform medical research by opening new avenues for understanding multiorgan diseases and for developing personalized treatments.

To further emulate the complexity of the human system *in vivo*, key elements of the immune, nervous, and vascular systems are being integrated into multi-OoC models.

The next generation of multi-OoCs will incorporate multimodal and real-time readouts in the form of on-chip chemical, physical, and molecular sensors, as well as online multiomic analysis.

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tissues release various molecules (miRNA, circulating tumor DNA, peptides, etc.), tumor-derived extracellular vesicles (tdEVs), and circulating tumor cells (CTCs) which play a central role in cancer metastasis and are key for cancer patient management [2,3].

All these examples illustrate that it is essential to include cross-organ communication and a systemic dimension, as depicted in Figure 1, which is most commonly achieved by using animal models. Nevertheless, *in vivo* models suffer from numerous limitations: high experimental costs, limited throughput, ethical concerns, and differences in genetic background. More importantly, they exhibit large physiological differences in terms of drug effects and/or disease phenotypes compared with humans, which explains the frequent failure of clinical trials [4]. Overall, animals do not allow analysis of inter-organ crosstalk, determination of quantitative pharmacokinetics (PK), or prediction of ADMET parameters, as recently highlighted [5]. Therefore, advanced *in vitro* approaches incorporating a systemic dimension and multiple organs must be developed to faithfully emulate human health and pathophysiology.

Previous efforts to study organ communication *in vitro* have employed either **conditioned medium** or cocultures in **Transwell** platforms. However, Transwell devices use large volumes

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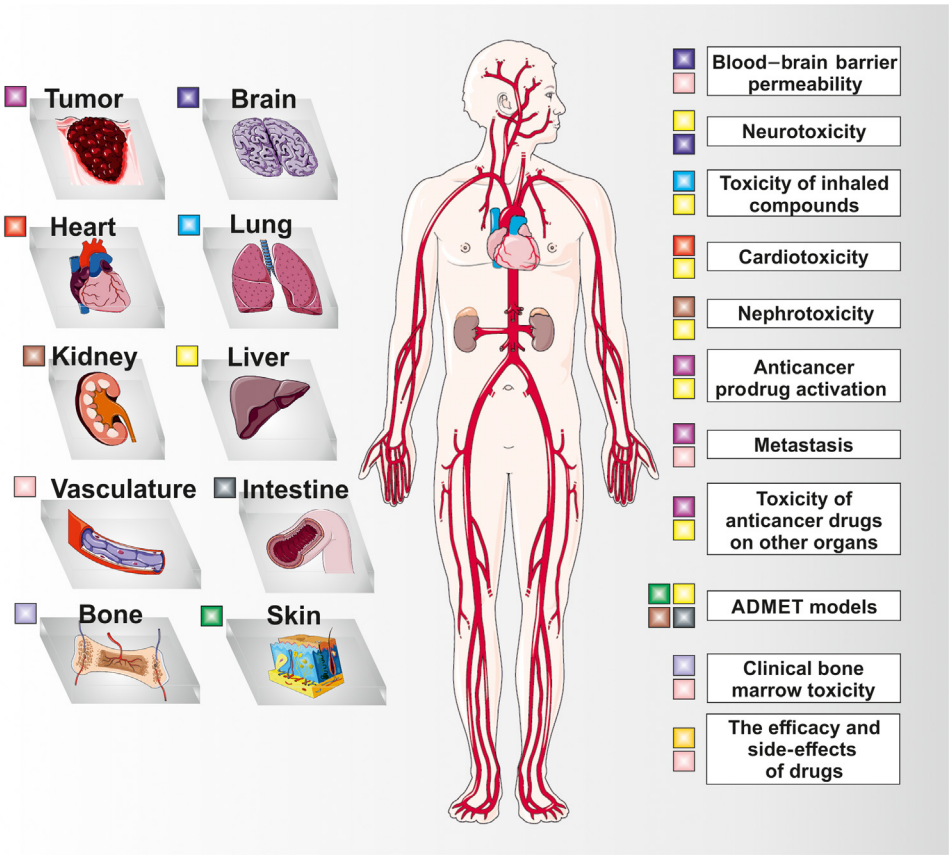


Figure 1. Various Multiorgan-on-a-Chip Combinations as a Model of Human Physiology and Pathophysiology in Different Biomedical Applications. For each application (right side), a minimal set of organs necessary to build an accurate systemic model is indicated, those organs being highlighted with colored squares (left side).

of liquid, and communication is therefore slow and low-concentration signaling factors are diluted, which altogether hampers studying cellular communication. Furthermore, the culture is entirely static, which precludes emulation of dynamic processes and the application of controlled cell biochemical and/or physical stimuli.

Using a microfluidic format can solve some of these issues by offering sub-milliliter volumes, dynamic culture, and exquisite spatiotemporal control over physical and chemical parameters in the cell/tissue vicinity. For instance, cell–cell communication has been studied in microdevices under continuous flow by using chambers separated by porous membranes [6], pillar arrays [7], or channels [8].

Building on these microfluidic cell cultures, **organ-on-a-chip (OoC)** devices aim to mimic the architecture and function of an organ by combining 3D bioengineered constructs (e.g., cell-laden hydrogels [9], differentiated epithelium [10,11], multicellular spheroids [12], and organoids [13–15]), *ex vivo* tissues (e.g., biopsies or explants) [16–18], recellularized scaffolds [19] and bioprinted constructs [20] with microfabricated structures [21], and possibly active stimulation (electrical, biochemical, or mechanical) [22–24]. The OoC field has been blossoming for a decade, and models have been proposed for virtually all organs and physiological barriers in the human body [21,25,26]. These OoC platforms are revolutionizing the field of *in vitro* experimentation and hold great promise for reducing animal testing.

Nevertheless, most OoC models are based on a single cell type or tissue, and lack both a systemic dimension and cross-organ communication. In a major recent breakthrough, multiple organs have been modeled in a single device as a **multiorgan platform** [13] (Figure 2). As detailed in Box 1, two major approaches are being pursued to realize multi-OoC platforms: coupling of single-OoC units and integration of multiple organs into one plate (multi-OoC plates).

In this review we first provide an overview of existing multi-OoC platforms and discuss combinations of organs that are best suited for particular applications. Specific areas of research are highlighted in which a multi-OoC approach brings superior information compared with single-OoC models. Finally, we discuss essential challenges remaining for the realization of multi-OoC platforms.

Latest Developments in the Multi-OoC Field

In the following section we review various multi-OoC applications. In each application we discuss the set of organs considered and highlight unique information provided by this multi-OoC approach. Selected examples over the past 5 years are summarized in Table 1 (Key Table).

Toxicity Screening

Toxicity is closely linked to metabolism by the liver, and multi-OoC approaches developed for toxicity purposes therefore include a liver model and at least one other (target) organ. For instance, to examine the acute and chronic toxicity of inhaled aerosols or drugs, human liver spheroids have been combined with a 3D lung epithelium model [27] (Figure 2C). For pharmacological studies an intestine model is typically added to this minimal liver–target organ coculture to mimic drug absorption [28]. Alternatively, when the undesired side-effects of a therapeutic treatment are being evaluated, both the target organ and the organ where side-effects are expected, for example, the kidney (nephrotoxicity), heart (cardiotoxicity) [29] or brain (neurotoxicity) [30], are modeled in the same platform. This approach has notably been used to assess the impact of anti-EGFR (epidermal growth factor receptor) treatment of a lung tumor on a skin model [31], of cyclophosphamide on heart [29], and the deleterious effects of 2.5-hexanedione on 3D neurospheres [30]. These studies collectively demonstrate the importance of coculture approaches for predicting compound safety and efficacy.

Glossary

Absorption/distribution/metabolism/excretion/toxicity (ADMET): the key processes that determine the safety, distribution, elimination, metabolic action, performance, pharmacological activity, and possible side-effects of a drug, food or/and additives therein, and environmental pollutants on a living organism exposed to them.
Conditioned medium: medium obtained from the culture of cells or tissues that contains biologically active substances released by these cells/tissues. This medium is used to stimulate the response of other cells/tissues in terms of cell physiological function such as growth, migration, etc.
Induced pluripotent stem cells (iPSCs): pluripotent cells generated from somatic human cells (fully differentiated adult cells, e.g., fibroblasts) that are reprogrammed by the introduction of three genes (encoding pluripotency transcription factors Oct4, Nanog, and Sox2) or four genes together with c-Myc. Of significance is their potential to create patient-specific cells, using a patient's own adult cells, that are extremely valuable for generating personalized tissues for disease modeling or transplantation therapies.
***In situ* analysis:** characterization of a sample directly in the (OoC) device without extraction from its location.
Multiorgan platforms: miniaturized microfluidic systems composed of several organ/tissue models that are either built from individual chip units connected by capillary tubing or integrated into a plate.
Offline measurement: analysis of a sample after it has been extracted from a (OoC) device.
Online measurement: analysis in a continuous and real-time way of samples eluted from a (OoC) device by using for instance tubing connected to a measuring instrument or assay.
Organ-on-a-Chip (OoC): a 3D engineered biological model implemented in a microfluidic format that mimics the structure, physiological function, and biomechanics of organs.
Pharmacokinetic/pharmacodynamic (PK/PD) analysis: modeling mathematical aspects to describe how fast and how completely a drug is absorbed into the body, distributed through the various tissues and fluids, metabolized, and eliminated from the body (via urine, feces, etc.).

Drug Metabolism

Multi-OoC platforms supporting liver–target organ communication similarly open new possibilities for testing prodrugs that only become biologically active following hepatic metabolism. This bioactivation process was successfully recapitulated for cyclophosphamide targeting of colorectal cancer by using spheroids cocultured under flow conditions in a 96-well format [32], and in a self-adjusting modular Tetris-like microfluidic platform (TILE) [33] (Figure 2H), where it was found to effectively overcome the 'apparent' resistance of metastatic oral squamous cell carcinoma (OSCC) tumor. The efficacy of multi-OoC was demonstrated for another prodrug, capecitabine, and its 5-fluorouracil metabolite in a liver–colorectal cancer coculture [34] (Figure 2E). Liver bioactivation was also examined for non-cancerous drugs. Both the hepatic metabolism of vitamin D and renal bioactivation of the resulting product were mimicked in a human liver–kidney multi-OoC [35] (Figure 2A), revealing enhanced expression of vitamin D metabolizing enzymes. The same liver–colorectal cancer coculture proved successful for the bioactivation by the liver of the pro-drug cyclophosphamide and enabled more potent suppression of the endothelial inflammatory response [36]. Finally, using a liver–immune system coculture, drug-induced skin sensitization was successfully predicted *in vitro* by modeling drug metabolism by 3D liver tissue and evaluating immune cascade activation by the resulting metabolites [37]. This last example further illustrates the importance of multi-OoC models for evaluating systemic drug effects that involve multiple processes and different organs.

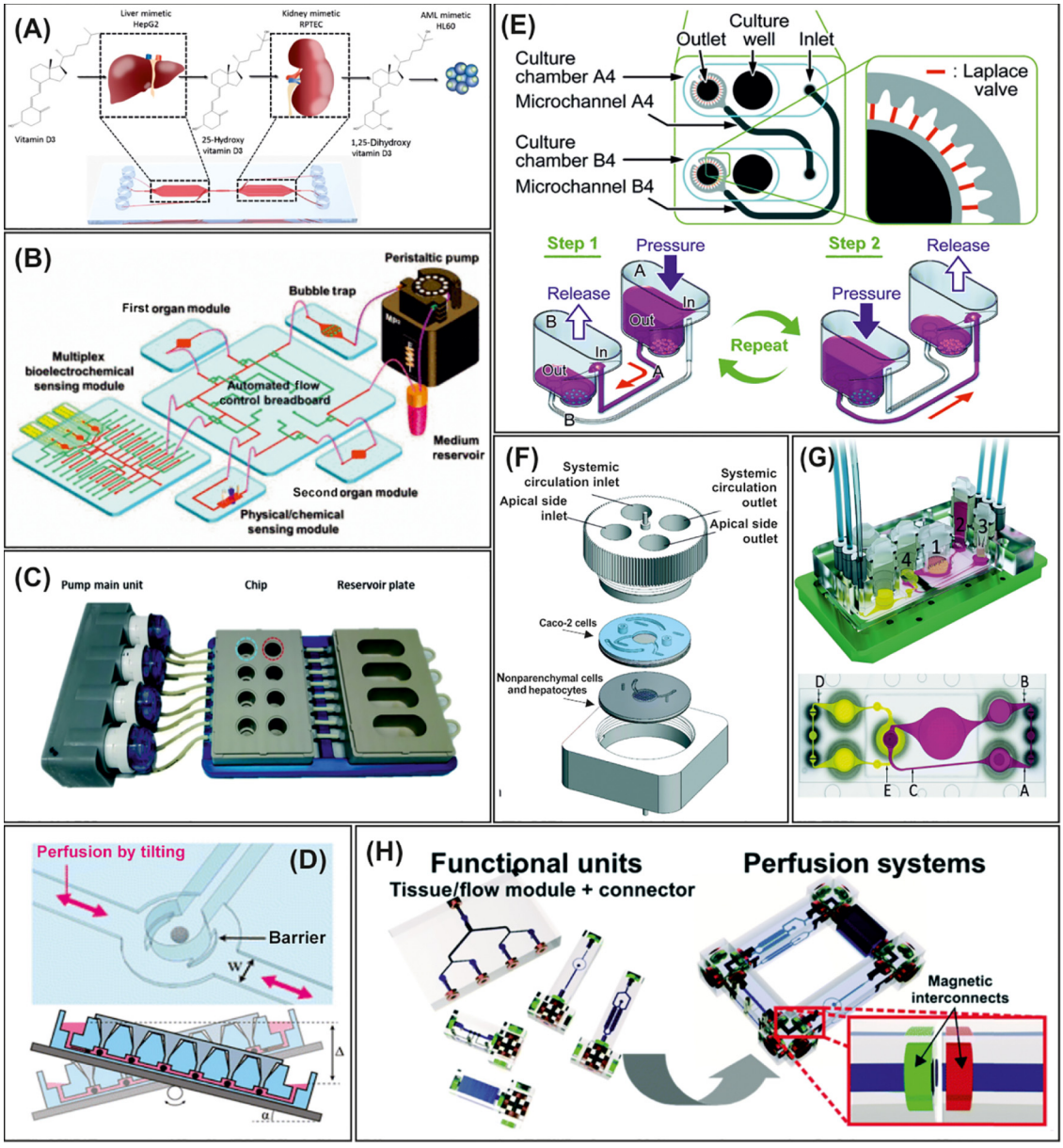
Pharmacokinetics

PK studies, that aim to understand and predict the biological effects (therapeutic or toxic) of xenobiotics on the body, require by definition modeling of various organs and their interactions. First, Skardal and colleagues emulated a drug response resulting from the crosstalk of heart, liver, and lung [38]. In another work, combining liver and lung with small intestine allowed evaluation of the PK of orally administered anticancer drugs [28]. Building further upon this approach, other organs were included in this multi-OoC platform, such as endothelium, brain, and testis [13], or liver, pancreas, gut, lung, heart, muscle, brain, skin, kidney, and endometrium [39], allowing very accurate **pharmacokinetic/pharmacodynamic (PK/PD) analysis**. Interestingly, several studies demonstrated that quantitative drug PK parameters, as measured in patients, can be predicted using a combination of a multi-OoC platform (liver–kidney or body-on-a-chip model) and PK modeling [40–42]. PK studies often suffer from the limited longevity of *in vitro* organ models, an issue which can be overcome by connecting single OoC models only once each organ culture has been established under optimized conditions [43].

ADMET Profiling

ADMET studies typically require all necessary organs to emulate the processes of absorption (intestine), distribution (blood circulation), metabolism (liver), excretion (kidney), and toxicity (a target organ). First, a modular two-organ platform combining 3D liver tissues and a differentiated gastrointestinal (GI) tract epithelium was proposed to emulate the absorption and metabolism of exogenous substances [44] (Figure 2F); both organ models were maintained for up to 14 days in this coculture configuration, and displayed enhanced cytochrome p450 (CYP) activities compared with liver alone. Next, in a quadruple coculture platform combining skin and intestine (to mimic topical and oral administrations) with liver and kidney (to reproduce drug metabolism and clearance), the integrity and functionality of all four organs were maintained for up to 28 days [45] (Figure 2G). Because each organ compartment in this platform was separately irrigated by medium, fluids could be collected at any time, which is essential for acquiring precise PK parameters such as the effective drug concentration and maximum tolerable dose. A brain model was added to the same platform, with all organs being built from **induced pluripotent stem cells (iPSCs)** derived from the same healthy donor, to produce an autologous system [46]. All organs were successfully cultured for 14 days using a common medium without any tissue-specific growth factor, and, except for the kidney, their differentiation was maintained.

Transwell: devices in which a semipermeable (porous) polymeric membrane delimits two chambers (upper and lower) in a microwell. These compartmentalized devices present basolateral and apical sides and are typically employed to model physiological barriers (e.g., skin, blood–brain barrier, vessels, etc.), to study cell–cell interactions, transport, or metabolic activities, or to monitor cell transmigration (e.g., intravasation or extravasation of cancerous cells during metastasis). Today it is used as a common name, often without its registered trademark, written 'transwell' or 'Transwell'.



Trends in Biotechnology

Figure 2. Multiorgan-on-a-Chip (OoC) Devices. (A) Multicompartment liver–kidney combination in a plate format for studying the metabolism of vitamin D3. Reprinted, with permission, from [35]. (B) Multi-OoC model comprising a microfluidic motherboard, an external peristaltic pump, and capillary connections for *in situ* monitoring of organoid behavior using integrated sensors. Reprinted, with permission, from [73]. (C) Two-way communication between lung and liver models for toxicity studies using a multi-OoC plate that incorporates liver spheroids and a differentiated lung epithelium cultured under air–liquid interface conditions. Reprinted, with permission, from [27]. (D) Tilting platform for dynamic medium perfusion in a multi-OoC plate containing spheroid models of liver and colorectal cancer. Reprinted, with permission, from [36]. (E) Multi-OoC plate with pressure-driven medium circulation between organs, applied here to two-organ (liver and colorectal cancer) and four-organ configurations (intestine, liver, tumor, and connective tissue). Reprinted, with permission, from [34]. (F) Modular multi-OoC platform for the coculture of a gastrointestinal (GI) tract epithelium and 3D primary liver tissues using gravity to actuate the flow. Reprinted, with permission, from [44]. (G) Multi-OoC plate combining four organs (intestine, liver, skin and kidney) for ADMET studies, with an on-chip peristaltic pumping module. Reprinted, with permission, from [45]. (H) Self-aligning Tetris-like (TILE) modular multi-OoC platform to study multiorgan interactions in a modular and flexible manner, with on-demand platform assembly and disassembly for analysis. Reprinted, with permission, from [33]. Abbreviation: AML, acute myeloid leukemia.

Box 1. Multi-OoC Typology and Applications

Multi-OoC devices can be classified into two main distinct types, this typology referring to the engineering approach used for their realization, namely through connection of single OoC units or by using a multi-OoC plate.

First, single OoC units are connected via capillary tubing or a microfluidic motherboard to reproduce the systemic interactions between two or more organ models (Figure IA). 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. By contrast, multi-OoC devices (Figure IB) integrate in a single-plate format all different organ models at different locations, where channels in the plate act as a vascular-like system to support inter-organ communication. This second approach is much akin to the human-on-a-chip or body-on-a-chip paradigm in which virtually all organs are modeled (Figure IC). Multi-OoC plates are more compact and user-friendly, they do not require manual and cumbersome connection, they limit the risks for leakage, and, in some cases, they can integrate a liquid actuation system. They are also advantageous for minimizing the total recirculation volume (see section on 'Circulation of Medium' in the main text). However, organ-specific vascularization is less trivial, and combining different organs modeled following various approaches see (section on .Organ Models. in the main text) may be more challenging.

These two different multi-OoC approaches are arguably better suited for specific purposes. The former 'Lego-like' approach is likely to be preferred for more fundamental research in an academic setting. However, they offer only low-to-moderate throughput, which is not ideal for preclinical, toxicity, or drug efficacy tests. By contrast, the more integrated and turnkey plate-based platforms offer higher throughput, and are hence more appropriate for the identification of biomarkers and therapeutic targets, and for the selection and optimization of drug candidates.

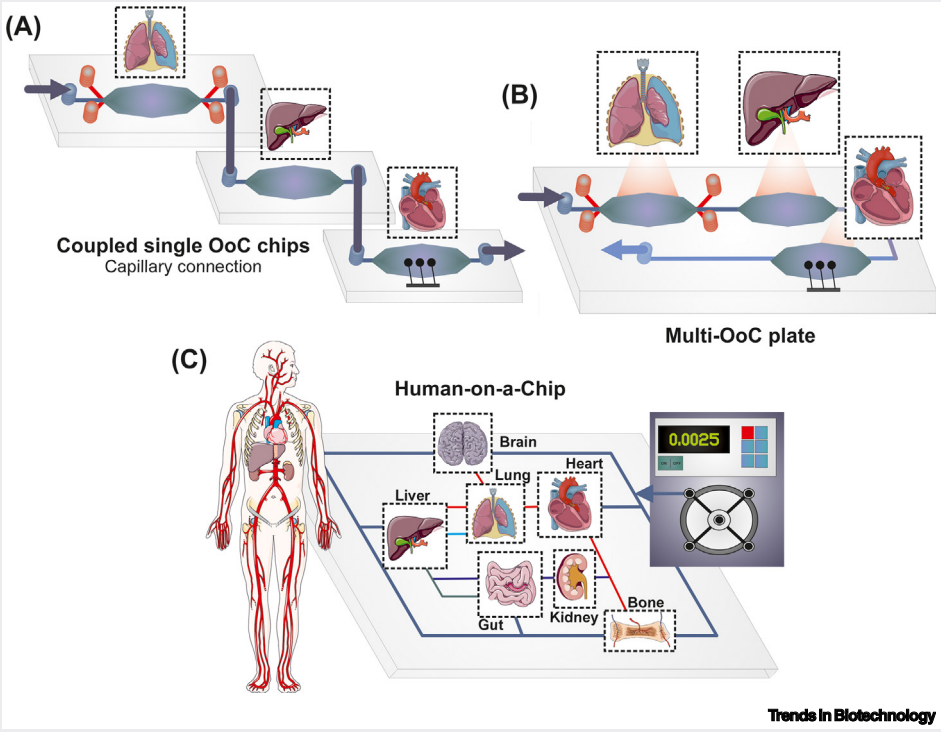


Figure I. Schematic Representation of the Two Main Approaches for Developing Multi-OoC Systems. (A) Through coupling of single OoC devices, each modeling a different organ, via capillary connection or a microfluidic motherboard (B); and (C) by integrating different organ models in a single plate, an approach that is more in line with the body-on-a-chip philosophy.

Multiorgan Metabolic Diseases and Reproductive Medicine

Modeling multiorgan diseases suffers from the poor accessibility of some organs and the fact that different cell types are involved in metabolic homeostasis. In this context, multi-OoC approaches can provide more complex disease models while giving access to key molecular mechanisms [47]. Recently, a multimodule system emulating different functions of the brain allowed the

Key Table

Table 1. Overview of Recently Reported Multi-OoC Platforms[a,b]

Classification	Application(s)	Organs	Organ modeling approaches		Vascularization (strategy)	Stimulation (biochemical, hormonal, physical)	Typology of system (see BOX 1)	Material	Medium composition	(Re-)Circulation (Yes/No/Not available (N/A)	Duration of the experiment	Analysis			Major outcome	Reference
												Mode	End-point	Assay / technique		
Toxicity screening	Evaluation of the potential toxicity of aerosols	Liver	Spheroids	HepaRG	N.A.	N.A.	Multi-OoC plate	PEEK	PneumaCult™ medium	Y	28 d	*In situ*	Characterization of the lung model	TEER and CBF (Cilia beating frequency)	Toxicity of inhaled compound reduced by liver tissue metabolism	27
												Off-line	Cell viability	ATP content (CellTiter-Glo®) LDH assay		
													Morphological changes	Immuno-staining		
		Lung	ALI	Normal human bronchial epithelial cells (NHBE)									Expression levels of phase 1 metabolism-associated genes	RT-qPCR		
													CYP enzymatic activity (liver)	Dedicated assay for CYP1A1/1B1		
													Metabolic and functional activity (Glucose consumption, lactate production, and albumin synthesis)	Dedicated assays		
	Evaluation of the toxic effects of anticancer treatment after activation by liver	Small Intestine	Monolayers on collagen coated surface	Caco-2	N.A.	N.A.	Multi-OoC plate	PDMS PET porous membrane	DMEM (+non-Essential AA Solution)	Y	≥ 3 d	*In situ*	Cell viability	Live/Dead staining	Successful replication of physiological circulation and organ ratio; new insights into the importance of small intestine and liver to evaluate the activity of anti-cancer drugs.	28
		Liver		HepG2												
		Lung Tumor		A549												
	Evaluation of neurotoxicity	Liver	Spheroids	HepaRG hHSteC (24:1 ratio)	N.A.	N.A.	Multi-OoC plate	PDMS on Glass	HepaRG medium	Y	14 d	Off-line	Cell viability	LDH assay	Enhanced sensitivity of the liver-Neurospheres model than single-tissue cultures	30
													Gene expression level (III-tubulin, Oct 4, Pol2, nestin, albumin, BSEP, CPS-1, Cyp 1A2, Cyp 2B6, Cyp 3A4, TBP)	RT-qPCR		
													Cell apoptosis and proliferation	TUNEL assay & Ki67 staining		
		Brain		NTera-2/cl.D1									Characterization of organ models (brain neuronal markers beta III-tubulin & MAP2 and pluripotent marker TRA-a-60; liver: Cyt P450 3A4, MRP-2, CK 8/18, Vimentin)	Immuno-staining on tissue cryosections		
													Cell metabolic activity (Glucose consumption, and lactate production)	Dedicated assays		

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Table 1 (*continued*)

	"Efficacy" assay to screen EGFR inhibitors in cancer treatment	Skin	*Ex vivo* human tissue Millicell insert		N.A.	N.A.	Multi-OoC plate	PDMS on Glass	E3 medium (+ glucose)	Y	5 d	*In situ*	ECM remodeling	Bright field imaging	Recapitulation of specific patterns observed in patients treated with Anti-EGFR therapy – such as release of inflammatory markers, and inhibition of the skin proliferative turnover	31
												Off-line	Tissue viability	LDH assay		
													Cell proliferation	MTT assay		
													Skin morphological change (Collagen IV, Vimention, E-cadherin)	Histology Immuno-staining		
													Cell apoptosis and proliferation	TUNEL assay and Ki67 staining		
		Lung tumor	Spheroids	hNCI-H292									Cytokine production	V-Plex Chemokine Panel I Human kit		
													Gene expression related to apoptosis induction, inflammation, differentiation	qPCR		
	Cardiotoxicity and the impact of hepatic metabolism thereon	Liver	Monolayer on collagen-coated glass coverslips	Primary human hepatocytes Hw36	N.A.	Electrical stimulation of the cardiac muscle	Multi-OoC plate	PDMS & PMMA	HSL2 (serum free, static culture) or HSL3 (serum free, housing-based experiments)	N	28 d	Online	Hepatic tissue state	Urea and albumin secretion (dedicated assays); CYP enzyme activity	Impact of the presence of liver on the effect of various drugs on the heart model.	29
												In situ	Characterization of organ models (albumin)	Immunochemistry		
													Electrical activity of the cardiomyocytes	MEA measurements		
													Cardiac contractile function	Cantilever-based force measurements		
		Heart	Monolayer on glass coverslips	Human iPSc derived cardiomyocytes								Off-line	Cell viability and metabolism	MTT assay and AlamarBlue assay		
													Drug metabolism	LC-MS/MS		
Cancer research - Metastasis	Evaluation of the invasion potential of lung cancer cells and associated fibroblasts in distant organs (brain, bone and liver)	Lung cancer	ALI	16HBE A549 (10:1 ratio)	HUVECs + macrophages (activated THP-1 and W138 cells) in basolateral compartment	N.A.	Multi-OoC plate	PDMS	N.A	N	-	*In situ*	EMT markers in a model and invasion in distant organs (E-cadherin, N-cadherin, Snail1, Snail2)	Immuno-staining	Reproduction of cancer growth and metastasis processes. Validation using a mouse model	54
		Brain	3D cell culture	Ha-1800									Lung model characterization Macrophage M2 marker CD206 + fibroblast marker a-SMA + lung cancer marker CEA			
		Bone		Fob1.1									Tight junction (E-Cadherin) in lung epithelium and endothelium			
		Liver		L-02									Cell apoptosis	Hoechst / PI staining		
													Tracking of lung tumor and stromal cells	Fluorescence microscopy (Cell Tracker dye)		
	Colon tumor metastasis in liver and drug screening	Colon cancer	3D cell culture in HA/ PEGDA / gelatin hydrogel	(RFP)-HCT-116 INT-407 (1:10 ratio)	N.A.	N.A.	Multi-OoC plate	PDMS	DMEM	Y	24 d	Off-line	Characterization of organ models (ZO-1, β-catenin, MMP 9, N-cadherin, PCNA, Vinculin)	Immuno-staining	Tumor cell migration influenced by the liver model mechanical properties and drug treatment	93
		Liver		HepG2								*In situ*	Tumor cell expansion and migration	Cell tracking		

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Table 1 (*continued*)

	Evaluation of metastatic preferences of CTC-like breast cancer cells under chemokine stimulation	Bone	Transwell-like support	rBMC	Vascular endothelial barrier (HUVECs grown in basolateral compartments)	Chemokine stimulation to trigger metastasis	Multi-OoC plate	PDMS on Glass	DMEM F12 (1:1 ratio) or L-15 medium	N	30 min	*In situ*	Breast tumor CTC-like cell invasion	Cell tracking	CTCs showed mainly metastatic potential to lung over muscle, and to liver then bone marrow over muscle in the microfluidic model Model and result validation using a mouse model.	53
													Characterization of the endothelium (ZO-1 marker)	Immuno-staining		
		Lung		Rat primary murine pulmonary cells									Cell viability	Live/Dead staining		
		Muscle		Rat primary murine muscle cells								Off-line	CXCL12 secretion from lung, liver, bone and muscle models	ELISA		
		Liver		Rat primary murine hepatocytes									Expression of CXCR4 receptor in CTC-like breast tumor cells under chemokine stimulation	Flow cytometry analysis		
	Evaluation of metastatic preference of colorectal cancer cells	Colon cancer	"organoids" 3D cell culture in hydrogel (Heprasil, Gelin-S and Extralink, ratio 2:2:1)	(RFP)-HCT116	Endothelium mimicked as an organoid not as blood vessels	N.A.	Multi-OoC plate	PDMS on glass	DMEM-10 EGM-2 (3:1 ratio)	Y	15 d	In situ	Cell viability	Live/Dead staining	Preferential colonization of colorectal cancer cells in liver and lung, as observed in vivo.	88
		Liver		HepG2									HCT116 cell tracking	Cell permanent labeling (RFP)		
		Lung		A549												
	Modeling tumor–lymph node interactions	Lymph node	*Ex vivo* mouse tissues (slices)		Innate vascularization (*ex vivo* tissues)	N.A.	Multi-OoC plate	PDMS PC porous membrane	RPMI-1640 (+mercaptoethanol, pyruvate, non-essential AA, HEPES)	Y	1 d	Off-line	Tissue viability	Live/Dead staining	Successful modeling of some features of the immune-tumor interactions	18
		Breast tumor											T-cell activity in lymph node slices (IFN-gamma)	ELISA		
Drug metabolism	Modeling bioactivation of nutraceuticals and anti-cancer prodrugs	Liver	Spheroids	HepaRG	Vascular endothelial barrier (HCAEC cells)	N.A.	Multi-OoC chip	PDMS Magnets embedded	Liver-endothelium HepaRG medium EGM2 (1:1 ratio) Liver-tumor RPMI1640 HepaRG (1:1 ratio)	Y	2 d	Off-line	Gene expression related to apoptosis	qPCR	Successful bioactivation of nutraceuticals and prodrugs Plug-and play and easily reconfigurable Multi-OoC platform	33
		Oral squamous carcinoma (primary or metastatic)		HN137 and OSCC								*In situ*	Characterization of organ models and inflammation of the endothelium (Vimentin, E-cadherin)	Immuno-staining		
													Cell viability	Live/Dead staining		
	Prediction of drug-induced skin sensitization using a liver-immune co-culture, and testing of three drugs known to cause cutaneous reaction	Liver	Spheroids	HepaRG	N.A	N.A	Multi-OoC plate	PDMS on Glass	Liver: HepaRG medium Immune cells: RPMI1640 (+ glutamine, HEPES, sodium pyruvate)	N.A.	2 d	Off-Line	Gene expression level (Liver: CYP1A2, CYP2A4, CBZ-E, 2-OH CBZ, 3-OH CBZ, p-HPPH, Oxipurinol; Immune cells for their activation: IL8, IL1β, CD86)	qPCR	Liver-immune co-culture system supporting organ culture and maintaining organ function. Successful triggering of APC activation response. Robust assay to assess the potential skin sensitization of systemically ingested drugs.	37
		Immune system	Cells in suspension	U937									Organ characterization (CD86 +)	Immuno-staining		
													Analysis of drug metabolites	LC-MS/MS		
	Activation of anti-cancer pro-drug by liver models	Liver	*Ex vivo* rate tissue	rLiMTs	N.A.	N.A.	Multi-OoC plate	PDMS on Glass	Proprietary liver microtissue medium	Y	8 d	Off- line	Albumin production	ELISA	Activation by the liver required for activation of the pro-drug cyclophosphamide.	32
													Quantification of prodrug and its metabolites	LC-MS/MS		
												In situ	Tumor spheroid size	Microscopy		
		Colorectal cancer	Spheroids	(eGFP)-HCT116									Characterization of organ models (DPPIV/CD6; actin/nuclear stain)	Immuno-staining		
													Cell viability	ATP assay (CellTiter-Glo) and PI staining		

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Table 1 (*continued*)

	Activation of anti-cancer pro-drug by liver models	Intestine	Monolayer on glass or Transwell	Caco-2	N.A.	N.A.	Multi-OoC plate	PDMS PC membrane Glass	Medium 670	Y	3 d	Off-line	Cell proliferation	AlamarBlue assay	Successful emulation of the processes of absorption (intestine), metabolism (by liver) and cell killing for tumor cells and connective tissues	34
		Liver		HepaRG									Drug concentration and its metabolites	LC-MS/MS		
		Colon cancer		HCT-116								*In situ*	Cell viability	Live/dead staining		
		Connective tissue		TIG-121									Quality of the intestine epithelium	TEER		
	Evaluation of the efficacy and side-effects of drugs on multiple organs	Liver	Organoids: HA/gelatine hydrogel with liver ECM solution	Human primary hepatocytes hHSteC Kupffer cells (80:10:10 ratio)	Endothelium (HMVEC-L) grown in lung module below the Transwell membrane	N.A.	Multi-OoC chip	PDMS on glass PET porous membrane Capillary connection	α-MEM	Y	9 d	Online	Stability of heart model	Beat rate analysis	Response to drug depending on tissue-tissue interactions; simultaneous effect of drug efficacy and side-effects on other organ	94
		Heart	Fibrin-gelatin hydrogel	Human iPSc-derived cardiomyocytes hPCF (90:10 ratio)								Off-line	Cell viability	Live/dead staining		
													Metabolic profiling	LC-MS/MS		
		Lung	Multi-layer culture on ECM-coated membranes	AMSC NHBE (layer by layer)									Secretion of inflammatory products (IL-8 & IL-1beta) by lung tissue	ELISA		
													Biomarker detection	Electrochemical impedance spectroscopy (EIS)		
	Proof-of-concept study to demonstrate the capability of a Lego Plug-and-Play system for Multi-OoC studies	Multiple heart models	Multilayer	Human iPSc derived cardiomyocytes	N.A.	N.A.	Multi-OoC chip	PDMS on glass	RPMI 1640 (+B27, Insulin)	N	3 d	*In situ*	Cell viability	Live/Dead assay	Successful maintenance of multiple heart tissues in one platform	43
													Functionality of the cardiac tissues	Bright field imaging		
	Patient-specific Multi-OoC model prepared using iPSCs from the same healthy donor	Intestine	Transwell spheroids	Human iPSC derived intestinal organoid Human iPSC derived stromal cells	N.A.	N.A.	Multi-OoC Plate	PDMS on glass PC membrane	HepaRG medium	N	14 d	Off-line	Characterization of organ models (Liver: albumin, ZO-1,4 alpha, SLC10A1, Cyt 8/18, Vimentin, Ki67; Intestinal: CdX2, Na+/K+-ATPase, Cyt 8/18, Vimentin, ZO-1, Ki67; Kidney: Cyt 8/18, Vimentin, Aquaporin 1, Na+/K+-ATPase, ZO-1, Ki67; Brain: TUBB3, PAX6, Nestin, TBR1, MAP2, ZO-1, Ki67)	Immunostaining	Patient-on-a-chip platform Successful maintenance of three organs in differentiated state for 14 days using one single medium; no differentiation of kidney model.	46
		Liver	Spheroids	Human iPSC derived Hepatocytes Human iPSC derived stromal cells									Gene expression level	qPCR		
													Tissue differentiation	RNA sequencing		
													Metabolic activity	Glucose assay		
		Brain	Spheroids on Transwell	Human iPSC derived Neurospheres									Tissue viability	LDH assay		
		Kidney	Monolayer on porous membrane	Human iPSC derived renal cells									Cell apoptosis and proliferation	TUNEL assay & Ki67 staining		

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Table 1 (*continued*)

	Reconstruction of complex metabolic interactions in a liver-kidney model, and the activation of leukemia cells by the metabolized drug	Liver	Monolayers on collagen coated surface	HepG2	N.A.	N.A.	Multi-OoC plate	COP	DMEM (high glucose no supplement)	N	1 d	Off-line	CYP mRNA expression analysis in all organ models	RT-qPCR	Successful emulation of metabolism of vitamin D3 by the liver and its bioactivation by the kidney; enhanced expression of Vit D3 metabolizing enzymes	35
													Differentiation of HL60 cells	Flow cytometry analysis		
													Analysis of Vit D3 metabolism and fate of its metabolites	LC-MS/MS		
		Kidney		RPTEC									Production of albumin by liver model	ELISA		
												In situ	Functionality of kidney model	Albumin uptake (FITC-albumin); fluorescence microscopy		
	Body-on-a-chip platform with pumpless medium perfusion	Liver	3D scaffolds	Human primary hepatocyte Non-parenchymal cells (primary human fibroblasts hHSteC, Kupffer cells, hLSMECs, vascular, biliary epithelial cells) (5:3 ratio)	Endothelial cells added to liver models	N.A.	Multi-OoC chip	3D printed Veroclear polymer PC porous membrane	DMEM # L3SNB-500 (1:1 ratio)	Y	14 d	*In situ*	GI tract epithelium function	TEER	Maintenance of tissue function in the liver-intestine model including enzymatic activity to response to toxicants	44
												Off-line	Liver cell viability	Level of aspartate aminotransferase		
		GI tract epithelium	Transwell-like support	Caco-2									Function of liver tissue	Urea and albumin synthesis		
													Liver tissue response to toxicants	Cyt P450 enzyme activity assay		
	ADME profiling with repeated dose systemic toxicity testing of drug candidates	Intestine	Reconstructed model (EpiIntestinal™)		Innate vascularization of skin (*ex vivo* tissue)	N.A.	Multi-OoC plate	PDMS on Glass PET porous membrane	Small intestine culture medium (+glucose, human serum) HepaRG medium, (+glucose, human serum) Proximal tubule cell medium (+glucose)	Y	28 d	Off-line	Cell viability	LDH assay	Reproducible homeostasis of all organs within 2- 4 days Maintenance of tissue functionality over a period of 28 days	45
													Cell metabolism (Glucose consumption & lactate production)	Dedicated colorimetric assays		
													Cell function (albumin synthesis)			
		Liver	Spheroids	HepaRG hHSteC (24:1 ratio)									Gene expression level (Intestine: SGLT1/SLC5A1, Na-K-ATPase, MDR1; Kidney: SGLT2/SLC5A2, Claudin 10, TJP3/ZO-3; Liver: Albumin, BSEP/ABCB11, GSTA2, CPY3A4, UGT1A1, MRP2)	RT-qPCR		
		Kidney	Differentiated "3D" epithelium	RPTEC/ TERT-1									Tissue-specific markers (Liver: Cyt P450 3A4; Skin: CK 10 & 15; intestine: CK 19)	IHC		
													Kidney epithelium: CK 8/18 & NaK-ATPase	Immuno-staining		
		Skin	*Ex vivo* human biopsy Air-liquid interface culture in a Transwell									*In situ*	Barrier function of the intestine	TEER		

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Table 1 (*continued*)

	Parallel assessment of drug efficiency (3-organ model) and toxicity on multi-organoid models (6-organ models)	Liver *3 and 6 organ platform*	Organoids in HA/gelatine hydrogel	Human primary hepatocytes hHSteC Kupffer cells LECs (75:10:10:5 ratio)	Vascularized organoids and endothelium in 6-organ model (H.UVEC cells in hydrogel)	N.A.	Multi-OoC plate	Adhesive film on glass	Testis organoid media EGM media (without FBS) (1:1 ratio)	Y	14 d	*In situ*	Cell and organoid viability	Live/Dead staining	Successful metabolization of the alkylating prodrug isfomamide by the liver to induce neurotoxicity	13
		Heart *3 and 6 organ platform*		Human iPSCs derived cardiomyocyte Cardiac fibroblasts Cardiac ECs (75:20:5 ratio)												
		Lung *3 and 6 organ platform*		A549 *(3-organ model)*												
		Testes *6 organ platform*		Lung fibroblasts HBEC (80:20 ratio)												
		Brain *6 organ platform*		SSC Leydig Sertoli cells (80:10:10 ratio)												
				Primary HBMEC HBVP, hA, HM, HO, HNC (30:15:15:5:1 5:20 ratio)												
	In vitro Multi-OoC model to provide quantitative PK/PD data	Liver	Transwell-like support	Human primary hepatocytes primary hLSMECs	Organ-specific microvasculature incorporated in all organs	N.A.	Multi-OoC chip	PDMS PET membrane	Common "blood substitute": DMEM F12 EGM-2 (+growth factors) Specific medium for each organ parenchymal compartment	Y	10 d	*In situ*	Barrier permeability	Translocation of fluorescent tracers	Excellent prediction of PK parameters for nicotine (oral administration) and the anti-drug cancer cisplatin (intravenous injection) Agreement with cisplatin PD data with data acquired on patients	40
		Gut	3D culture (villi) on porous membrane	Caco-2 BBe										TEER		
		Kidney	Transwell-like support	primary hRPTECs								Off-line	Liver function (Albumin secretion)	ELISA		
													Cytochrome P450 CYP3A4 activity	Dedicated assay		
													Quantification of CYP2A6 and P-glycoprotein	Western-Blot BCA assay		
		Bone marrow	3D cell culture in fibrin gel (apical)	Human primary bone marrow CD34+ progenitor cells									Analysis of bone marrow cells	Flow cytometry		
													Quantification of nicotine and its metabolites	LC-MS/MS		
	Establishment of a Multi-OoC platform with 4, 7 or 10 organ models, or physiome-on-a-chip for quantitative pharmacology study	Liver /Immune *4, 7 and 10 organ platform*	3D culture on scaffold	HPH Kupffer cells (10:1 ratio)	N.A.	N.A.	Multi-OoC plate	PSF on acrylic PU membrane	Mixed medium (N/A ratio)	Y	14 d 21 d 28 d	Off-line	Organ function	Liver (albumin); endometrium (IGFBP-1); pancreas (C-peptide); muscle (myostatin) ELISA	Maintenance of phenotypic markers for 2 weeks (*4 organ platform*) Robust operation and maintenance of phenotypic function for 3 weeks (*7 organ platform*) Maintenance of phenotypic function for 4 weeks and PK analysis (*10 organ platform*)	39
		Lung *4, 7 and 10 organ platform*	ALI	NHBE												
		Gut /Immune *4, 7 and 10 organ platform*	Transwell	Caco-2 or C2BBe1 HT29-MTX-E21 (9:1 ratio) Dendritic cells (basal side)									Brain function (N-acetyl-aspartate)	LC-MS/MS		
		Endometrium *4, 7 and 10 organ platform*	Multilayer culture tHESCs in PEG hydrogel, Ishikawa cells on hydrogel Transwell	Ishikawa cells tHESCs									Pharmacokinetics of anti-inflammatory drug and its metabolites			
		Brain *7 and 10 organ platform*	3D culture *(7 organ platform)*	NPCs								*In situ*	Cardiomyocyte beating frequency	Video microscopy		
			Transwell *(10 organ platform)*	Human iPSCs derived astrocytes and neurons												
		Heart *7 and 10 organ platform*	Transwell	Human iCell cardiomyocyte 2												
		Pancreas *7 and 10 organ platform*	3D culture PS scaffold *(7 organ platform)* Alginate hydrogel *(10 organ platform)*	Rat pancreatic islets												
		Kidney *10 organ platform*	Transwell	RPTEC									Epithelial barrier integrity (Gut, lung, skin and kidney)	TEER		
		Skin *10 organ platform*	Culture on collagen matrix Air-liquid interface	Human keratinocytes												
		Skeletal muscle *10 organ platform*	Transwell	Human primary skeletal muscle myoblasts												

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Table 1 (*continued*)

Metabolic and multi-organ diseases & Reproductive medicine	Evaluation of the ovarian hormone control of downstream human female reproductive tract and peripheral tissues (liver)	Ovary / follicle *1, 2 and 5 organ platform*	*Ex vivo* tissues	Mouse tissues	Innate vascularization (*ex vivo* tissue)	Hormone stimulation (Continuous perfusion of prolactin during luteal phase day 0-14)	Microfluidic motherboard	-	αMEM F-12 (+BSA, BF, Insulin, Transferrin, Selenium) (1:1 ratio)	Y	28 d	Off-line	Stability of organ models Morphology analysis	Histology	Emulation of endocrine loops between organs. Murine ovarian follicles able to reproduce the 28-menstrual cycle.	51
		Fallopian tube *1, 2 and 5 organ platform*		Human tissues									Oocyte spindle morphology Chromosome alignment	Immuno-staining		
		Uterus *1, 2 and 5 organ platform*											Tissue fonction (Endometrium Ki67, CK, ER, PR; Cervix, PR & Ki67)			
		Cervix *1, 2 and 5 organ platform*											Stability of fallopian model (OVGP1 and alpha-tubulin)	Immunoblot analysis		
		Liver *1 and 5 organ platform*	Microtissues in alginate or 3D-printed on gelatin scaffolds	Human primary hepatocytes Non-parenchymal cells (Kupffer cells, hLSMECs, hHSteC)									Monitoring of fallopian epithelial cilia beating			
													Hormone production (E2 - oestradiol, P4 - progesteron, inhibin A, inhibin B, FSH, hCG)	Immuno-assays (e.g., ELISA, chemilumi-nescent assays)		
													Chemokine (IL8, VEGF-A)			
													Liver function (albumin)			
	Model for type 2 diabetes	Pancreas (islets of Langerhans)	Human pancreatic islets of Langerhans		N.A.	High glucose stimulation	Multi-Ooc plate	PDMS on Glass	HepaRG medium (without insulin)	Y	15 d	Off-line	Measure-ment of pancreatic (insulin, glucagon and CK8/18, vimentin and albumin) and liver function (CK8/18, vimentin, albumin, CYP3A4)	Immunohisto-chemistry	Establishment of a functional coupling, with release of insulin in response to glucose stimulation and enhanced glucose uptake in presence of insulin.	50
													Insulin receptor expression level	RT-qPCR		
		Liver	Spheroids	HepaRG phHSteC (24:1 ratio)									Glucose concentration	Dedicated assay		
													Insulin production albumin expression and AKT expression (phospho-rylated *vs.* non-phospho-rylated)	ELISA		
	Modeling of the gut-liver-immune axis in a Multi-OoC platform while mimicking ulcerative colitis (UC)	Liver	3D culture on PS scaffold	Human primary hepatocytes Kupffer cells (10:1 ratio)	N.A.	Short chain fatty acids (SCFAs): acetate, sodium propionate, sodium butyrate	Multi-Ooc plate	PSF PET membrane	William's E medium (+cell maintenance supplement pack, IL-2, Hydrocortisone, glucose, insulin)	Y	4 d	Off-line	Liver function (albumin production)	ELISA	New insights into the link between UC, liver function and SCFAs. Impact of SCFAs on UC positive or negative depending on the activation state of the immune system.	47
													Metabolomic analysis; analysis of SCFA concentration	LC-MS/MS		
		Gut	Transwell	Colon organoids prepared from patient biopsy (apical side) Monocytes-derived dendritic cells and macrophages (basal side)									Cytokine and chemokine analysis	Dedicated multiplexed assay		
													Influence of the organ model interaction and the SCFAs on gene expression level	RNA sequencing		
		Immune system	Cell suspension	CD4+ Treg Th17 (2:1 ratio)									Characterization of organ models (F-actin, CD14))	Immuno-staining		
												In situ	Gut model integrity	TEER		

(See table footnote at the bottom of the next page.)

contributions of different cells to the function of the entire organ to be dissected, and revealed metabolic coupling between neurons and microvascular cells of the blood–brain barrier (BBB) [48]. Metabolites of the drug methamphetamine, that are produced by vascular cells, were found to directly increase the synthesis and secretion of neurotransmitters by neurons. Connecting the liver, gut, and circulating immune cells brought new insights about the role of short-chain fatty acid (SCFA) metabolites in liver and inflammatory gut diseases (e.g., inflammatory bowel disease and ulcerative colitis) and the immune response [47]. Hematopoietic dysfunction [49] and diabetes type 2 mellitus [50] have also been modeled using multi-OoC approaches. For instance, cocultures of human pancreatic islets and liver spheroids successfully maintained postprandial glucose concentrations in the circulation, thereby mimicking the feedback loop that controls glucose consumption and insulin secretion, whereas glucose levels remained elevated in both organ modules when cultured separately. In a final example, *ex vivo* tissues of all organs of the female reproductive tract (ovary, fallopian tube, uterus, and cervix) were cocultured with liver organoids and recirculated medium, and emulated the endocrine loop through timely hormonal stimulation to successfully reproduce the 28-day human menstrual cycle [51].

Cancer Metastasis

In cancer, cross-organ communication can lead to disease metastasis, which is the main cause of cancer mortality [52]. Metastasis, which is driven by CTC intravasation and their colonization of other organs, is known to occur in preferred niches. To understand the processes involved in this metastatic cascade and to design new treatments, multiorgan models that combine the tumor and potential metastatic niches are highly desired. Coupling 3D colorectal cancer and liver models in a two-organ plate, with real-time monitoring of cancer cell migration, revealed the formation of metastatic clusters in the liver [38], as well as the importance of the mechanical properties of the liver microenvironment for cancer spreading. The preference of cancer cells for homing to different organs was studied in a four-organ plate, and demonstrated that breast CTCs in the perfusion invaded lung, bone, and liver, but not muscle [53], in full agreement with animal studies. Perfusion of metastatic inhibitors stopped the invasion process, as in animal models. Spreading of lung tumor cells into different distant organs (brain, bone, and liver), that were all equipped with a microvasculature, was similarly examined in a multi-OoC plate [54], and demonstrated metastasis of cells undergoing EMT (epithelial–mesenchymal transition) to all three target organs. These examples illustrate the potential of complex *in vitro* multi-OoC models for predicting cancer metastasis and testing antimetastatic treatments.

What Are the Current and Remaining Challenges in the Multi-OoC Field?

In this final section we discuss the challenges we have identified for building multi-OoC platforms (some of which are also valid for single OoCs) regarding the specificity and constraints of each

[a]Abbreviations: A549, human non-small cell lung cancer cells; AA, amino acid; ALI, air–liquid Interface; AMSCs, airway stromal mesenchymal cells (donor derived); APCs, antigen-presenting cells; BCA, bicinchoninic acid; BF, bovine fetuin; BSA, bovine serum albumin; Caco-2, heterogeneous human epithelial colorectal adenocarcinoma cells; ECM, extracellular matrix; FBS, fetal bovine serum; Fob1.19, human osteoblast cells; hA, human astrocytes; HA, hyaluronic acid; HA-1800, human astrocyte cells; 16HBE, human bronchial epithelial cells; HBMECs, human brain microvascular endothelial cells; HBVPs, human brain vascular pericytes; HCT-116, human colon cancer cells; HepaRG, human hepatic stem cells; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer; HepG2/C3a, human hepatocellular carcinoma cells; hHSteC, human hepatic stellate cells; HL60, human leukemia cells; hLSMECs, human liver sinusoidal microvascular endothelial cells; HM, human microglial; HMVEC-L, human lung microvasculature endothelial cells; HNC, human neural cells; hPCF, human primary cardiac fibroblasts; hRPTECs, human renal proximal tubule epithelial cells; HUVECs, human umbilical vein endothelial cells; Hw36, human primary hepatocytes; Kupffer cells, stellate macrophages; IFN, interferon; IL, interleukin; L-02, human hepatocyte cells; LC, liquid chromatography; LDH, lactate dehydrogenase; MBA-MD-231, human breast cancer cells; MCF-7, human breast cancer cells; MDCK, human Madin–Darby canine kidney cells; MEA, measurements of neurons using the Maestro™ MEA (Multi Electrode Arrays) system; NHBE, normal human bronchial/tracheal epithelial cells; NPCs, neural progenitor cells; NTera2/cl.D1, pluripotent human testicular embryonal carcinoma cells; PEEK, polyetheretherketone; PEGDA, poly (ethyleneglycol) diacrylate; PET, polyethylene terephthalate; PI, propidium iodide; PSF, polysulfone; RPTECs, human primary renal proximal tubule epithelial cells; RPTEC/TERT-1, human immortalized renal proximal tubule cells; SSCs, spermatogonial stem cells; THP-1, human monocyte cells; TIG-121, normal human diploid fibroblast cells; Treg, regulatory T cell; UC, ulcerative colitis. Cell lines preceded by (GFP) or (RFP) indicate that they have been engineered to express GFP/RFP.
[b]References [13,18,27–35,37,39,40,43–47,50,51,53,54,88,93,94] can be found in the reference list at the end of the paper.

organ environment, inter-organ coupling strategies from a biological and engineering point of view, and how to stimulate individual organs and measure cross-organ communication.

How Simple Is Complex Enough?

The biological question to be addressed or the physiological process to be modeled drive the design of multi-OoC models, notably in terms of the type and number of organs. In some cases a simple engineering approach is sufficient to mimic an organ function, for example, by integrating a peristaltic micropump to mimic pulsatile blood flow [55]. In sharp contrast, studying the systemic toxicity of drug candidates or deciphering disease etiology requires dynamic crosstalk between several organs [25,39,56–58], and, in turn, specialized microenvironments and interconnecting flows to provide more physiological conditions [59].

In Which Environment to Build Models?

A multi-OoC platform connects different organ models, and therefore a first essential question is – what is the best approach and environment for building each organ model, from both a physiological and a platform point of view? The first element is already becoming challenging when parenchymal tissues (e.g., fat, kidney, heart, adrenal glands, liver, spleen, and pancreas) are combined with physiological barriers (e.g., BBB, skin, GI tract, and lung) whose modeling requires entirely different engineering and perfusion strategies.

Physiological barriers are typically created using compartmentalized devices in which different cell types are cultured on different sides of a porous membrane [45]. To ensure full differentiation of the epithelial layer into a stratified and properly functioning structure, continuous perfusion is applied [60], but two independent perfusion lines with different media are required, versus one only for parenchymal tissues. Barrier models are used to evaluate drug or toxicant translocation through the BBB [48], intestinal [61], or blood–alveolar barriers [62], through the skin [31], and their elimination in the kidney [63], as discussed earlier.

Parenchymal tissues are best modeled using 3D culture approaches, possibly mimicking the *in vivo* architecture and complexity, as well as combining multiple cell types. Various 3D culture strategies have been proposed. Tissue biopsies or explants emulate the full complexity of the tissues; however, they are restricted in terms of supply and inter-donor variability [26,31], and they are also often too large to easily be incorporated into microfluidic devices. 3D bioengineered constructs are therefore preferred. Of these, multicellular organoids and tumoroids [2,9,13,14,16,29,34] require appropriate environments for self-organization and differentiation [15] that can be provided by adequate matrices. Natural matrices are mostly employed, such as Matrigel [6,7], collagen [10,27,64], hyaluronic acid [11,13,33], and gelatin [64]. Nevertheless, organ-specific decellularized matrices [65,66] more faithfully reproduce the *in vivo* environment. Alternatively, synthetic hydrogels (e.g., polyacrylamide, polyethylene glycol–fibrinogen, and polylactic acid) offer more controlled, tunable, and reproducible environments; they support animal-free experimentation [65,67]; and can be engineered to include molecular cues for cells to adhere, differentiate, mature, and sustain proper functionality over time [67–69].

Materials

As for any microfluidic device, the choice of material(s) from which multi-OoC platforms are fabricated is crucial. PDMS (polydimethylsiloxane) remains the number one material in the academic community: it is gas-permeable, optically transparent, easy to process, and its elastomeric properties are advantageous for integrating valves and/or pumps, and for mechanically stimulating cells [70,71]. Nevertheless, significant concerns have been raised about PDMS because it is a porous hydrophobic material that is prone to absorb small hydrophobic molecules

(such as drugs and hormones) and could possibly release uncured oligomers that can interfere with the experimental outcomes [72]. PDMS is also incompatible with large-scale fabrication and device commercialization. Recently, nonabsorbent elastomeric polymers (e.g., styrene–ethylene/butylene–styrene polyurethane elastomers) have been developed for OoC applications [73,74]. Other promising alternatives are inert thermoplastic polymers (polymethyl methacrylate, PMMA), polycarbonate (PC), polystyrene (PS), cyclic olefin (co)polymer (COC/COP), polyetherimide (PEI), and polysulfone (PSF/PSU) [75] that are used as porous membranes in compartmentalized devices. More biomimetic solutions using soft and/or curved substrates are currently being developed to build barrier models [76]. 3D-printed materials have entered the OoC field [77], with the promise of offering both faster turnover in fabrication and easier realization of multimaterial platforms with integrated sensors [78]. However, 3D-printed materials are often nontransparent, which precludes *in situ* imaging, and may release toxic compounds that could possibly act as endocrine disruptors [60]. Similarly, 3D bioprinting allows processing, in a single step, of multiple materials together with different cell types to yield precisely controlled (tissue) architectures [79]. In this approach, smart materials with changeable shapes or functionalities can introduce a fourth 'temporal dimension' of stimulus-responsive structures to better mimic organ function [80,81].

Scaling

Extreme miniaturization of *in vitro* organ and OoC models, without appropriate scaling, can cause significant structural reorganization and changes in organ proportions [82], and this is particularly important for toxicity and drug screening assays, metabolic studies, and PK/PD modeling [83,84]. However, scaling remains a significant challenge. The size of the organ, the flow and shear in each organ module, and the total volume of medium must all scale to physiological dimensions. Disproportionately scaled multi-OoC devices do not properly replicate organ–organ interplay [75], and affect the residence time of medium in the recirculation, thus introducing a bias into the experimental outcome [85]. Various scaling approaches (proportional, allometric, and functional scaling) have been introduced in the OoC field, as discussed in a recent review [86]. Nevertheless, none of them correctly emulates all the *in vivo* features in mini-organ models.

How to Combine Models Prepared Using Different Approaches?

Medium Composition

To support the growth, long-term viability, and function of all the organs in a multi-OoC platform, appropriate medium must be supplied to fulfill the requirements of each organ. Consistent with the strategy of engineering a multitissue microenvironment, a universal blood substitute should feed all organ compartments in a multi-OoC platform. Human serum could ideally fulfill this role and maintain the physiological function of all cell types because it nourishes the entire body *in vivo*. However, synthetic strategies in the form of 'blood surrogate' have been preferred (see later) to avoid infection risks, inter-sample variability, and administrative constraints linked to donor consents. Arguably, this human serum approach still holds promise for the creation of patient-specific models.

Culture media typically contain animal-derived serum that presents inter-batch variability. Chemically well-defined serum-free basal media have been developed, for example, consisting of a 50:50 DMEM:Ham's F-12 mixture [40] or a high-glucose medium [35], that were nevertheless able to maintain the functionality of only a few connected organs (cardiac, muscle, neuronal, and liver modules) over several weeks [87]. Noteworthy, this one-medium approach is even more challenging when using more sensitive primary and/or immune cells [48].

To feed all organs in multi-OoC models, common media (MEM, DMEM, or William's medium) have been supplemented with specific hormones [47,50], growth factors [31], fatty acids and lipids [47], vitamins [31], or trace elements [51] to provide organ specificity [27,30,33,45,53]. However,

tedious optimization is necessary to include all the required supplements while ensuring that they are not detrimental for any other organ. Alternatively, common media have been prepared by mixing individual media in specific ratios [39,44,49,51,59,88], possibly using *in situ* computer assistance [39]. In this latter scenario, culture is typically initiated using organ-specific media before inter-organ communication is established and medium composition is optimized [39,89,90]. Ultimately, to identify ideal common media, biological optimizations must be combined with engineering efforts to support precise medium exchange and/or the integration of mixing units [90].

In a final but more flexible approach, organs are cultured as physically separated entities in compartmentalized devices. The communication is occurring through either porous membranes or an endothelium in the case where each organ is 'equipped' with a blood vessel. Both approaches allow altogether media to be independently tailored [56,91,92].

Circulation of Medium

How OoC models are coupled typically depends on the specific purpose of the study. Medium can be perfused in one direction to study the influence of one organ on other(s) [29,35,43,49,53,54] or recirculated to emulate reciprocal interactions [18,27,28,30,32–34,41,44,45,51,88,93–95] (Figure 3) and better reproduce the *in vivo* situation.

As highlighted in Box 1, multi-OoC models can be created (i) by using capillary tubing to connect single-OoC modules [94,95] (Figure 3), (ii) by attaching them to a microfluidic motherboard that includes all microfluidic connections, and possibly sampling and sensing units [33,43,44], or (iii) by using a user-friendly plate approach [27,29,30,45,93] (Figure 3) in which one connecting channel acts as a vasculature-like system. In a recent modular and entirely reconfigurable approach, 'Interrogator', medium was transferred between single-OoC devices using liquid-handling robots [41]. Finally, some OoC devices, for example, models of physiological barriers,

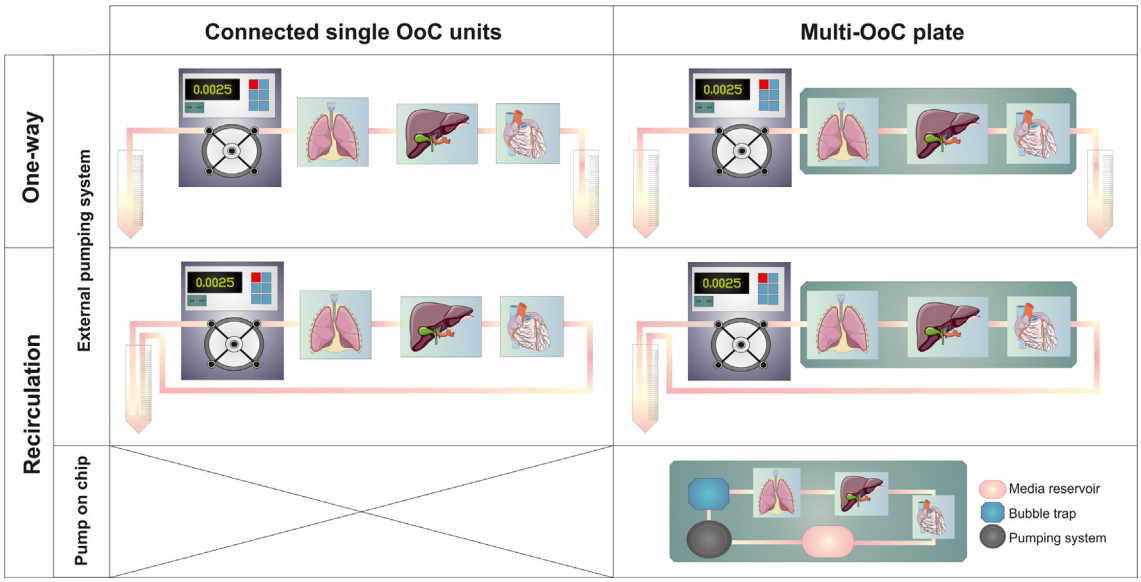

Figure 3. Various Strategies for Establishing Communication between Different Organ Models in a Multiorgan-on-a-Chip (OoC) Platform. Connected single OoC units and integrated Multi-OoC plates can reproduce one-way communication from organ A to organ B (top row) or two-way communication (recirculation) between organs A and B (bottom row). Flows are generated using an external pump or an on-chip pumping system in a multi-OoC plate, also including bubble traps (bottom, right).

involve two independent perfusion lines [48,60–63], which necessitates the inclusion of multiple fluid circuitries in multi-OoC platforms [45,46].

In all the scenarios discussed here, the microfluidic circuitry and perfusion parameters must be properly designed to both support and measure organ communication. Specifically, the flow rate should be optimized to ensure that secreted factors achieve a given threshold concentration to affect the next organ [96], while being measurable. Similarly, in a recirculation loop, the total volume of medium must be adjusted to prevent extensive dilution of the components of interest while ensuring that sufficient nutrients are available and that harmful components are properly removed [45,46].

Box 2. Vascularization of OoC Models: Importance and Strategies

The vascular system connects all organs in the human body and plays an essential role in the physiology of each organ. Specifically, blood vessels ensure proper delivery of nutrients and oxygen to all organs, allow removal of waste products, and support inter-organ communication through soluble and lipid-encapsulated factors in the form of exosomes and extracellular vesicles. All molecular exchanges between blood and the different organs occur through the endothelium.

Different strategies have been proposed for engineering a vascular system, depending on the type of blood vessels to be modeled – from large structures to capillaries – and the context of the research. Endothelia in physiological barriers such as the blood–alveolar barrier [54,62,94], the gut [33,47,99], and the BBB [48,114] are typically created by growing endothelial cells on one side of a porous membrane (Figure IA). However, this approach does not capture the intrinsic curvature of the vasculature. By contrast, cylindrical structures in hydrogels or channels [115] incorporate this curvature. For instance, endothelial cells (ECs) have been seeded in lumens previously formed in a hydrogel matrix using (i) needles, metal rods, or fishing lines [116] that are removed after gelation, (ii) sacrificial materials such as gelatin or 3D-printed carbohydrates [117], or (iii) the viscous finger patterning technique (Figure IB). In an alternative approach, perfusable capillaries have been generated through the self-assembly of ECs in a hydrogel matrix [118] (Figure IC), possibly under external stimulation by soluble factors e.g., vascular endothelial growth factor (VEGF) [101] perfused or secreted by fibroblasts in another channel (Figure ID), to yield a complex 3D vascular architecture.

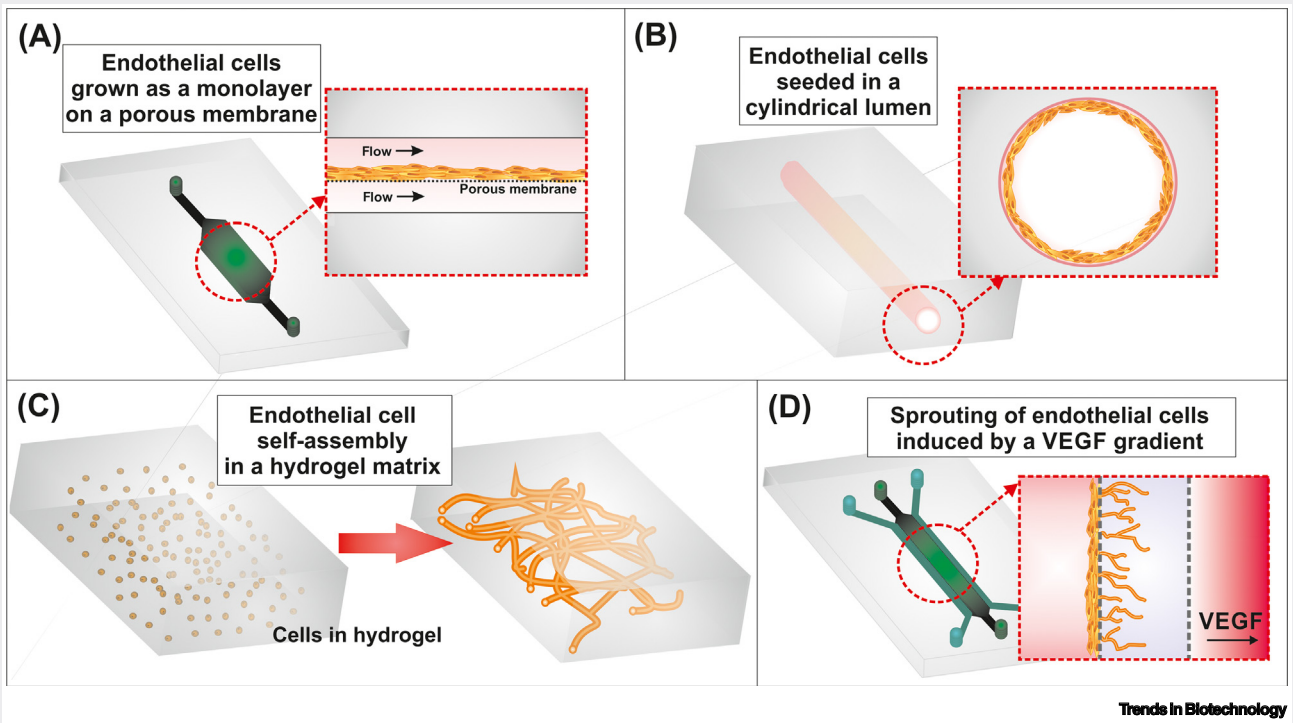

Figure I. On-Chip Vascularization of Organ-on-a-Chip (OoC) Models. (A) Endothelial cells (ECs) are seeded onto a porous membrane to emulate a physiological barrier (in most cases, epithelial cells of the modeled barrier are grown on the other side of the membrane). (B) Lumen created in a hydrogel matrix before being lined with ECs to yield a cylindrical blood vessel. (C,D) Self-assembly of ECs in a hydrogel matrix, either spontaneously (C) or through exposure to external soluble factors such as VEGF (vascular endothelial growth factor) (D).

Vascularization or No Vascularization?

Organs are connected *in vivo* through a vascular system (Box 2) that is essential in multi-OoC models to support inter-organ communication. Pathologies are also often characterized by alterations in the vasculature: for example, malignant cancer [97], cardiovascular diseases, thrombosis [98], diabetes, rheumatoid arthritis, and central nervous system diseases [99]. Although innovative approaches for vascularization have been developed for OoC platforms (Box 2), in multi-OoC platforms the 'vasculature' is often merely modeled using tubing (Figure 3), as discussed in the previous section, without including any endothelium. The endothelium can be modeled using the well-established human umbilical vein endothelial cells (HUVECs) [33,47,48,54,62,94,99,100] that express important endothelial markers and signaling molecules associated with vascular homeostasis regulation. However, the vascular barrier is characterized by an organ-specific morphology (architecture), cellular composition, and function [99]. Ideally, organ-specific microvasculature endothelial cells should be used to faithfully model the physiology of each organ. Such organ-specific endothelial cells are not always commercially available; therefore, they must be isolated from biopsied tissues or produced by the differentiation of human iPSCs or mesenchymal stem cells [97].

How Can Models Be Stimulated?

In vivo, organs are constantly exposed to various stimuli (mechanical, electrical, bio/chemical, etc.) that are crucial for proper development, functioning, and physiology. Conversely, abnormal stimulation can trigger some diseases (e.g., neurodegenerative, metabolic, and cardiovascular diseases). Mechanical stimulation plays a key role in the development, function, and maintenance of articular cartilage [102] and the blood–alveolar barrier [103]. Similarly, electrical stimulation is vital for the conductive and contractile properties of the heart, and the concomitant action of electrical, mechanical, and chemical stimuli is central to the homeostasis of the nerve–muscle junction. Physiological flow and associated shear ensure proper expansion of the endothelium in blood vessels and arteries. Moreover, reproductive organs and pancreas are tightly regulated by timely hormonal exposure, as is the entire male/female physiology [64,104], and this also modulates non-reproductive organs such as liver and kidney [60]. Therefore, timely hormonal cues must be incorporated when designing sex-specific multi-OoC models [60]. All these cues are difficult, if not impossible, to incorporate into conventional *in vitro* models, partly because of their pulsatile, chronic, or periodic nature. However, using microfluidic technology, virtually any stimulus can be included, with accurate control over their spatiotemporal character. Examples of mechanical stimulation include shear-induced flow, surface strain [105], the combination of surface strain and fluid-flow shear [106], compression [107], and the combination of compressive and bulk shear forces [70]. Noteworthy, many of these modalities utilize physiological-like deformation of an elastomeric PDMS membrane. Electrical stimulation is typically produced by integrated electrodes that have supported heart tissue differentiation and cardiomyocyte contraction [29,108]. Finally, to emulate hormonal, (bio)chemical stimulation, or exposure to drugs or toxicants, the perfused medium must be supplemented with those soluble stimuli.

How to Measure Cross-Organ Communication?

An associated challenge is to measure cross-organ communication. To that end, the same strategies and techniques can be applied as those for single OoC devices [109], with ***in situ* analysis** [pH, oxygen levels, beating frequency, TEER (trans-epithelial electrical resistance) measurements, and cell labeling and tracking], **online analysis** using spectroscopic techniques or classical biochemical assays, and **offline analysis** after collection of circulating culture medium and/or retrieval of cellular models from the device for further analysis, as detailed in Table 1.

Organ-specific phenotypes are mostly characterized *in situ* using (fluorescence) microscopy, possibly after cell fixation. This approach is limited to end-point measurements and only a few read-out parameters [e.g., live/dead and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling] assays; characterization of targeted functional and structural proteins). Furthermore, *in situ* imaging is highly challenging owing to the 3D nature of the cellular models and the use of multiple materials, which are possibly opaque. Therefore, advanced imaging techniques are employed such as light-sheet and two-photon microscopy [110], or wavefront shaping [111,112]. Alternatively, the cellular models are extracted from the device for high-resolution confocal microcopy imaging, possibly after histological sectioning or tissue clearing. Next, offline comprehensive -omics analysis or flow cytometry, both after dissociation of the cellular model, bring insightful information on organ status and communication, although they use sacrificed samples. Finally, offline measurements can be conducted on intact samples or effluent medium collected manually or automatically, regularly or at given timepoints, to assess organ function, targeted or comprehensive metabolism, inflammation (cytokine production), viability, or drug and toxicant metabolism, by using dedicated assays or mass spectrometry (MS)-based analysis.

Ideally, real-time information should be continuously acquired to follow dynamic inter-organ interactions and monitor the function of each tissue. To that end, on the one hand, (bio)sensors or electrodes are integrated into organ-specific modules for *in situ* monitoring of specific culture parameters (e.g., pH, oxygen), organ metabolism, the integrity of physiological barriers via TEER measurements, or cell beating. Of particular interest are nanoplasmonic sensors, an approach that has recently been explored for multiplexed analysis of inflammatory cytokines in an adipose tissue-on-a-chip model [113]. On the other hand, online molecular analysis can be performed using spectroscopic techniques or standard biochemical assays. A significant challenge for the latter molecular analysis scenario is to optimize the amount of sample collected to meet the sensitivity of the analytical assay without dramatically perturbing the multi-OoC micro-environment. Similarly, online analysis calls for minimal or no sample preparation, and this can hamper the detection of low-abundance species in complex matrices. All these factors explain why this information-rich and virtually noninvasive approach is scarcely pursued, and why most analyses are still conducted offline at the end of the experiments.

Concluding Remarks and Future Perspectives

Although tremendous efforts have significantly improved the complexity, quality, and robustness of OoC models, recent initiatives are now bringing this technology to the next level by generating multi-OoC platforms that aim to emulate entire biological processes that are seldom limited to a single organ. Multi-OoC technology can simulate human physiology at the level of the whole organism, offering excellent accuracy and model complexity, as well as new opportunities in multiple fields, while supporting the implementation of the '3Rs' (replacement, reduction, and refinement of animal models) and the paradigm of personalized medicine. However, multi-OoC devices currently remain only complementary to animal models, and there is still a long way to go before they are fully adopted. To achieve this Holy Grail, key challenges remain in maintaining the homeostasis of multiple organs and in incorporating all the essential cues, including hormonal stimulation, the immune system, the lymph, the microbiome, and organ innervation and vascularization, that have been scarcely explored to date. As a next step, multimodal real-time analysis should be implemented in multi-OoC platforms through the integration of multiple sensors and coupling to online spectroscopic analysis (see Outstanding Questions).

To build patient-specific multi-OoC models, iPSC- and/or patient-derived organoids or *ex vivo* tissues are both equally promising. These personalized models will open new avenues to capture specific features of a person's disease, predict the response of a patient to given treatment, and

Outstanding Questions

What is the best compromise between simplicity in operation and the complexity of a model? Which minimal sets of organ models and stimulatory elements are necessary to build an accurate systemic model for given purposes and diseases? Similarly, are tissue vascularization and innervation, and the incorporation of an immune component, essential to properly emulate physiological inter-organ communication?

Which combination of innovative materials (e.g., biomimetic hydrogels) and engineering processes (e.g., micro-structuring, shaping to include curvature, and so on) will be instrumental in building the next generation of multi-OoC models?

How can we formulate universal culture media for multi-OoC platforms that typically require different types of nutrients and supplements for each organ model?

How far are we from a worldwide adoption of multi-OoC platforms as an alternative to animal experimentation? Which developments are most pressing to promote these advanced models for fundamental, medical, pharmaceutical, and toxicity research?

How can all relevant platform parameters (respective organ size, flow rate, channel dimensions, etc.) be appropriately scaled to best emulate *in vivo* physiological conditions?

detect possible long-term and/or side-effects of drugs, thereby supporting the concept of personalized medicine. This patient-specific approach can also capture the inherent diversity in a population in terms of genetic and ethnic background, gender, and age [25].

So far, OoC technology has made significant progress in an academic setting, but many technological hurdles limit its full deployment in an industrial environment. One expected breakthrough is in the pharmaceutical industry where OoC technology could help to reduce the elevated failure rate of drug development. First, and above all, multi-OoC models must prove that they can faithfully reproduce the *in vivo* environment, the evolution of a pathology, adverse effects of drug candidates, or therapeutic outcomes. This still limited demonstration of their benefits compared with more commonly used 2D and animal models explains why various stakeholders do not yet recognize (multi)-OoC models as reliable humanized *in vitro* models. One promising approach to validate multi-OoC models is to combine them with *in silico* modeling [40–42]. Furthermore, and finally, to be more widely adopted as routine bench-tools, multi-OoC systems should be easy to use, plug-and-play, reconfigurable on demand according on the targeted application, highly multiplexed, fully automated, and compatible with standard laboratory practice.

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