Organ-on-Chips: trends and challenges in advanced systems integration

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Abstract

Organ-on-chip platforms combined with high throughput sensing technology allow bridging gaps in information presented by 2-D cultures modelled on static microphysiological systems. While these platforms do not aim to replicate whole organ systems with all physiological nuances, they try to mimic relevant structural, physiological, and functional features of organoids and tissues to best model disease and/or healthy states. The advent of this platform has not only challenged animal testing but has also presented the opportunity to acquire real-time, high-throughput data about the pathophysiology of disease progression by employing biosensors. Biosensors allow monitoring the release of relevant biomarkers and metabolites as a result of physicochemical stress. It, therefore, helps conduct quick lead validation to achieve personalized medicine objectives. The organ-on-chip industry is currently embarking on an exponential growth trajectory. Multiple pharmaceutical and biotechnology companies are adopting this technology to enable quick patient-specific data acquisition at substantially low costs.

Keywords: organ-on-a-chip, biosensors, disease models, microphysiological systems, microfabrication

Introduction

The organs-on-chips (OOC) market is expected to reach $350.8 million by 2030, with a Compound Annual Growth Rate of nearly 60% since 2015[1]. This constant market growth has been driven but not limited to the global demand to ban cosmetic animal testing. As evidenced by the EU directive 76/768/EEC[2], Mexican general law of health directive 465 Bis[3], the US Food and Drug Administration Modernization Act of 2021, and the Humane Research and Testing Act (HR 1744)[4]. Recent advances in microfluidics, additive manufacturing, and 3-D cell culture have significantly affected the OOC technological development and expansion. The growing number of partnerships and collaborations between pharmaceutical companies and OOC manufacturers to develop high-throughput and scalable drug testing platforms to minimize financial losses due to late-stage drug failure has accelerated efforts to design and develop OOC platforms[1]. The Pharmaceutical industry is afflicted with the limitation of high throughput screening and clinical scalability of contemporary drug discovery procedures. Therefore, it is pertinent to try to reproduce patient-specific tissues and organ systems in vitro and integrate them with high throughput sensing platforms.

This perspective highlight the most relevant advances in the last few years on biosensor integration in OOC platforms to achieve sensitive and real-time monitoring of metabolites and relevant biomarkers. The perspective also discusses potential challengesand opportunities to achieve clinically scalable, high-throughput multiorgan-on-a-chip systems with real-time monitoring using novel integrated imaging techniques and novel optical biosensors.

1. OOC general overview and state of the art

Each year the pharmaceutical industry spends billions of dollars on a single drug candidate to get it from the bench to the market. The conventional route of drug discovery includes testing for safety and efficacy in animal models, which offers negligible to minimal reproducibility in humans [5]. About 60% of animal-tested drugs are rendered inefficacious in clinical trials. Typical examples include vaccines for tuberculosis, Hepatitis C, and Hu5c8 monoclonal antibodies, which passed the animal testing phase but failed to reproduce similar efficacy in humans or elicited a toxic response [6,7]. Equally disturbing is that multiple drugs

that could have been efficacious in humans never made it to clinical trials. Due to this weak predictive power of animal testing and the high costs incurred, the need for alternatives became a severe concern for the pharmaceutical and biotechnology industry. Attempts were then made to establish mammalian cell cultures with nutrient provisions. These static 2-D cell cultures were, after that, extensively used to study underlying pathophysiological cues which determine drug responses but eventually were also proven incapable of accurately replicating the *in vivo* biochemical and physiological environment *in vitro*[7]. OOC technology rapidly developed and occupied an important niche in personalized medicine and drug development (see Figure 1). It uses different types of cells withdrawn from human donors to encapsulate them in a suitable biomaterial, thereby allowing them to proliferate and differentiate in a suitable microphysiological system[8]. Therefore, the technology presents different techniques for co-culturing cells to form organoids or placing tissues together with suitable barriers to study crosstalk between tissue or organ systems.

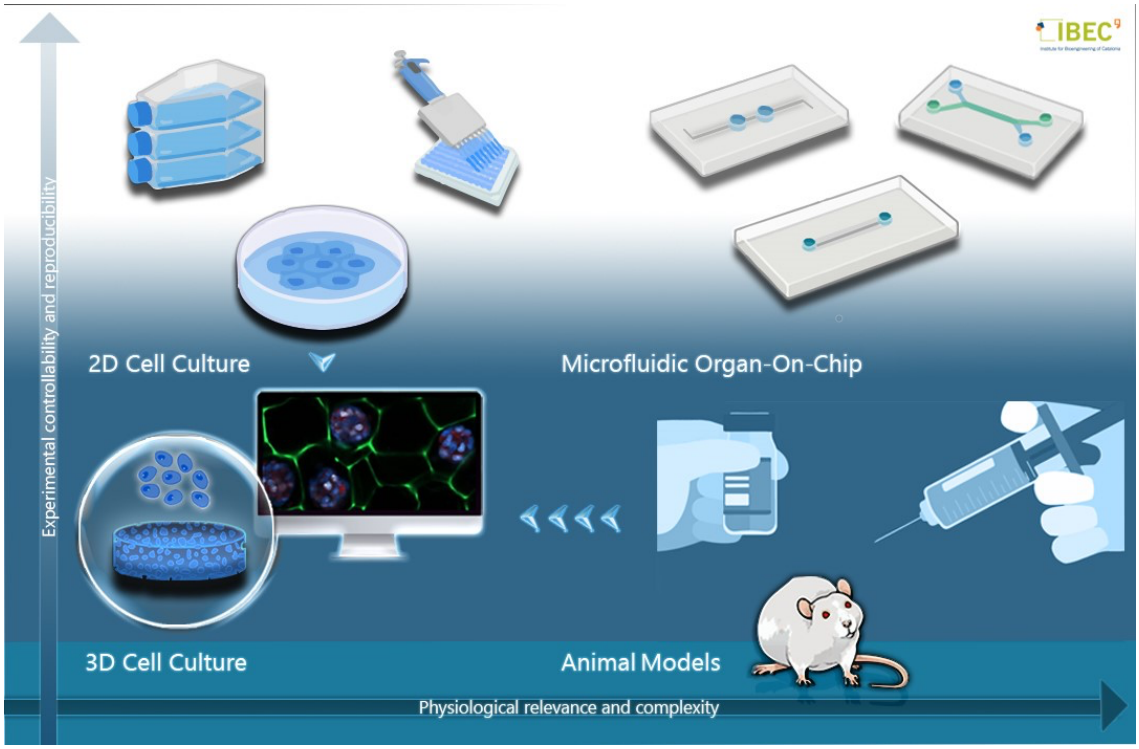

Figure 1. Relevance of OOC devices as platforms to replicate human biology. Preclinical studies employ in-vitro cell cultures and in-vivo animal models for drug testing. 2D models represent fast and high-throughput tools for drug testing. However, 3D models try to mimic advanced physiological tissue environments. Therefore, it is expected to be more accurate on drug responses. Animal models represent the gold standard in drug testing, but differences in physiological mechanisms between animals and humans promote a lack of accuracy and reproducibility of results. Microfluidic OOC devices allow controlling cell culture parameters to mimic organ microenvironments. As a

consequence, provide a more physiologically relevant environment to interrogate human biology. Adapted icons BioRender.com

The choice of a suitable biomaterial considers different physical-chemical characteristics:diffusion to and from the environment, biocompatibility, topography, and micromechanical properties such as porosity, swelling, topography, and stiffness[9]. Once mature and functional, these organoids can be assembled on microfluidic sensing platforms to allow for *in situ* biosensing applications. Microfluidic platforms allow maintaining the organoid in environments similar to their natural cellular microenvironment[10]. Initially, the efforts focused on designing and developing the right microfluidic system ensuring appropriate perfusion and tissue functionality. However the objective has since evolved to bring the models as close as possible to replacing animal models[7,9]. Whether the aim is to reproduce the respiratory crackle sounds or establish an oxygen gradient across tissue interfaces, single and multiorgan on-chip platforms have currently occupied a top niche in research on interdisciplinary science.

2. 2D Vs. 3D In Organs-on- Chip, State of the Art

2.1. 2-D Culture

Beginning from Wilhelm Roux's *"Entwicklungsmechanik"*in 1885*,* developmental biology has undergone multiple transformational phases to be what it is at the moment[11]. The term *"Entwicklung"* stood for *" development by natural causation",* which favors the functional attributes of biological development over time in the presence of natural biological cues. The last set forth the precedence of 2-D culture to study a plethora of biochemical and functional changes accompanying growth in a specific cell type. In Roux's words, *"We now wish to learn what this extensive play of changing shapes is good for"* [11].

A 2-D culture involves adhering to and maintaining a proliferative mono-layer cell culture[12]. It is sometimes also pivotal to keep cells at a certain specific maturation stage to ensure proliferative growth. However, stimulation of proliferative growth to keepthe proliferative factors is also associated with a

decline in tissue-specific functions, leading to questionable data. For a wide variety of cells sourced from solid tissues, adherence to the substrate is a common and critical step before initiating proliferation[12]. For anchorage, cells release matrix proteins that attach to the conditioned plastic substrate, followed by receptor-mediated cell adherence[13]. It is important to mention that different cell types produce several matrix elements that contribute to the extracellular matrix (ECM). For example, epithelial cells release laminin while fibrocytes release collagen type I and fibronectin. This ECM is subsequently responsible for regulating the phenotypic expression of cells[13]. The complex and dynamic composition of the ECM is, therefore, an important variable to consider while controlling a specific cellular phenotype. The lack of a suitable perfusion system results in nutrient depletion and accumulated toxic waste over time which must be frequently replenished and removed[9,10].

2.2. Microphysiological Systems and 3-D Organoids

The need to better sustain functionality over extended periodsand achieve biomimicry, gave birth to micro-physiological systems that allowed the development of 3-dimensional encapsulation of tissues in hydrogels or materials mimicking the ECM[14–16]. These scaffolds provide essential support to the cells and help restore and repair damaged tissues[17]. The employed biomaterials used for making these scaffolds are porous and permeable materials to confer flexibility and easy diffusion. Due to this porosity, they help release biological cues and bioactive molecules such as cytokines, antibiotics, inhibitors, stimulators, and other externally added drugs[15]. The scaffold's charge also plays anessential role in improving the proliferation capacity. For example, increasing the positive charge helps the cells spread and proliferate due to their negatively charged membranes. The elastic modulus of the scaffold is another important property that determines the ease of adhesion, differentiation, and overall morphology of the tissue construct. These scaffolds can be made artificially or sourced from natural sources such as the IKVAV, YIGSR laminin-derived sequences, and self-assembling peptides[18,19]. The ability of hydrogels to mimic viscoelastic and topographical cues makes them the material of choice for developing scaffolds.

3-D tissue constructs grown in a static environment can mirror histological and functional attributes, particularly for drug metabolism studies. In order to develop

organ systems or organoids *in vitro*, multiple types of tissues or cells must be co-cultured. The first-ever organ on chip recapitulated the lung alveolus using soft lithography inspired by the microchip industry. This chip's concept comprises two-channeled PDMS structure separated by a porous membrane[20]. This porous partition was coated with the ECM to allow the growth of human alveolar epithelial cells on one side and vascular endothelial cells on the other. It helped to mimic not only vascular perfusion but also the liquid-air interface present in the lungs. From there on, multiple organ chips were developed using different microengineering approaches. For instance, the most convenient format to develop tissue-tissue interfaces are trans-wells with a porous partition serving as a barrier between two cell types. The well with co-culture is usually accompanied by a reservoir and a flow channel beneath the chambers to help with nutrient perfusion[21]. A more extensive fluidic coupling can also be installed for multi-fluid transfer and collection[21,22]. To facilitate high-resolution imaging and visualization, the material of these chips is preferred to be transparent.

Since the first use of human lung alveolar epithelial cells to develop the lung alveolus chip, the model has been used to study bacterial infections, toxic exposure to nanoparticles, perfusion of chemotherapeutic drugs such as IL-2, and pulmonary edema toxicity due to it[23]. Observations from these experiments shed light on the importance of mechanical stimulation and thereby the inference that dynamic conditions best mimic pulmonary toxicity disease conditions compared to static[20]. Therefore, work on multiple axes has aroused growing interest in organ-on-chip devices, such as developing models for the brain and blood-brain barrier in conjunction with gut and microbiota to study the dynamic relationship between gastrointestinal microbiota and the gut-brain axis. The overall system has three types of cells to mimic brain function: neurons, glial cells, and astrocytes. Caco-2 cells performed the gut function, and for the immune system, they used macrophages and lymphocytes. Meanwhile, endothelial cells recapitulate the blood-brain barrier[24].

2.2.1 Single Organ-on-chip Models

2.2.1.1 Tumor-on-Chip

OOC platforms have been popular for studying cancer's underlying mechanisms and multifaceted disease pathology. In particular, these models help to mimic the tumor microenvironment in solid and liquid forms. The tumor microenvironment includes chemokines, stromal cells, and immune suppressor cells[25]. For example, a single organ-on-chip was designed to mimic pancreatic cancer to investigate the interaction between the ductal adenocarcinoma, vascular system, and activin signaling. This model helped explain hypo-vascularity, which leads to low drug delivery and poor chemotherapeutic outcomes for an aggressive type of cancer. To develop tumor biomimetic models several different factors have to be considered such as the oxygen gradients responsible for inducing *in vivo* intravasation and optimum perfusion. Sung's group developed 3-D tumor models based on spheroids to model breast cancer invasion by employing the technique of surface tension pumping[26]. This allowed them to bring about sequential loading of cells at different time points. Thereby providing an important model to study the spatial and temporal cues that determine metastasis, tumour invasion and cell migration. More recent attempts have developed a 3-D model with controlled perfusion systems to model the microvascular system of the tumour microenvironment. The Lung Tumor chips developed by Hassell et al., using the human NSCLC cells helped to explain cancer dormancy and resistance to tyrosine kinase-based inhibitors[27]. Brain tumor chips developed using U87 glioblastoma cells allowed screening drugs such as pitavastatin and irinotecan. These and multiple other tumor models exemplify that the OOC technology can be used to study signaling mechanisms, the mechanobiology of disease progression, and immune suppression leading to poor therapeutic outcomes. In parallel, the pharmacokinetic (PK) and pharmacodynamic (PD) studies of different drug candidates on the disease models can help understand their dose-response relationship and their administration dosage. PK and PD studies using multiorgan systems have been carried out for nicotine, fluorouracil, cisplatin, amiodarone, and several other drugs [7,28].

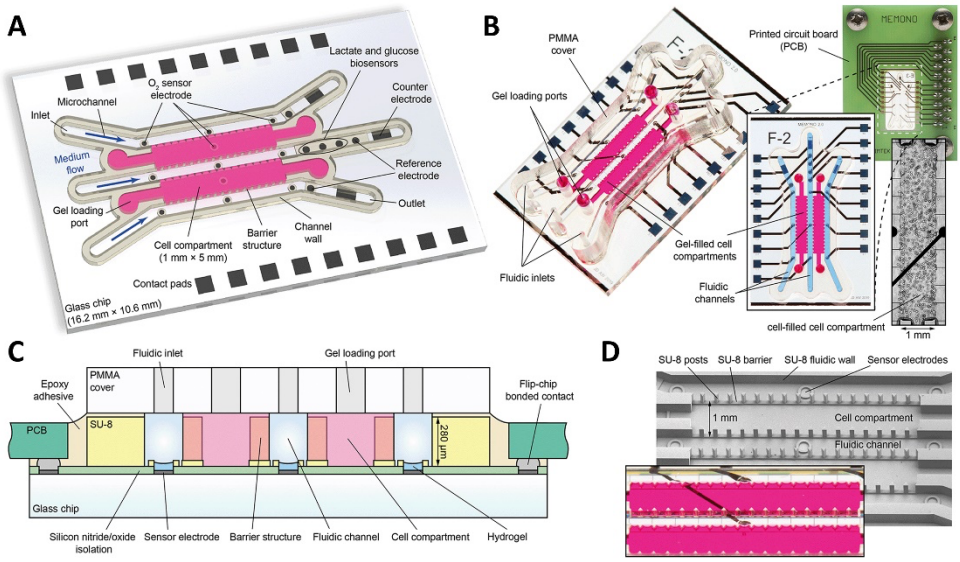

Figure 2: Overall design of the system, arrangement and fabrication A) Top-view of the glass cell culture system for the production of spheroids with microchannels for nutrient supply and fluidic control, in conjunction with electrochemical metabolite sensors for oxygen, glucose and lactate to enable *in situ* monitoring B) An assembled device C) Cross-sectional schematic D) SEM micrograph of patterned SU-8 structures[29].

2.2.1.2 Hepatic Models

Ex-vivo human hepatic models allow for studying drug metabolism and pathology of drug-induced damage by mimicking the spatiotemporal characteristics of the organ[30]. In addition to the conventional 2-D micropatterned systems using a combination of hepatocytes and 3T3-J2 fibroblasts[31], 3-D designs have been developed that employ static hepatic spheroids in conjunction with perfusion systems to introduce nutrients, withdraw metabolites in real-time, establish oxygen gradients, and shear stress[32]. One important biomarker to study the proper functioning of ex-vivo hepatic models is the cytochrome P450[30]. This enzyme helps synthesize cholesterol, steroids, and prostacyclins, and the source of hepatocyte retrieval has influenced its activity. For example, primary human hepatocytes and cryopreserved human hepatocytes had higher CYP450 activity than animal and iPSC-derived hepatocytes[33]. Some non-perfusion hepatic systems include HepatocPac, PDMS Stencil, Microarray chip, micropatterned fibrous mat, RegeneTox, and GravityTRAP[34]. On the other hand, perfusion-based systems include DILI Train, HUREL Tox, Flux, and Viral chips. The HUREL chips have eight microfluidic channels arranged in parallel while two are connected in series. The material of the biochip is polystyrene which allows cell seeding to develop mono and co-cultures. The flow rate within the chip was

optimized to 4.5uL/min/chip using peristaltic pumps[35]. Under flow conditions, the hepatocytes exhibited higher metabolic activity compared to static cultures. The platform can also reduce unwanted adsorption of hydrophobic drugs, often a common problem in PDMS platforms[34,35]. While these advancements contribute to technology, there can still be architectural improvements in the microstructure of hepatic models and the incorporation of fluorescent biomarkers within cells to provide an automated quantification of requisite outputs *in situ*.

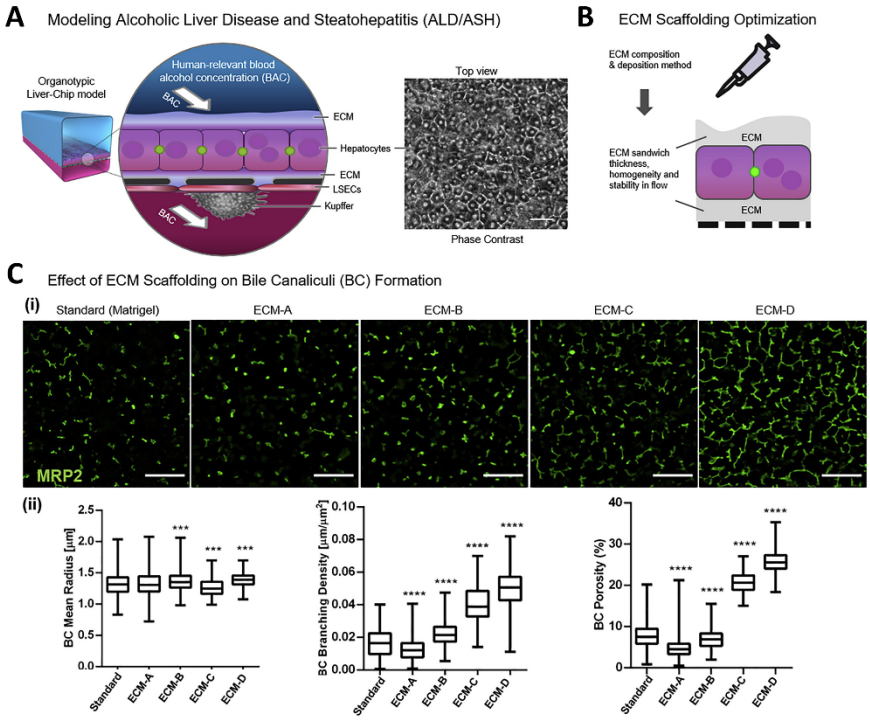

Figure 3: Alcohol associated Liver Disease Liver-Chip [36] [37]¡

[7]. 2.2.1.3 Skeletal Muscle Models

Contemporary gold standard techniques for *in vitro* characterization of muscles are based on studying the expression of myogenic markers and fusion indices. While this information is indispensable for observing and studying differentiation in culture, it does not help in understanding the physiological processes such as the force of contraction, opening, and closing of the Ca^{2+}-gated channels. Progressively degenerative diseases such as muscular dystrophies, amyotrophic lateral sclerosis, atherosclerosis, and other inflammatory illnesses affecting muscles have been studied using muscle-on-chip platforms with the requisite perfusion strategy. Platform design, choice of biomaterials, and integration with

the proper testing platforms can help to model and study the pathology of multiple diseases such as diabetes mellitus, implicating skeletal muscles. The most widely used approaches for generating *in vitro* skeletal muscles include micropatterning and microcontact printing, bioprinting, and electrospinning. Other techniques include E-field assisted printing, Microfluidic extrusion, droplet-emulsion assisted patterning, and dielectropatterning. Geometric cues such as anisotropy of scaffold fibers help form better-aligned sarcomeres and enhance cell maturation. Electrical stimulation with varying frequencies can also be used to micropatterned substrates to enhance myogenic differentiation further. Biomaterials such as PEG-DA, GelMA[38], CMCA, PEDOT[39], and Matrigel-Fibrinogen have been used to micropattern and caste hydro or cryogels for cell encapsulation to eventually form biochemically and physiologically viable *in vitro* muscle organoids[40].

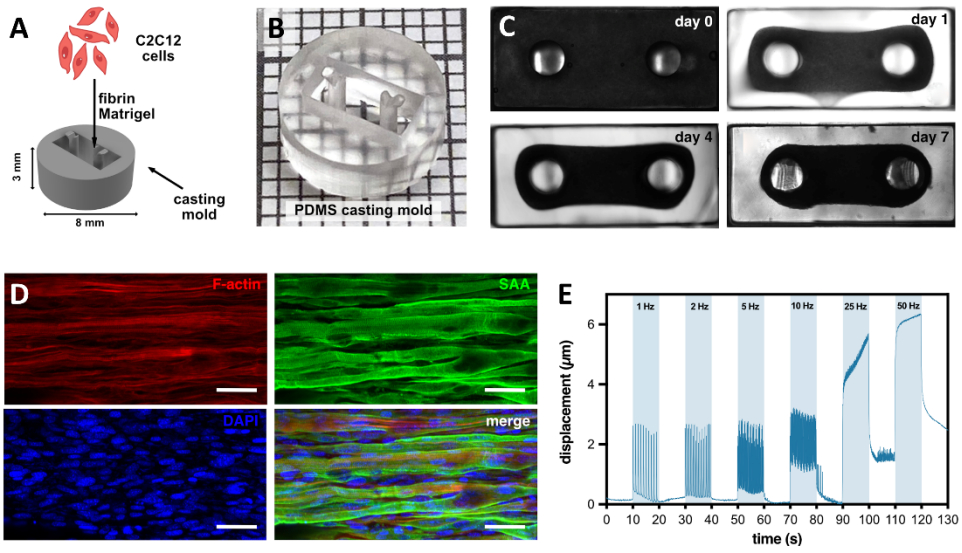

Figure 4: 3D bioengineered skeletal muscles[40]

2.2.1.4 Other Models

Induced pluripotent stem cell-derived cardiomyocytes cultured in ECM were grown within a single-channel microfluidic system on micro-array electrodes to record the electrophysiological responses *in situ*[33]. This platform helped to evaluate the cardio-toxic response to drugs such as terfenadine and doxorubicin. Other models utilized micropatterning through soft lithography, microcontact printing, electrospinning and 3D bioprinting to patterns HUVECs, rat-derived cardiomyocytes and fibroblasts. The materials employed by these models include PAAM, Collagen, GELMA and MeTro. All of these models helps to understand

the effect of material properties such as stiffness on cellular attachment, proliferation, alignment and communication, An important component of the heart-on-chip models is the microactuator which allows introduction of external stimuli to enable cell maturation. These actuators can be electrical or mechanical. In the case of electrical actuators multiple types of electrodes can be used, such as indium tin oxide, gold, graphite, titanium and platinum. There are two types of mechanical microactuators; pneumatic and electromagnetic. Mihic et al., discovered that cyclic stretching helped drive the maturation of stem cell-derived cardiomyocytes and the formation of 3-D tissue[41]. To induce stretching they introduced a non-contact electromagnetic force by fixing custom-built stainless-steel clamps at both ends of the tissue construct, it was possible to control the frequency, pattern and time duration of the stretching cycles.

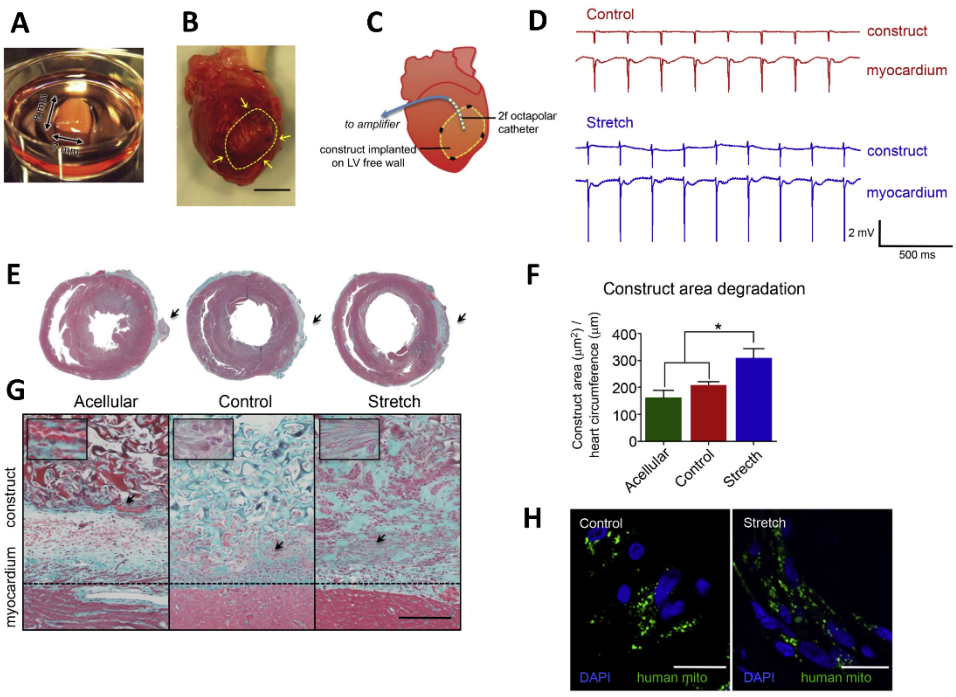

Figure 5: Implantation and characterization of hESC-CM constructs on Rat Epicardium[41].

Optimizing microfluidic parameters to promote villi formation for developing intestinal models also warrants significant mention. Efforts have been made to create intestinal models with or without endothelium. Compared with static flow, dynamic flow has been found to assist in the development of villi and in increasing the number of goblet cells and secretion of the mucous membrane. Increasing or

decreasing receptors such as ACE-2, mucous production, and associated morphological and inflammatory changes in response to external stimuli or treatment can help understand disease progression[7].

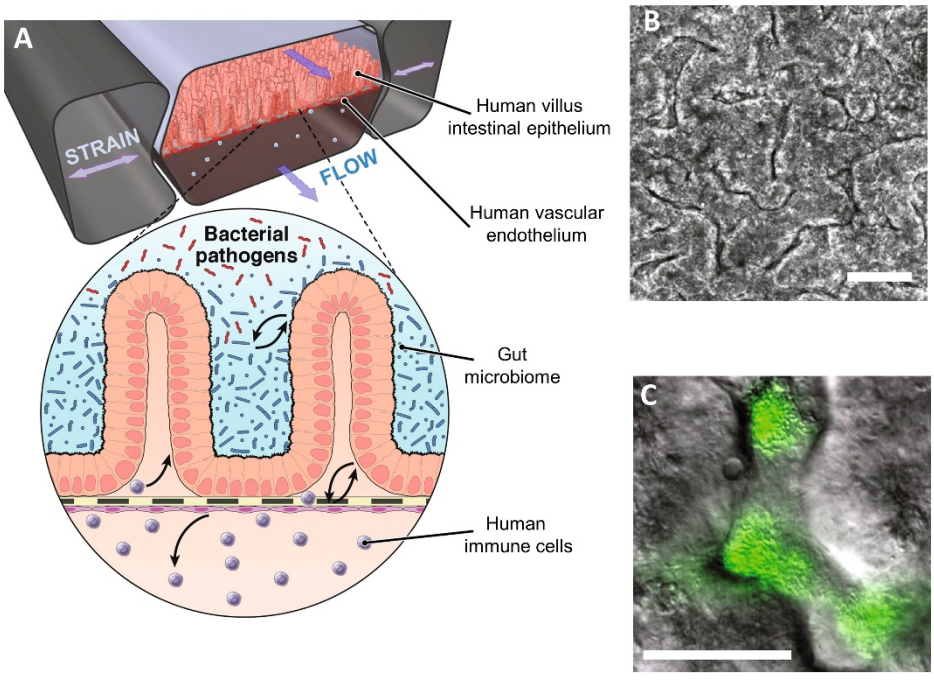

Figure 6: Intestine-on-a-chip[42]

2.2.2 Multiorgan-on-chip models

While single organoids models can provide important insights into disease progression as evidenced in the previous section, to accurately model and study multi-factorial or multi-organ-based disease pathophysiology, it is essential to allow physiological communication between two or more different types of organs. Efforts have been made to establish co-cultures, vasculature, and multiorgan on-chip (MOC) platforms with the recirculating vascular flow. Every organ in the body functions within its physiological boundaries. Therefore, while designing multiorgan platforms, it is essential to maintain and preserve the independent physiological space of each organ[43]. Therefore, establishing endothelial barriers and promoting organ-organ crosstalk through the vascular fluid is critical to achieving functional integration. When designing endothelial barriers, shear stress is an important hemodynamic parameter. Adequate shear stress is essential to maintain tight barrier function and to form gap junctions. The

barrier thus formed allows tissues to communicate through the release of cytokines and exosomes through the vascular fluid. Ronaldson-Bouchard et al. interlinked hepatic, cardiac, bone, and skin tissues on a multiorgan platform and studied their crosstalk via recirculating vascular fluid across endothelial barriers[43]. This approach allows the development of personalized disease models for each patient to identify early disease biomarkers and establish patient-specific toxicity profiles. Mechanistic models to study pharmacokinetics are limited by flow or permeability rates. To simulate mechanistic PK models for multi-compartment/ organ systems, it is, therefore, essential to model vascular flow as a closed loop, the endothelial barrier as a porous membrane, and the topography as close as possible to real-time organ morphology. Assuming a closed-loop vascular system, it is also important to consider the conservation of mass and volume in mechanistic studies. Multiple studies are continuing to explore possibilities to improve technical and physiological nuances in disease models and eventually move towards personalized treatment approaches.

Even physically distant, the interaction between organs is essential for the function of the human body. Chemical factors are released from one cell to another to maintain the body's homeostasis. This continuous feedback loop system is tightly regulated at local (i.e., paracrine signals) and systemic levels (i.e., hormones). With the advance in technology applied to human health, it was possible to identify players that affect other organs using blood and lymphatic flow, such as miRNA, extracellular vesicles, cytokines, peptides, etc. When this communication is not correct, many diseases can result. A dysfunctional organ typically occurs in the early stage of the disease, but when it reaches a more advanced stage, the involvement of multiple organs occurs [44]. The understanding of cross-organs communication must be pursued to decode the mechanisms underlying the disease to find potential biomarkers and targetable players for diagnostic and therapeutic purposes. A tremendous leap forward has been accomplished in replicating *in vitro* many pathological processes in OOC devices on such a smaller scale by using the organ-to-organ communication information gathered. These systems can be employed to demonstrate the causality of an organ on a different organ during disease development [45]. Initially, these systems were used for toxicity studies, but over the years, they became an

essential tool for personalized medicine [46]. The complexity of these systems is determined by organ-to-organ biology that must be replicated. MOC can include cell lines [45], Induced pluripotent stem cells (IPS) [47], and primary cells [48] representing different organs. The next step of complexity is reached when cellular integration is considered. The cells can be either cultured in 2D [49], in circulation [47], encapsulated in specialized scaffolds [45], separated by a barrier [50], or including an air-liquid interface condition [51]. Since there are no standard rules, principles, or a clear outcome, all these MOCs present various limitations that must be solved before this technology can be integrated for diagnostic or treatment reasons.

Primarily, the MOCs have a wide range of measurable outcomes such can be mechanostructural (i.e., force [52] and architecture [53]), physicochemical (i.e., oxygen [54] and charge [55]), biochemical (i.e., hormones [56] and cytokines [57]), and particles production (i.e., extracellular vesicles [58]). However, the results are difficult to reproduce since the MOC only works using the setup and sophisticated instrumentations present in the laboratory where it was developed. Second, most publications do not show the variability in the fabrication process and its impact on the microfluidic of MOC, which impacts the experimental success ratio and, more importantly, the cell phenotype. Third, MOCs are built using no Good-Manufacturing Protocol (GMP) processes, which prevents them from being applied to patients. Obtaining GMP certification is a long and expensive process that few laboratories can afford. Fourth, the cells, tissues, organoids, and 3D printed tissues employed to build a MOC have their own set of supplements in the medium (i.e., glucose content, amino acids pool, insulin, transferrin etc.) designed for the specific organ. However, when multiple organs are co-cultured, such organ-specific supplements can affect the metabolism of the downstream organ. Fifth, intensive collaboration is essential between engineers, biologists, and computational scientists to analyze and interpret the results. Overall, the MOCs have a disruptive potential to give us more information about drugs and compounds, reduce animal experimentation, speed up the diagnostic and drug discovery pipeline, and unveil important cues about human biology and disease progression. With the reverse-engineering approach, MOCs allow us to progressively exclude both players to reproduce in vitro a specific pathologic

phenotype and biases introduced by operator sampling, genetic components, and physical variables.

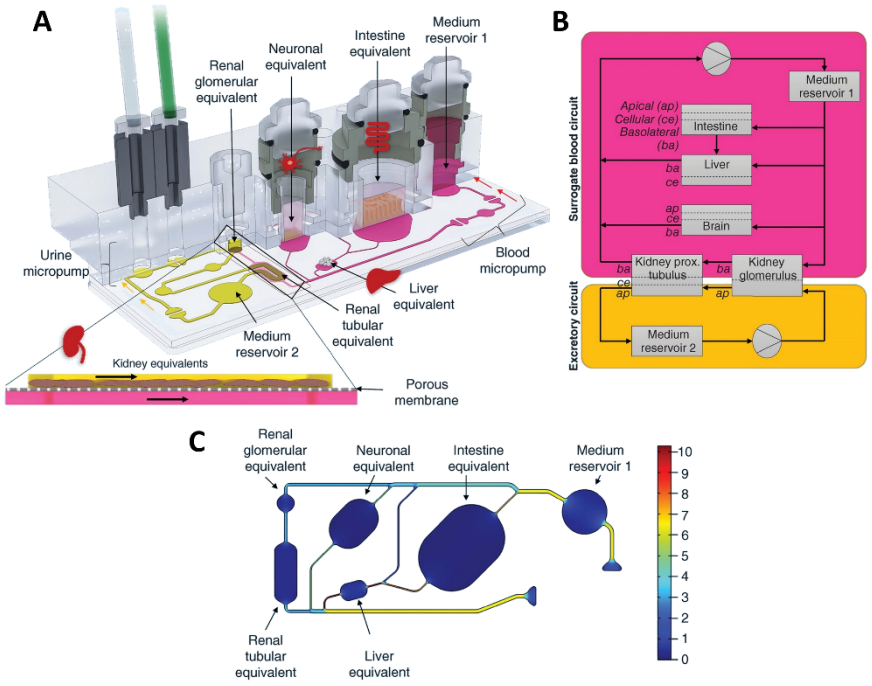

Figure 7: The Four-organ-on-chip Microfluidic Model schematic A) Physiologically relevant model of the four-organ-on-chip B) Yellow: Excretory system and Pink: blood circuit C) Distribution of the wall shear stress[59]

3. Biosensing integration in OOC

In 2009, the first microfluidic perfusion system was designed to conduct functional analyses and quantify insulin production from pancreatic beta cells following a glucose challenge through ELISA[60]. Within an organ system, at a specific time, multiple factors are at play, such as the pH, oxygen gradient, temperature, chemicals such as hormones, cytokines, and metabolites such as glucose, lactate, and calcium[61]. These culture parameters offer the diagnostic potential to monitor cell behavior changes using various techniques. Biosensors can be briefly defined as integrated analytical tools that allow the quantitative analysis of biochemical interactions with high accuracy in a few minutes with minimal sample volume and pretreatment. Biosensors have high miniaturization and integration potential for developing Lab-on-a-chip devices[62]. Consequently, they are highly suitable to be integrated for real-time monitoring in 2D cell

culture[63] and OOC devices[64]. A biosensor usually uses antibodies, aptamers, or enzymes as bioreceptors/biorecognition elements. Among the different transduction methods for biosensing in OOC devices, mainly electrochemical and optical transducers have been reported due to their well-known potential for direct and label-free sensitive biodetection.

Over the last few years, the number of biosensors to detect relevant analytes and biomarkers secreted by OOC devices has steadily increased. However, there have been limited advances in integrating biosensors with OOC devices to achieve fully integrated and autonomous platforms (see Figure 2). Although the integration in a whole single chip of biosensors and 3D organoids would be desirable and would represent an evolution in the technology, there are still different challenges to surpass. We have observed over the last years systems that include biosensors integrated into the OOC device or in a modular way interconnected in line with the organ on chip. The selected integration is highly related to the transduction method, the type of analyte/biomarker to detect, and the physicochemical stability of the biorecognition elements.Among the challenges to surpass, microfluidics is an indispensable module for performing simultaneous analysis with low sample consumption. Consequently, an appropriate design of microfluidics pathways, chambers, etc., can influence the speed of the reactions, sensitivity, and mass transfer[65]. On the other hand, surface functionalization that allows the target's sensitive and selective bio/chemo-recognition while decreasing non-specific binding from different molecular elements present in cell-culture media is highly desirable[66]. Also, it is crucial to consider that a biosensor with potential recycling/regeneration[67] or a wide working range for multiple biodetection cycles would be ideal.

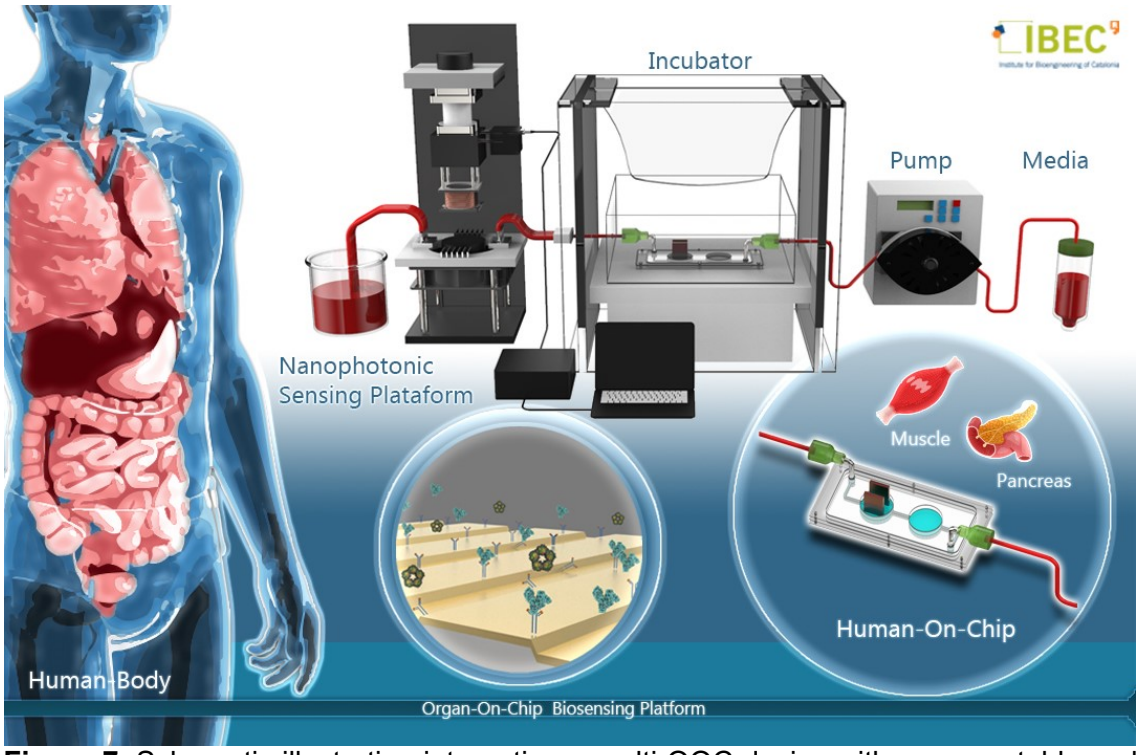

Figure 7. Schematic illustration integrating a multi-OOC device with nanocrystal-based plasmonic biosensors for direct, label-free, and real-time biodetection of relevant biomarkers. Integrated platforms must mimic relevant environmental conditions while providing opportunities to couple novel sensing technologies. Microfluidic devices with the potential for multiorgan coupling for crosstalk are desirable.

3.1 Breakthroughs in Biosensing integration in OOC

As mentioned before, there have been limited advances in integrated biosensing platforms for *in situ* biodetection of segregated biomarkers or metabolites from OOC devices; these are described mainly in a modular integration in line with the organoids rather than full integration in a chip. Due to the wide availability and variety of commercial electrodes, most technological advances have been achieved using electrochemical biosensing. Recently, the Ramon-Azcon group has presented a multiplexed modular in-line platform based on gold electrochemical biosensors for the biodetection of IL-6 and TNF-α secreted by electrical and chemical stimulation on a muscle OOC platform using monoclonal antibodies as biorecognition elements[68]. Although the electrochemical biosensors achieved sensitivity in the order of ng/mL, the biodetection was based on a "sandwich" assay using a secondary recognition antibody, increasing the complexity and not being a direct assay. The Khademhosseini research group has developed other examples of modular in-

line electrochemical biosensors in OOC platforms. One first approach allows the multiplexed monitoring of Creatine Kinase, Albumin, and GST-α on a heart-liver OOC model using monoclonal antibodies as biorecognition elements with a sensitivity in the order of ng/mL[69]. A subsequent approach allowed the monitoring of Creatine Kinase in a heart OOC model using aptamers as biorecognition elements. The electrochemical biosensors based on aptamers achieved a superior shelf life and biosensing performance (in the order of pg/mL) compared to those based on monoclonal antibodies[70]. In the case of metabolites, Weltin et al. presented a brain cancer OOC device with integrated on chip electrochemical biosensors for the multiplexed biodetection of glucose and lactate using glucose and lactate oxidases immobilized in a membrane and detecting the formation of hydrogen peroxide[71]. Both biosensors achieved sensitivity in the µM range and allowed them to obtain a measurement in less than a minute with high stability, selectivity, and reversibility. More recently, Bavli et al. integrated on chip enzyme-based commercial electrodes to integrate them and monitor glucose and lactate on a liver OOC model with sensitivity near µM[72]. The biosensing system was automated to perform the perfusion of the biosensors with the potential to achieve multiplexed measurement every 200 seconds.

Finally, considering the potential benefits of optical biosensors in terms of direct, label-free, and real-time multiplexed biosensing Ramon-Azcon group has presented different optical biosensing platforms based on plasmonic metamaterials for monitoring multiple biomarkers secreted from OOC. In a first approach, rod-like gold nanoantennas obtained by electron-beam lithography were used to monitor insulin secretion from a pancreas OOC model under low/high glucose concentrations[64]. The proposed optical biosensor allowed direct, label-free, and multiplexed in-line monitoring of insulin secretion from OOC. However, the achieved sensitivity was in the order of ng-µg/mL, unsuitable for detecting other biomarkers, usually in the order of pg-ng/mL. In a recent approach, nanograting-like plasmonic metamaterials based on commercial Blu-Ray optical discs[73] were used to detect in-line the IL-6 and Insulin secretion in a pancreas-muscle OOC device simultaneously. The proposed biosensor platform not only achieved sensitivity in the pM range but also provided direct, label-free, and multiplexed biosensing with a high-throughput, lithography-free

fabrication method[64]. **Table 1** summarizes the main relevant integrated biosensors applied to OOC platforms, including information like detection method, detected biomarker, sensing performance, integration level, cell culture type (2D-3D), and the features of the proposed biosensors.

Table 1. Research papers overview using integrated biosensors in OOC platforms.

OOC platform Biosensor type	Bioanalytes	Integration Level and Features
Brain Cancer Electrochemical[74]	Glucose and Lactate	**Integrated on Chip-(2D cell culture)** Engineered enzymatic electrodes with LOD in µM range
Liver Electrochemical[75]	Glucose and Lactate	**Modular interconnected-(3D cell culture)** Commercial enzymatic electrodes with LOD in µM range and fully automatized
Muscle Electrochemical[76]	IL-6 and TNF-α	**Modular interconnected-(3D cell culture)** ommercially available electrodes with LOD in nM range and multiplexed detection with indirect assay (sandwich)
Liver-Heart Electrochemical[77]	Creatine Kinase, Albumin, and GST-α	**Modular interconnected-(3D cell culture)** Engineered electrodes with monoclonal antibodies biorecognition elements in nM range and fully automatized
Heart Electrochemical[78]	Creatine Kinase	**Modular interconnected-(3D cell culture)** Improved shelf life and biosensing performance using aptamers with LOD in pM range
Pancreas Optical[79]	Insulin	**Modular interconnected-(3D cell culture)** Direct, label-free, and multiplexed optical biosensing with high-resolution lithographic fabrication and LOD in µM order
Muscle-Pancreas Optical[80]	IL-6 and Insulin	**Modular interconnected-(3D cell culture)** Direct, label-free, and multiplexed optical biosensing with lithography-free fabrication and LOD in pM range
Breast Cancer Electrochemical[29]	*Oxygen, Glucose and Lactate	**Integrated on Chip-(3D cell culture)** Engineered enzymatic electrodes with LOD in µM range, system combine bio and chemosensors

Abbreviations: Limit of detection (LOD)*Oxygen detection based on chemosensing

As previously described, in most examples of biosensors integration in OOC devices, biosensors are modular in-line interconnected to the OOC device. The selection of modular platforms can be highly correlated in some cases with the complexity of integration of the transduction method and the physicochemical stability of the biorecognition interfaces. On the other hand, different sensors and chemosensors integrated into chips have been described considering that external stimuli or internal abnormalities can manifest in several ways: cell confluency or adhesion, temperature changes, pH, oxygen and carbon dioxide levels, among others.

Integrated electrical sensors based on the detection of impedance are mainly reported. The electrical impedance measurement in trans-epithelial electrical resistance (TEER) is widely used to evaluate the integrity and differentiation of 2D in-vitro cell cultures; the electrical impedance across the epithelium is correlated to the junction forces between neighboring cells in a cell monolayer. Odijk et al.[81] and Henry et al.[82] have reported the development of integrated electrodes for impedance measurement over several days and weeks in 2D-based intestine and lung-on-chip, respectively. Collagen was selected in both models to promote cell adhesion to electrodes. On the other hand, oxygen and nitric oxide sensing integrated on-chip has been reported based on electrochemical sensing and optical chemosensing. Li et al.[83] used core–shell titanium carbide/carbon nanowire arrays for highly specific nitric oxide electrochemical monitoring in a 3D blood vessel on-chip model. The sensor achieved a LOD in the nM level and a response time of miliseconds. In oxygen sensing, Grant et al.[84] integrated optical chemosensors based on commercial nanoparticles engineered to measure oxygen gradients on an intestine on-chip model. The oxygen monitoring was based on a commercial fiber coupled detection system and allowed continuous detection over the 72 hrs duration of the experiment. Finally, Dornhof et al.[29] integrated bio and chemosensing on a single chip using electrochemical sensing in a 3D breast cancer model. In one part, the enzyme detection of glucose and lactase; in the other part, the amperometric oxygen sensor proposed was based on the electrochemical reduction of oxygen species in a platinum electrode. All the sensors achieved a LOD in the µM range. Considering the vast number of materials for sensing and chemosensing purposes, we can expect, as in the last example, a hybrid combination of bio/chemosensors in organ on chip platform for a multiparametric monitoring.

3.2 Perspective in biosensing and imaging for OOC integrated platforms

As mentioned, real-time monitoring of biomarkers, metabolites, and morphological changes is desirable to study the influence of physical and chemical stimuli in 2D and 3D cultures and crosstalk between the different cell types (see Figure 2). Thoroughly integrated platforms combining real-time biosensing and imaging under relevant cell-culture conditions are required for this aim. Although, as mentioned previously, all integrated on-a-chip systems would be desirable and a technological breakthrough, the complexity, associated fabrication costs, and fixed design would be relevant limitations. However,

developing modular/brick systems (LEGO-like) would be a high-throughput, versatile, cost-effective solution.

As demonstrated in the last years, evanescent-wave optical biosensors (especially those based on plasmonic metamaterials) offer indisputable advantages in terms of sensitivity (up to aM-fM level), miniaturization, and multiplexing capabilities (multi-spot array capabilities) over other technologies [[85] in developing fully integrated OOC real-time biosensing platforms [[64]. The latest advances in nanolithography have exploded the development of meta-plasmonic biosensors with many potential materials and designs. However, in most nano lithographic technologies, there are still challenges and questions about their potential to achieve large-scale and high-throughput novel devices outside the laboratory. As we recently proposed, lithography-free methods like glancing angle deposition and thermal dewetting can help surpass these challenges and, even more, enhance the capabilities of conventional lithographic techniques [[86].

In the case of the biorecognition layer, new biofunctionalization strategies are required, considering that a biosensor's final performance implies a direct correlation between the transducer and the biorecognition elements. Traditional biofunctionalization methods are truly time-consuming and require multiple steps. Engineered biorecognition elements with high specificity, long-term stability, the potential for direct attachment, and several detection cycles like aptamers would be ideal. These specific biorecognition elements must be accompanied by antifouling features that minimize non-specific absorptions from the cell culture media components. It looks like engineered polymer brushes and other blocking elements like serum from different host species can significantly decrease non-specific absorptions and cross-reactivity issues[87].

Additionally, to biosensing platforms, with the latest advances in high-performance CMOS image sensors with affordable magnification elements (between 500-1000x) in handheld digital microscopes, it could be feasible to achieve integrated OOC platforms that combine real-time microscopy and biosensing information. Under relevant culture conditions, it is possible to study the morphological changes and biomarkers released from cells to drugs and physicochemical stimuli. As a first step, conventional broadband bright/dark-field can provide relevant information from cell morphological changes. However, new

techniques like multispectral imaging[88], where certain compounds in cells have a high absorption from light, can bring to light information complex to detect by the human eye, improving the contrast of details and the real-time tracking of physiological events[89]. Finally, the massive amount of information generated in real-time monitoring allows machine learning to be a handy tool in collecting and analyzing data. While also, with feedback from sensors, intelligent control of culture or experimental conditions can be possible.

[43][43][44][45][46][45][47][48][49][47][45][50][51][52][53][54][55][56][57][58][85][64][86][87][88][89]4. High throughput, clinical applicability scalability

Despite their numerous benefits, organ-on-a-chip platforms are complex systems with significant biological and technical complexities, which have constantly increased over the past two decades[90]. To reproduce biological models that resemble native conditions, OOCs require the coordination of individual multidisciplinary elements. For instance, the material choice, sterilization technique, surface modification, and peripheral equipment (pumps, incubators, sensors, among others) are tied to one another[91]. Similarly, the phenotypes observed during an experiment run inside an OOC are the outcome of the interaction between single elements, each adding variability and propagating the experimental error[90]. Due to this modular nature, OOC devices deal with numerous sources of variability that influence reproducibility. OOC devices, therefore, show low reproducibility, like many other disciplines[92]. A significant challenge lies in reducing background variability from multiple elements to detect and measure the systems' response to the treatment of the study.

The manufacturing process can be optimized at different stages, and there is still massive room for improvement if OOCs to reach commercial availability[93]. Tools like checklists that help verify the dimensions and features of the device have been suggested to improve reproducibility in OOC fabrication. This could help researchers to assess weak spots in their fabrication processes. Additionally, commonly used prototyping materials like PDMS have poor scalability, and it is necessary to transfer initial designs to automated fabrication.

Large-scale manufacturing employs techniques like injection molding, and alternative materials compatible with these technologies are often expensive.

Recently has been substantial efforts to standardize the experimental work in OOC devices. Technology-related, production-related, and market-enabling aspects of OOC can be used to determine the main tasks towards standardization in fields like metrology, performance characterization, and quality [94]. Therefore, scientists must collate the primary sources of variability and prioritize standardization tasks according to importance and feasibility. Researchers should, for instance, aim to standardize fluid, gas, and temperature control, which is a highly feasible and essential task. The recommendation of optical readouts and the definition of quality controls for ECM fabrication (concerning mechanical properties, composition, and batch-to-batch variability) are further examples of highly feasible and vital tasks in OOC technologies[94].

One of OOC technologies' most interesting potential applications is the drug discovery screening process. However, drug screening needs high-throughput (HT) platforms to assay a significant number of compounds simultaneously, which has not been already achieved for the OOC. HT assays require pharmacological relevance, reproducibility, and low-cost, high-quality outputs. For example, standard HT drug screenings using cell cultures imply the use of up to 1536 well plates that allow to assay 10000-100000 compounds in 24 hours. The small number of HT-OOC platforms available to date reflects that several challenges are still present in OOC technology and must be addressed to establish real HTS platforms[95–97]. These challenges include the limitation in the fabrication scale-up process due to the chip materials, the automatization of the injection of media and drugs, and the integration of robust analytical devices as real-time biosensing platforms. The fabrication processes must be standardized to achieve reproducible protocols using materials that permit multiplexing.

Moreover, media and compounds must be automatically and precisely introduced and extracted in the HT-OOC to allow the parallelization of the assays while reducing variability. To achieve this, complex microfluidics designs must be implemented in HT-OOC devices. Finally, the outputs of the drug screening must be robust by integrating the analytical devices that allow multiplexing readouts, including optical, electrochemical sensors, fluorescence markers, and

biochemical readouts. HT-OOC devices integrating these biosensing platforms would enable the assay of compounds in biomimetic systems, which would be a step forward in the field of drug discovery.

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