Sensing and Bio-Sensing Research 33 (2021) 100443

Contents lists available at ScienceDirect

Sensing and Bio-Sensing Research

journal homepage: www.elsevier.com/locate/sbsr

Gut-on-Chip microphysiological systems: Latest advances in the integration of sensing strategies and adoption of mature detection mechanisms

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ARTICLE INFO

Keywords:
Gut
High-throughput monitoring of bio-physical-chemical parameters
Organ-on-Chip
Sensors integration

ABSTRACT

Synergic efforts in microfabrication processes, cells culture and tissue engineering promoted extraordinary progress in Organ-on-Chip (OoC) technology, leading to the development of *in vitro* microphysiological models able to recapitulate the microenvironment and key biochemical, functional, structural and mechanical features of specific tissues and living organs. In order to assess the functionality of these cell cultures with every increasing biological complexity, it is also important to equip OoCs with miniaturized sensing devices able to monitor key physical and chemical parameters related to the culture microenvironment and to pathophysiological cell-cell interactions. Gut is one of the most interesting and studied human organs: it performs multiple fundamental body functions, from transport, absorption and metabolism of nutrients and drugs, to the maturation of the immune system and host protection from pathogens and infections. In this Review, an overview of Gut-on-Chip (GoC) systems is provided, with a special attention focused on the most relevant sensing strategies integrated into GoC, aimed at monitoring *in situ* the microphysiological parameters related to intestine functionalities. Advantages and limitations associated with currently integrated physical, chemical, and biochemical sensors are discussed, together with the challenges that the technology still faces, and the possible adaptive solutions coming from other developed OoC models. Finally, we focus the attention on how gut microbiota connect to other organs of the human body and on the role of gut in the understanding of the progression of many diseases, such as the most recent pandemic infection caused by SARS-CoV-2 virus.

1. Introduction

The recent pandemic event related to Covid-19 has shown how clinical research and synergic collaboration around the world can provide decisive support, for instance in the discovery of new vaccines and clinical treatment strategies. However, within the drug development process, the clinical trial stages are still extremely expensive and time consuming, leading to the risk of making the tested drug already obsolete before its validation is completed and its marketing authorization is obtained [1]. Failure of several *in vivo*, *in vitro* and clinical trial steps in accurately mimicking human pathophysiology and response means that many diseases still lack an efficient therapy [2]. Finding alternative ways to model human diseases *in vitro* would accelerate the development of new drugs and promote advance in precision medicine, which aims at identifying the genetic, molecular or environmental causes of a specific disease and develop treatments specific to the single individual.

From this perspective, Organ-on-Chip (OoC) devices represent a new kind of systems useful for the understanding of fundamental mechanisms relevant to cell biology, diseases and drug action. They permit to accelerate healthcare, reducing costs considerably and overcoming limitations related to the use of either animal models or two-dimensional (2D) *in vitro* models [3]. In particular, 2D *in vitro* cell culture models fail to reproduce both the complex structure of a human tissue or apparatus, which is characterized by continuous stimulating agents (impulses, hormones, chemical and mechanical signaling), and its interaction with other organs [4], whereas animal models lead to ethical problems and often have poor testing efficiency due to the differences existing between animal and human body [5]. In contrast, OoC devices are advanced microfluidic systems that faithfully recapitulate the microenvironments and the fundamental functions of a specific human tissue or a network of functional organs. In such systems, the physiological microenvironment of an organ can be recreated exploiting

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https://doi.org/10.1016/j.sbsr.2021.100443
Received 4 May 2021; Received in revised form 6 July 2021; Accepted 19 July 2021
Available online 21 July 2021

the synergic combination of microfluidic and microfabrication technologies; moreover, the cellular microenvironment of micro-engineered cell culture models can be monitored by miniaturized and automated assays, based on high resolution techniques; finally, some key parameters, such as concentration gradients, shear force, cell patterning, tissue-boundaries, and tissue–organ interactions, can be regulated using microfluidics [6–10].

The quality of an OoC device in emulating *in vivo* environment is largely dependent on its architecture and complexity. To this aim, several microfabrication techniques have been exploited, yet some of them - such as microcontact printing, soft lithography and replica molding [11] – have led to drawbacks mainly related to the preparation of the biological structure [12]. New methods aimed at overcoming these limitations have been established; for instance, 3D printing technology enables the realization of multiscale cell patterning using customized high-resolution scaffolds that precisely determine the spatial distribution of cells, biomolecules and biomaterials layers [13]. One of the most interesting advantages of this technology is the versatility in the definition of the digital masks used for the cell pattern, which represents a very critical task for the *in vitro* reconstruction of the cellular microenvironment [14]. Another recent approach is based on scaffold-free models, using cells suspensions to fabricate spherical micro-tissues; this method exploits a variety of cell types and techniques, such as ultralow-adhesion plates, hanging drops, bioreactors, and automated high-throughput bio-printing [15–19].

Parallel to the requirement of cell cultures with increased biological complexity for the development of faithful OoC models, there is an increasing need for assessing the functionality of the OoC by monitoring physical and chemical parameters of the culture microenvironment and pathophysiological cell-cell interactions. At the moment, such monitoring is mainly based on optical and fluorescence microscopy, and on conventional laboratory bench-scale methods that can be hardly integrated. Thus, interest towards the development of miniaturized sensing platforms showing high sensitivity and selectivity, as well as high-throughput capabilities, is growing. These would allow *in situ* and real-time assessment of OoC functions, with continuous and long-term monitoring of bio-physical-chemical parameters. Micro/nano systems integrating actuators, sensors and microfluidics on the same chip offer many opportunities to closely monitor cell-level and tissue-level events in cell cultures. At present, a major challenge for these multi-sensor platforms is ensuring biocompatibility, sensitivity, stability, and reproducibility under different cell culture conditions. Together with non-invasive miniaturized biosensors, imaging techniques are required, too, for a complete monitoring of the microphysiological systems. This approach has been successfully tested in [20], where phenotypic and genotypic data in both cell cultures and engineered tissues were used to establish a direct correlation between the variation of physical/chemical parameters and the identification of physiological performance markers. Obviously, as many technologies as possible should be exploited to achieve a complete monitoring of OoC devices, both exploiting several sensing mechanisms (*i.e.* thermal, acoustic, optical, chemical, and electrical ones), and implementing reliable sensing strategies for the detection of different parameters of interest. The integration of biosensors in OoC has great potential in applications such as drug screening, for the detection of metabolites in the circulating medium, and for the monitoring of interconnected individual organs (Body-on-Chip) [21], as summarized in Fig. 1.

Advancements in microfluidic technologies and the fast pace of MEMS technology application in the biomedical field represent a revolutionary opportunity for many biological applications, such as manipulation of single cell or molecules, high-throughput drug screening and delivery, advanced therapeutics, biosensing and point-of-care diagnostics [22]. A common goal in these research fields and applications is to explore and investigate the mechanisms leading to the

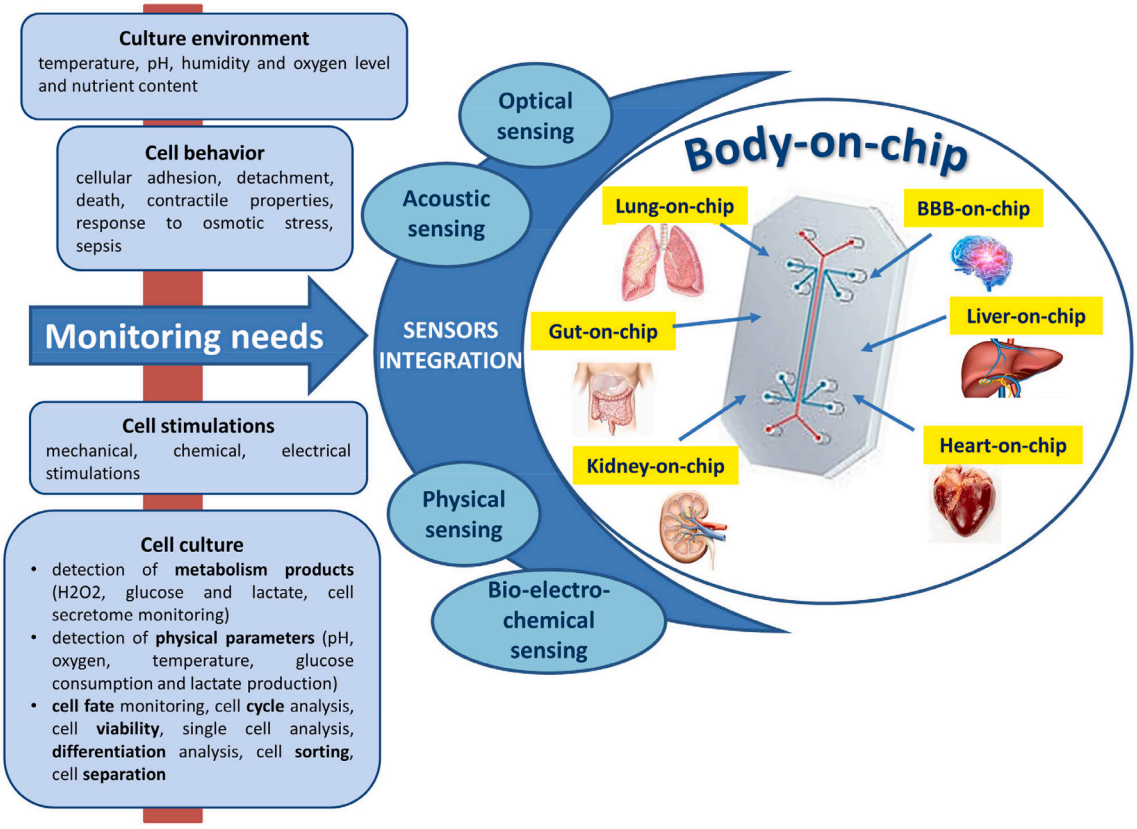

Fig. 1. Schematic illustration of the central role of sensors integration in Body-on-Chip for a real-time and continuous monitoring of microphysiological parameters. Right side of the picture, source: findings from Wyss Institute.

physiological and pathophysiological states induced by the most infectious and chronic diseases of the world. It has been shown that, in most of them, human intestine plays a complex role and function; for this reason, gut is one of the most interesting and studied human organs. It is responsible for several fundamental body functions, mainly concerning the transport, absorption and metabolism of nutrients and drugs across the highly polarized epithelial cell layer and the intestinal mucosa. Other physiological gut functions include adaptation of immune responses and protection of the host from pathogens and infections. Such important role in immunity has been discovered by studying the probiotic bacteria present in the human microbiome, an intestinal flora considered decisive in the physiological balance and in the interaction with pathogens. Gut communicates with other organs too, such as liver and pancreas, *via* the portal flow, and it contains an enteric nervous system that forms a part of the gut-brain axis [23]. Any disequilibrium of the basal microbiome is generally associated to diseases of the gastrointestinal tract, in spite of all the involved reactions causing these pathologies are difficult to pinpoint. Due to the complexity of the human host-gut microbiome, a faithful reproduction of all the main related processes by using conventional *in vivo* and *in vitro* models of the gut is challenging. Moreover, as an additional challenge, a realistic model should also consider the mechanical peristalsis movements of the gastrointestinal tract. Some of these limitations have been addressed using Gut-on-Chip (GoC) systems containing microchannels continuously perfused and covered by different intestinal cell types, arranged to form tissues mimicking the *in vivo* morphology. Researchers have also developed multiple organs-on-chip connected through common vascular channels that mimic the connectivity between the gut and other organs within the human body, in order to better predict human Physiologically-Based PharmacoKinetics and PharmacoDynamic (PBPK/PD) responses to drugs *in vitro*. As a matter of fact, while OoC systems can be useful independently, each organ model plays a distinct role in contributing to the development of a physiologically relevant Body-on-Chip model [24,25].

This review discusses the state-of-the-art in GoC devices focusing on: i) microphysiological gut models and the evolution from cells-on-chip to GoC to enable clinical translation into effective therapeutics processes; ii) latest trends in integrated sensors for monitoring the human physiology in GoC systems; iii) sensors integration, limitations and strategies to overcome them; iv) open challenges about on-chip integrated sensors for high-throughput systems. Furthermore, the latest advances in GoC developed within the frame of the global diseases and pandemic infections will be presented.

2. Important tools in a Gut-on-Chip model: developmental biology and microenvironment

In this section, we will focus on the more recent progresses done in microelectronics, microfluidics, and developmental biology to recapitulate the gut microenvironment through suitable microphysiological systems.

The intestine is the biggest organ in the human body. The intestinal epithelium is composed of a single layer of columnar cells on the luminal surface of the organ and has the capability to rapidly self-renew within few days. These cells are tightly packed and polarized, and organized according to two distinct configurations, called villi and crypts. Villi are composed of several differentiated cell types (absorptive enterocytes, Paneth cells producing antimicrobials, goblet cells producing mucus, and enteroendocrine cells producing hormones), and are shaped as finger-like protrusions located at the apical part of the epithelium [26]. Fig. 2 shows a schematic representation of the intestinal epithelium with its surrounding tissue [27]. The crypts, containing intestinal stem cells, are invaginations located at the basal part of the intestinal epithelium in direct contact with the basement membrane. The tight junction (TJ) between epithelial cells acts both as a physical barrier, which lines the cavities of the intestine, and as a defensive layer which protects the organ from external chemical, physical, and microbial attacks, determining gut permeability, and regulating the mucosal immune system.

The apical side of epithelial cells, facing the luminal surface of the organ, is characterized by a cell arrangement that differs in composition and shape from that of the basolateral side [28]. By covering the mucosa of the small intestine, the villi increase its absorbing surface thus improving the permeability to nutrients, while forming a defensive barrier against any pathogens. The peristaltic movements of the intestinal muscles favor the passage of the chime towards the ileum, where a massive absorption of digested nutrients takes place. Digestion in the

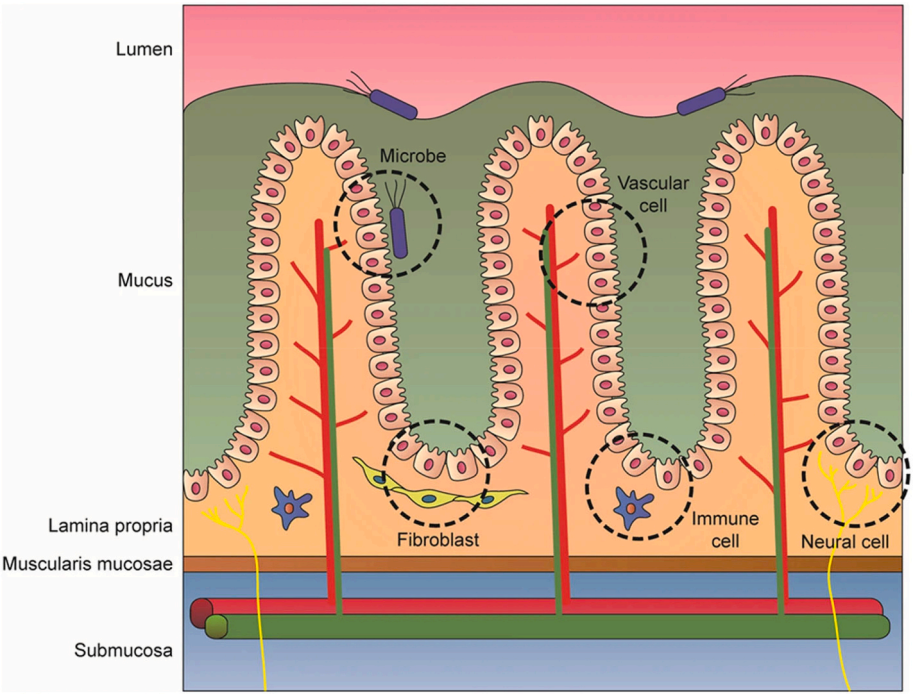

Fig. 2. Representation of the intestinal epithelium with the surrounding tissue. Intestinal epithelium image is reproduced from [27], CC-BY-4.0.

large intestine continues with the absorption of what remains of the ingested food, namely water, electrolytes, and fibers. In spite of the multiplicity of gut functions is now recognized, a large number of the involved mechanisms remains only partially understood. Several *in vitro* models were developed in the last decades mimicking intestinal functionalities in a more or less complex manner. In the following discussion, the evolution of cell culturing platforms and *in vitro* microphysiological models for gut organ will be presented, outlining the main conceptual steps from cell-on-chip to OoC.

2.1. Two-dimensional cell cultures

In spite of cell biology is paramount in the path to organ chips, developmental biology has been a determining factor in the understanding and control of some fundamental mechanisms involved in cell fate and tissue morphogenesis. The exploitation of inert surfaces is a physiologically relevant control element to limit the distortion of cell shape. Over the last twenty years, the most widely used cells for *in vitro* intestinal models have been intestinal immortalized cell lines, notably Caco-2, derived from human colon carcinoma, because of their availability, transfectability, and ease of culturing [29]. When cultured on flask and on flat Transwell inserts, these cells differentiate in less than twenty-one days, forming a monolayer of columnar cells exhibiting TJs. When translated in 3D culture systems, they mimic some of the physical and physiological properties seen *in vivo* [30]. In spite of immortalized cells represent the gold standard for biological characterization studies, they do not fully reproduce the *in vivo* system. In contrast, the use of primary patient-derived cells or adult stem cells allows to obtain a better physiological representation of the organ function, because these cells retain many of the functions and functional markers seen *in vivo*. Moreover, they exhibit a significant donor to donor variability that could be exploited for the development of personalized medical solutions [31]. Yet they also show some disadvantages, such as senescence, poor proliferation, complex protocols for cell culturing and limited potential for self-renewal and differentiation, lacking in intestinal epithelial subtypes [32]. Some of these drawbacks can be overcame using induced Pluripotent Stem Cells (iPSCs), which are patient-derived cells, reprogrammed for reaching a stem cell-like behavior. Both healthy and diseased states can be reproduced using iPSCs-based *in vitro* models [33]. HT29 [34] and T84 [35] are two examples of pluripotent intestinal cell lines, used to investigate the mechanisms behind intestine functions. IPSCs use suffers from several disadvantages but the most relevant one is the need to adopt specific cytokine-supplemented media and complex culture conditions for their culturing. In any case, depending on the research question, the cell model should be selected to ensure, as much as possible, biological authenticity and experimental reproducibility, at the same time guaranteeing a reasonable compromise between cost, availability, and throughput. For these reasons, despite primary and stem cells models have been developed, human Caco-2 immortalized cell lines still represent the preferred standard for the prediction of drug intestinal permeability in humans by pharmaceutical companies and regulatory authorities [36]. In fact, the first *in vitro* intestine models were obtained seeding immortalized human intestinal epithelial cell lines on porous membranes coated with ExtraCellular Matrix (ECM) within Transwell plates.

In a 2D culture system, cells grow as a monolayer on a flat surface by receiving a homogenous amount of growth factors and nutrients through the medium. To extend their application to high-throughput screening of new compounds, new methods for faster Caco-2 culturing have been also developed [37,38]. 2D co-culture models have been developed to better mimic cell-cell interactions and the complex composition of the intestinal barrier. A cell model based on the co-culture of Caco-2 and mucus producing HT29 cell lines was shown [39] to provide more predictable experimental results by mimicking differentiated intestinal cell types, *i.e.* both enterocytes and goblet cells, thus allowing to observe the role of mucus on drug transport too. A human cell line-based co-culture model of differentiated Caco-2 cells and macrophages (differentiated THP1 cells) was developed to mimic the intestine in homeostatic and inflamed states [40]. Gut physiology together with the interactions occurring between intestinal epithelium and macrophage and the host responses to enteric pathogens were studied through the development of the first primary human macrophage-enteroid co-culture system [41]. However, one of the main limitations of 2D cultures, including those based on co-cultures, is represented by the unnatural flat morphology in which cells are constrained, which affects numerous cellular mechanisms, *i.e.* cells proliferation, interactions between epithelium and stroma, and co-culture of human intestinal cells with commensal microbiome. As a result, 2D models fail to reflect the *in vivo* physiology and metabolism, or to recapitulate the state of some diseases or intestinal disorders. Furthermore, even though the Caco-2 cell line is able to differentiate and to create a functional barrier monolayer, the lack of a significant mucosal layer has a great impact on the permeability of drugs and molecules, influencing the compound solubility at the cell surface [42]. These limitations have motivated the research for more complex cellular models.

2.2. Three-dimensional cell cultures: from organoids to Organ-on-Chip

The self-renewal and differentiation capabilities of multipotent stem and progenitor cells, and their ability to self-organize into complex structures when cultured *in vitro* under selected growth conditions, allow for the reproduction of intestinal stem cell niche in three-dimensional (3D) *in vitro* culture systems, called intestinal organoids, or also “mini-gut” [43]. A key principle behind organoid engineering is that when different cell types are placed in the correct microenvironment, they spontaneously accumulate, switch on developmental programs and self-organize. This results in the formation of highly polarized and differentiated intestinal epithelial tissue structures that closely resemble those seen *in vivo*, such as mucus and 3D finger-like intestinal villi [44]. Organoid systems provide the conditions to recreate physiologically relevant microenvironment and human tissue architectures [27,45–47] and permit to recapitulate the biological response to several stimuli induced through changes in the homeostasis of the microphysiological system [48]. Several recent approaches have developed tissue-specific organoids, created by isolating intestinal stem cells from patient biopsies [49–51]. Advances in reprogramming the genome of adult stem cells to obtain iPSCs made a new type of stem cells available for *in vitro* culture into human intestinal organoids, besides primary tissues and embryonic stem cells [52,53]. These 3D *in vitro* models recreate several architectural and physiological features of the human tissue, with various differentiated intestinal cell subtypes, included into the polarized epithelial layer surrounding the intestine surface. An Intestine-on-Chip, containing all the intestinal epithelial subtypes, polarized and biologically responsive to exogenous stimuli, was developed by incorporating iPSCs derived by human intestinal organoids into small microengineered chips [54,55]. Compared with Caco-2 chip-derived monolayers, iPSCs-based ones showed a relevant induction in interferon-γ downstream gene targets, with a better capability to model the normal and pathophysiologic responses of the intestinal epithelium [56]. In spite of small intestinal epithelial–like cells can be generated with high efficiency from human iPSCs, the clinical relevance of the results coming from experiments on iPSCs-based models remains still under investigation, because these cells generated *in vitro* retain embryonic- or fetal-like characteristics [57].

The approaches to recreate a 3D microenvironment *in vitro* can be distinguished in two main groups: scaffold-free and scaffold-based cell culture models. In a scaffold-free system, cells proliferate and migrate starting from a building block that can be a single cell suspension, spheroid shaped multi-cellular aggregates, cell sheets or tissue strands [58–61]. In these models, cells are able themselves to produce their own ECM components. In a scaffold-based culture model, cells grow by attaching and colonizing a porous matrix of organic or synthetic

material, in the form of hydrogel or solid scaffold [62]. Natural materials are biocompatible and contain cell adhesion sites, but their biodegradability is hardly controllable and, in some cases, may compromise cell activity. Furthermore, natural materials suffer from limited mechanical properties and an intrinsic lot-to-lot variability. Synthetic materials allow to overcome these limitations, as they are designed to be inert, reproducible, and with bespoken mechanical and biodegradability properties. However, they contain a scarce amount of cell adhesion sites and often they need to be coated with ECM proteins to overcome this limitation. Hydrogels consist of elastic and cross-linked networks capable to maintaining a high water content. Cells can be encapsulated inside the gel using several methods, before gel curing with UV light. However, this is a very delicate step that risks to damage cells. Solid scaffolds allow to obtain an organized and reproducible cell arrangement into fibrous or porous matrices, which can be fabricated using different techniques and materials. Ovsianikov and co-authors proposed an interesting discussion on the main features of scaffold-based and scaffold-free approaches, and on how a promising strategy combining the advantages of both approaches is emerging [63].

Numerous methods have been reported to recreate the 3D micro-environment *in vitro*, from the use of multilayers models, where cells are deposited in different sheets [64,65], to more engineered systems, in which porous scaffolds recreate a realistic mucosal architecture with villus-like projections, for the growth and differentiation of epithelial cell types in a manner similar to *in vivo* intestinal tissues. Some examples are briefly mentioned below. An artificial 3D scaffold reproducing the dimensions and architecture of intestinal crypts and villi was obtained by using a photo-polymerizable hydrogel and high-resolution

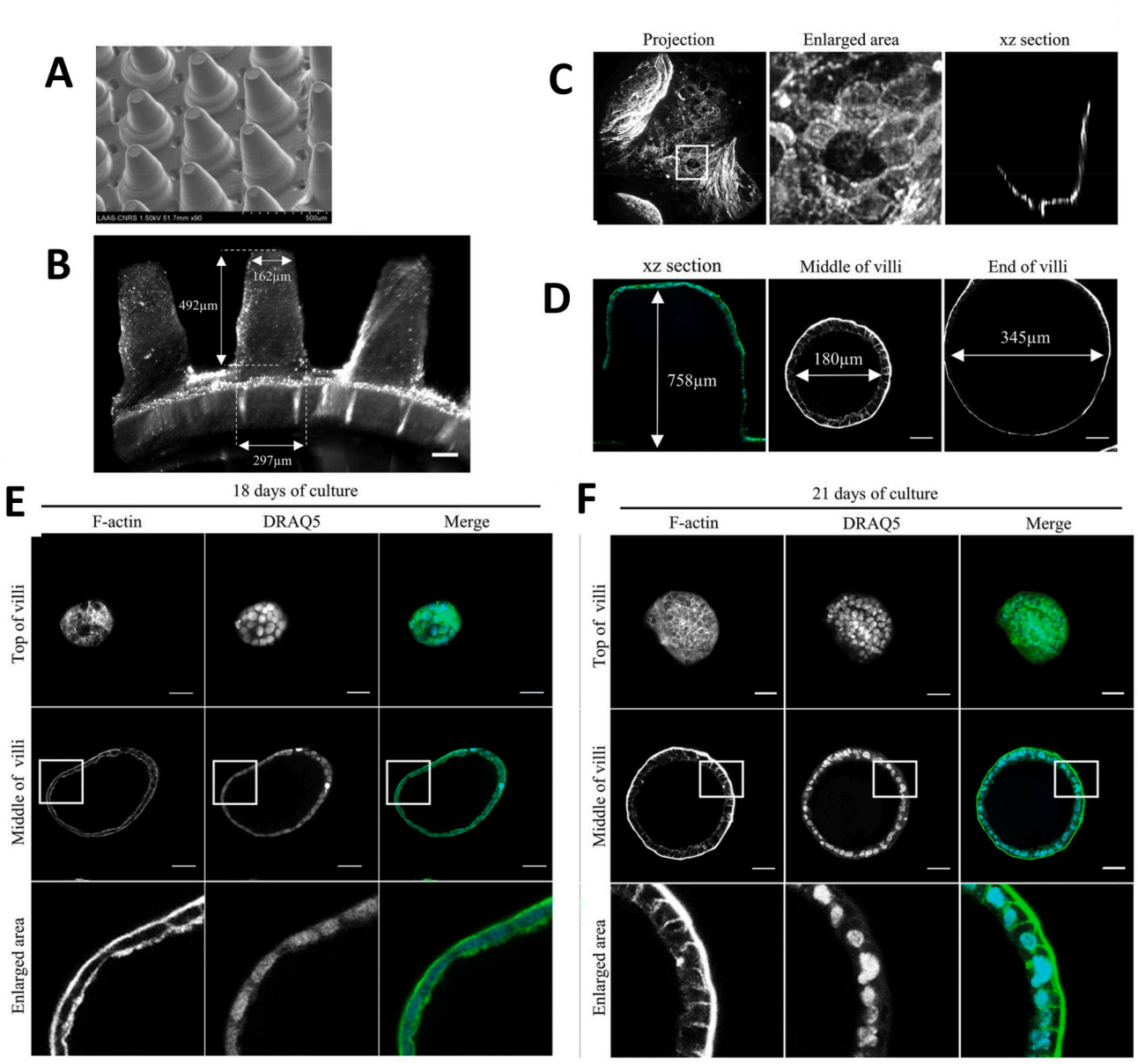

Fig. 3. Polarized epithelium formed by Caco-2 cells on 3D scaffold. A. 3D scaffold observed by SEM. B. Side view of the scaffold observed by Light Sheet Fluorescence Microscopy. C. 3D reconstruction of the scaffold by high-resolution dynamic speckle illumination imaging (left), focus on crypt level (middle) and xz section (right). D. Confocal imaging of villi; left image shows xz villi section, middle and right images depict xz sections of the middle and base of a single villus, respectively. *E*-F. After 18 or 21 days in 3D scaffold, cells were fixed and stained for nucleic acid (DRAQ5, blue) and F-actin (Phalloidin-FITC, green). The images show cellular morphology and actin polarization after 18 and 21 days, respectively, by performing experiments on 3 independent scaffolds. (scale bar = 50 µm; for interpretation of colour in this figure, the reader is referred to the Web version of [66]. Overview figure is adapted from [66], CC-BY-NC-ND 4.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stereolithography 3D printing [66]. This 3D culture model was able to support the growth and differentiation of Caco-2 cells for 3 weeks (Fig. 3).

In [67] Sung et al. fabricated micrometric hydrogel structures with high aspect ratio and curvature using laser fabrication and sacrificial molding; Caco-2 cell line was seeded onto the 3D scaffold and cultured for 3 weeks, until the intestinal villous-like structures were fully covered with epithelial cells, as shown in Fig. 4.

The same method proposed by Sung et al. was used in [68] to realize a microfluidic GoC with an integrated collagen-based scaffold reproducing 3D villi structures, upon which Caco-2 cells were cultured in the presence of fluidic shear obtaining further differentiation, improved physiological relevance and metabolic activity. Yu and co-authors proposed a method for testing drug permeability of human cells cultured on this *in vitro* 3D model [69]. Experiments demonstrated that cell differentiation varies along the villous length, with cells resulting columnar and more polarized at the top and less differentiated near the villous base. However, culture times longer than 14 days using this 3D model resulted in shorter villous heights and penetration of the villus scaffold by multiple cell layers. Esch and *co*-workers fabricated and used flat and three-dimensionally structured microporous polymeric membranes for culturing human Caco-2 cell lines, thus obtaining a more faithful architecture of the intestinal epithelium *in vitro,* with villi and TJs [70]. Recently, strictly related to the 3D scaffold requirements, conducting polymer (CP) scaffolds made from poly(3,4-ethylenedioxythiophene) doped with poly(styrene sulfonate) (PEDOT:PSS) demonstrated a more reliable integration with 3D cell cultures, adding optimized cell-to-electrode coupling, required for better low-level signal transduction. These materials have shown interesting performance as tissue engineering substrates for hosting a variety of cell lines and tissues [71]. In addition to providing a 3D template for cell growth, the CP scaffolds and PEDOT:PSS layers can be used as transparent and photo-definable electrodes to electrically monitor cell growth [72]. A recent related application of the CP for OoC investigation has been proposed by Moysidou et al. [73]; a tubular CP-based scaffold was used to mimic the 3D luminal architecture of the human gastrointestinal tract. Porous CP scaffolds within T-shaped tubes were fabricated, and a hollow channel was incorporated in the center of the scaffold to mimic the gut lumen. The proposed device was exploited both as engineered substrate for the co-culture of Caco-2 cells and HT29-MTX cells, and as new device structure, called L-Tubistor, for non-invasive and real-time monitoring of cell activity and tissue formation, for a period of 1 month (Fig. 5).

An interesting feature of the proposed design is that the device sensing components were expected to be incorporated inside the tissue, so that the L-Tubistor could work in dual-mode, both as electrode for electrochemical impedance spectroscopy measurements used to quantitatively monitoring the status of the cell culture system, and as transistor for transconductance measurements that qualitatively assess tissue formation. The observed stability of the above reported L-Tubistor devices was in good agreement with previous studies of Dijk et al. [74] on PEDOT:PSS-coated gold electrodes, reporting stable electrical behavior after 4 months in cell culture medium. Another interesting example is HuMiX [75], that is a modular microfluidics-based human-microbial co-culture platform developed to study the host–microbe molecular interactions in the gut under anaerobic conditions. In this system, three stacked and parallel microfluidic channels were used as microbial, epithelial, and perfusion microchambers. The microchannels were separated by a semipermeable polycarbonate membrane that

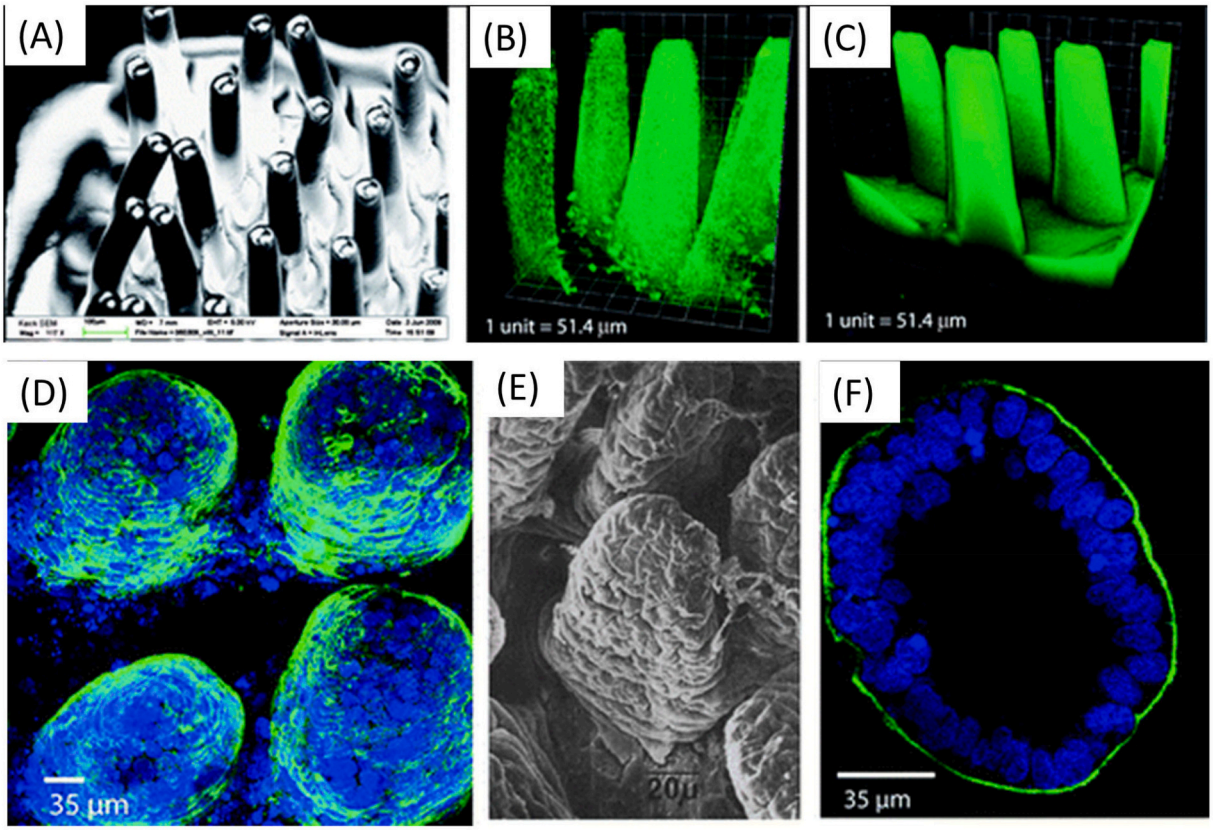

Fig. 4. Microscale 3D hydrogel scaffold for biomimetic gastrointestinal (GI) tract model. A. The view PDMS villi structure observed by SEM. B-C. Image of the collagen scaffold (B) and of the PEG scaffold (C) after 3-D rendering, captured by Confocal microscope. D. Caco-2 cells on collagen scaffold observed by Confocal microscope after staining for actin (green) and nucleic acid (blue). E. SEM image of human jejuna villi. F. Confocal microscope image of x − y slice of Caco-2 cells on the collagen scaffold, stained for actin (green) and nucleic acid (blue). This figure is adapted from [67]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

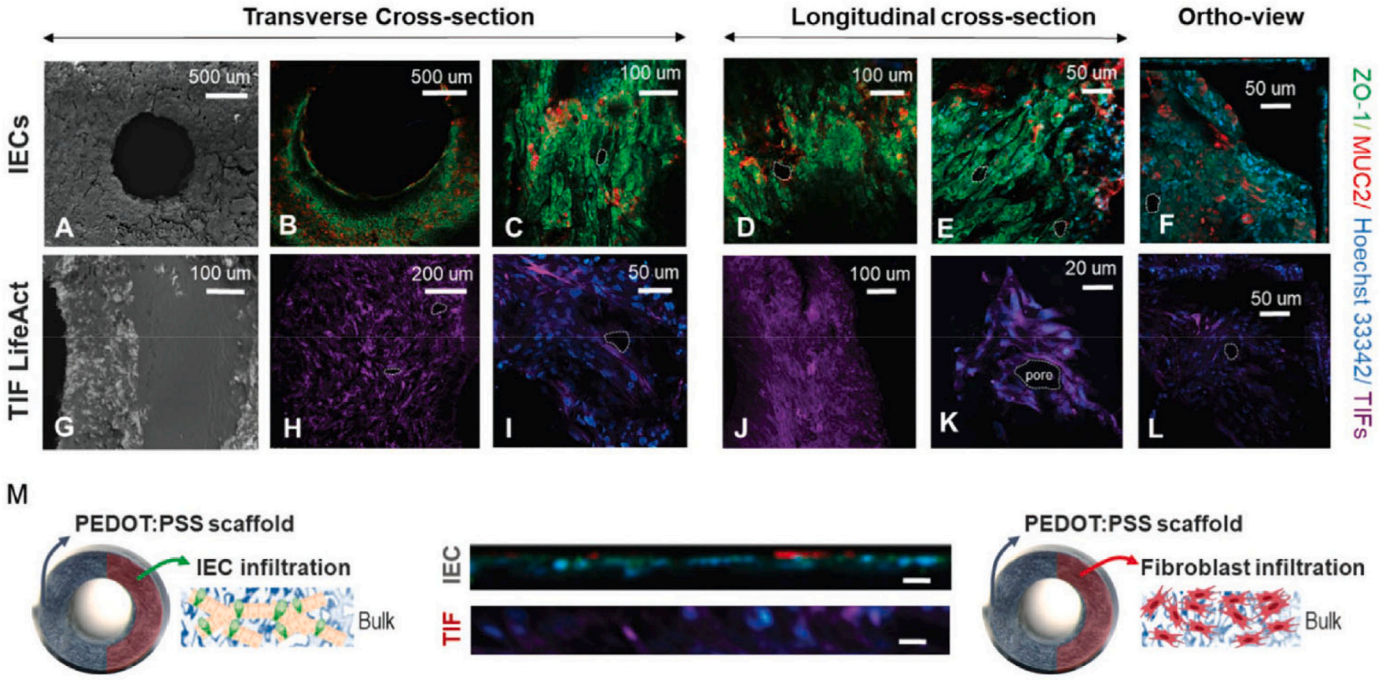

Fig. 5. The 3D bioelectronic human intestine hosted in L-Tubistors. A. SEM image showing the coverage of the scaffolds by the tri-culture cell model. B-F. Immunostained transverse, longitudinal, and orthogonal sections of the 3D intestinal model, confirming the capability of enteric cells to form a continuous epithelial coating on the lumen layer supported by the lamina propria sheet present in the bulk compartment of the L-Tubistor scaffolds. The tight junctions, mucin secretion and nuclei can be observed in green, red, and blue, respectively. Image F shows the x/y (center), x/z (top), and y/z (right) views of the sample obtained by the z-stacked confocal images. G. A schematic picture of the bioelectronic platform hosted human intestine model (left). Magnified x/z orthogonal views (right) of the samples obtained by z-stacked confocal images, showing the fibroblasts and IECs arrangement in the scaffold microenvironment. Overview figure is adapted from [73], CC-BY-4.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

avoids the infiltration of microorganisms from the microbial chamber into the epithelial one, ensuring the unidirectional diffusion of the cell growth medium into the epithelial chamber. The growth medium was perfused only during incubation using a pump. However, the experimentation was carried out in the absence of mechanical peristalsis. Organoid-on-Chip devices as biomimetic tissue models can be useful for studying several physiological/pathophysiological states and for evaluating the drug therapy. 2D monolayer gastric organoids generated from 3D gastric organoids of the antrum could reproduce a portion of the gastric mucosa, producing mucus after 13 days post seeding. For this purpose, some features have to be considered: the intestinal crypt-villus axis for the organoid cultivation; the accessibility to the apical and basal chip surface for an homogenous confluent monolayers formation; the scaffold system compartmentalization for a luminal space creation, that is important for mimicking the native intestinal mucosa [76]. Studies on organoids-on-chip have shown the potential of this technology in the field of drug discovery, creating patient-specific disease models, and also in personalized and regenerative medicine, providing models of expanded organoids transplanted *in vitro* and able to repair damaged organs [77,78]. Yet, organoid-on-chip technology is still far from mimicking the human *in vivo* development and functionality of a tissue/organ. To fulfill this gap, microenginering techniques are being explored, in combination with improvements in organogenesis and in 3D imaging techniques to better monitoring organoid development. From this viewpoint, it is expected that advances in microengineered culture devices instrumentation could also reduce the observed organoids variability present in *in vitro* cultures, probably derived from chip architecture and structures that affect the organoid cultivation. At the same time, variability is also an intrinsic characteristic of organoids development when they are cultured *in vitro* [79]. Even though organoids are able to exhibit organ-specific morphology and cellular organization, they have some drawbacks: i) the 3D closed geometry hampers direct access to the apical region of the epithelium, preventing studies on nutrient transportation, microbiome−intestinal epithelium interactions and drug absorption [80]; ii) the absence of fluid flow and peristalsis movements prevents the use of important cell types like immune cells and blood vessel-forming endothelial cells; iii) the heterogeneity in shape and size of organoid cultures causes an inevitable variability in experimental results. The genetic differences, variance in size, shape and functional features of organoids could be alleviated by the automation of several steps, thus favoring reproducible and accurate manipulation in organoids based-culture protocols. To achieve an automated organoid 3D culture, it is necessary to integrate sensors, and in particular biosensors, inside on-chip cell cultures. They could promote long-term and real-time monitoring, thus enabling screening at different levels of the OoCs [81]. In the most common configuration, an OoC platform comprises a lower chamber, containing endothelial cells, and an upper one, containing cells of the tissue or organ of interest. They are separated by a flexible porous membrane upon which cells adhere, that allows communications between the two compartments. Fig. 6 provides a clear description of these geometries, representing the complex interactions between different specialized cells arranged in well-defined spatial distributions and microenvironments, creating specific interfaces and promoting organ functions [82].

This technology allows culturing of different human intestinal cell populations on engineered platforms, designed to properly mimic the structural, mechanical and physiological properties of the human intestine [83]. The most recent technologies have provided several effective tools to promote the development of more overspecialized GoCs. This process was initially implemented in standard 2D cultures on microfluidic systems mimicking transwell cell culture inserts that enable the culturing of intestinal epithelial barriers. In [84] Caco-2 cells were cultured into a microdevice composed of a porous flexible membrane coated with ECM and placed between two microfluidic channels. Cells

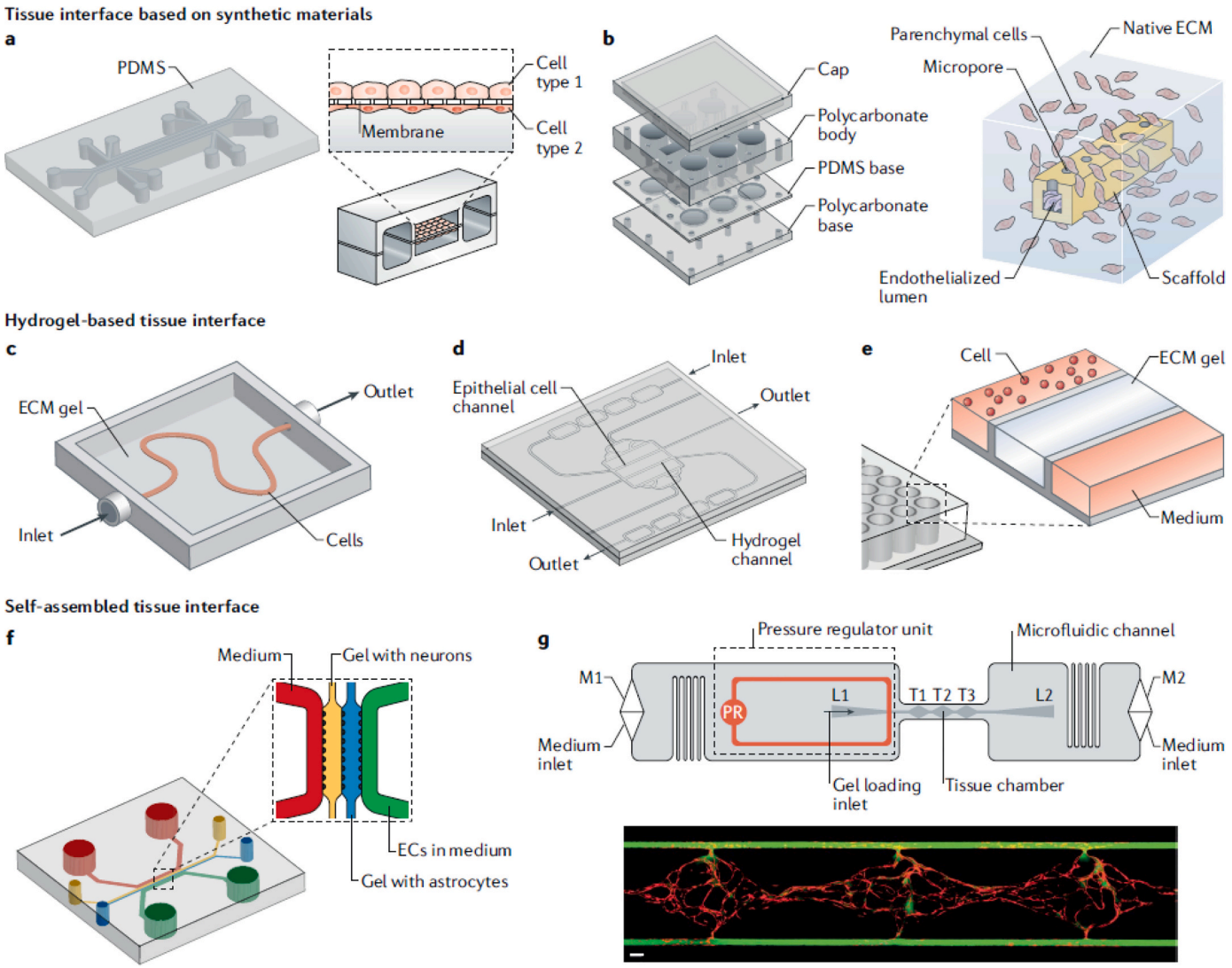

Fig. 6. (a,b) Representation of the tissue barrier function. Tissue interface realized by synthetic materials (tissue model by PDMS flexible membrane (a), microfabricated scaffolds (b)). (c-e) Tissue interface based on hydrogel (3D printing of hydrogel (c); moulded hydrogels (d); phase-guided hydrogels (e)). (f,g) Self-assembled tissue interface (flow-driven cellular self- assembly (f); perfusion-driven vasculogenesis (g)). The micrograph shows the formation of a vascular network within one tissue unit. Scale bar = 100 μm. From [82].

were cultured under luminal fluidic flow and peristaltic motions, growing to form a polarized columnar epithelium that spontaneously developed into folds, recapitulating the structure of intestinal villi with a high integrity barrier. In this microdevice, microbial flora was successfully co-cultured on the luminal surface of the epithelium for more than a week, preserving the epithelial cell viability and the normal barrier functions, with an improvement of the barrier integrity over time. A further and more detailed discussion was proposed by the same authors in [85]. They demonstrated that, under fluid flow and peristaltic movement, the cultured tumor-derived Caco-2 cells recreate undulating human intestinal villi, surrounded by basal proliferative crypts along the crypt–villus axis and populated by the four main types of differentiated small intestinal epithelial cells (absorptive, mucus-secretory, enteroendocrine, and Paneth). Exploiting the same microfluidic architecture, consisting of two independent channels and a semipermeable separation membrane, Kimura and co-authors [86] developed an advanced microfluidic device, in which Caco-2 cells were cultured under continuous perfusion for more than thirty days (Fig. 7). A stirrer-based pumping system ensured the circulation of the culture medium and the uniform distribution of nutrients and metabolic products inside the device. An optical fiber system embedded in both apical and basolateral compartments of the culture chamber allowed to perform fluorescence measurements on chip. In [49] epithelial cells isolated from healthy regions of intestinal biopsies were cultured into a primary human small Intestine–on-Chip: after expansion in the form of a 3D organoid, primary epithelial cells were dissociated and cultured on a porous membrane under fluid flow and cyclic deformation. A parallel microchannel within the same microdevice was used to culture a human intestinal microvascular endothelium. The study demonstrated the formation of epithelium with villi-like projections and polarized multi-lineage differentiated epithelial cells, apically exposed to the open lumen, and interfaced with endothelium. The 3D intestinal villi-epithelial barrier with the consequent mucus production also showed a digestive capacity, evaluated by measuring the activity of the brush border enzyme, through the transcriptome analysis that revealed the expression of genes related to digestion. This primary Intestine–on-Chip enables cell proliferation on an ECM-coated porous membrane, providing nutrient uptake, a defense response to infection, much closer to the *in vivo* characteristics of an adult human duodenum than the only organoids that were used to plate the chips or Caco-2 GoC [49]. Costello et al. realized a small intestinal bioreactor using 3D printing and polymeric porous villous scaffolds made of a not biodegradable polymer, poly-

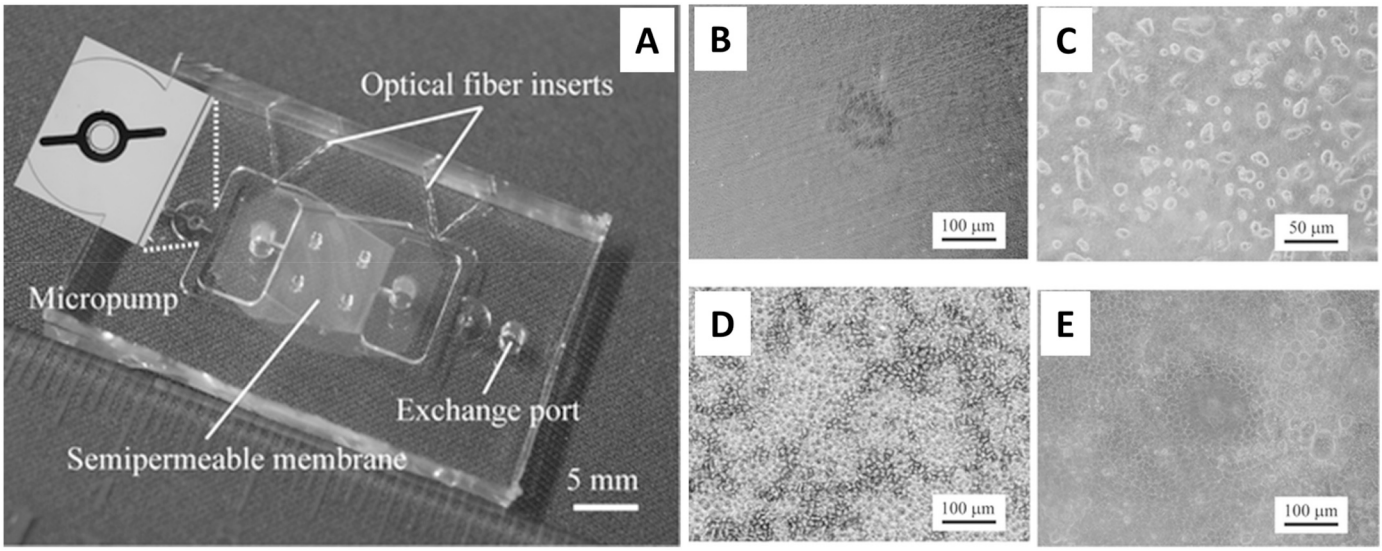

Fig. 7. A. Photograph illustrating the microfluidic device, where Caco-2 cells were cultured by Kimura et al. [79]. Figs. B-E show the semipermeable membrane: B) before Caco-2 cells seeding; C) after cells attachment on day 1, and confluent conditions; D) on day 9 and E) on day 21. Figures adapted from [86].

ethylene-*co*-vinyl-acetate (PEVA), to mimic the 3D intestinal topography [87]. The intestinal fluid flow was reproduced by a peristaltic pump and Caco-2 cells were cultivated on villous scaffolds for more than 3 weeks, obtaining interesting results. In [88] a perfusable tubular scaffold composed of a mixture of type-1 collagen and Matrigel was used to culture stem cells, obtaining an intestinal epithelium arranged in crypts and villi-like structures, and with accessible lumen. The microdevice consists of a central hydrogel compartment for cells culture, two basal side medium reservoirs placed laterally to the hydrogel compartment, and inlet and outlet medium reservoirs for microchannel perfusion. A GoC model composed exclusively of patient-derived human primary material was proposed in [89] seeding human biopsy-derived epithelium in the upper channel of the membrane-free 3-lane OrganoPlate platform under fluid flow, to model inflammatory bowel disease condition.

3. Sensing system integration in Gut-on-Chip

The control and assessment of microenvironment biophysical and biochemical parameters are very critical aspects which corroborate the capability of an OoC device to recapitulate the human organ faithfully. To this aim, automated and non-invasive sensor systems are strongly desired for a real-time and long-term monitoring of cells vitality, as well as of their dynamic response to external stimuli. Key parameters to be monitored are cellular adhesion, detachment, death, response to osmotic stress, sepsis on chip, osmolarity, pH value, and so on. An array of multifunctional sensors integrated on the OoC platform may enable the realization of closed-loop feedback on the culture system, bypassing the need for several off-line measurements [90]. Fig. 8 shows the main types of active and passive sensors that can be integrated into an OoC platform.

This section reviews the integrated sensing strategies exclusively adopted in Gut-on-Chip platforms to control the intestinal barrier integrity and the evolution of physical/chemical parameters. The study of the intestinal barrier integrity helps to recognize dysfunctions and gastrointestinal diseases, while the monitoring of physical/chemical parameters in environment-sensitive cell cultures promotes stable, controllable and reproducible culture conditions.

3.1. Oxygen monitoring in Gut-on-Chip

Reactive Oxygen Species (ROS) and O_2 concentration are parameters strictly connected to gut functionality. Intestinal Epithelial Cells (IECs) are organized as a cell monolayer that forms a continuous functional barrier, which preserves gut structural integrity, separating the bowel wall from microbes and toxins [91]. In a healthy intestinal mucosa, the

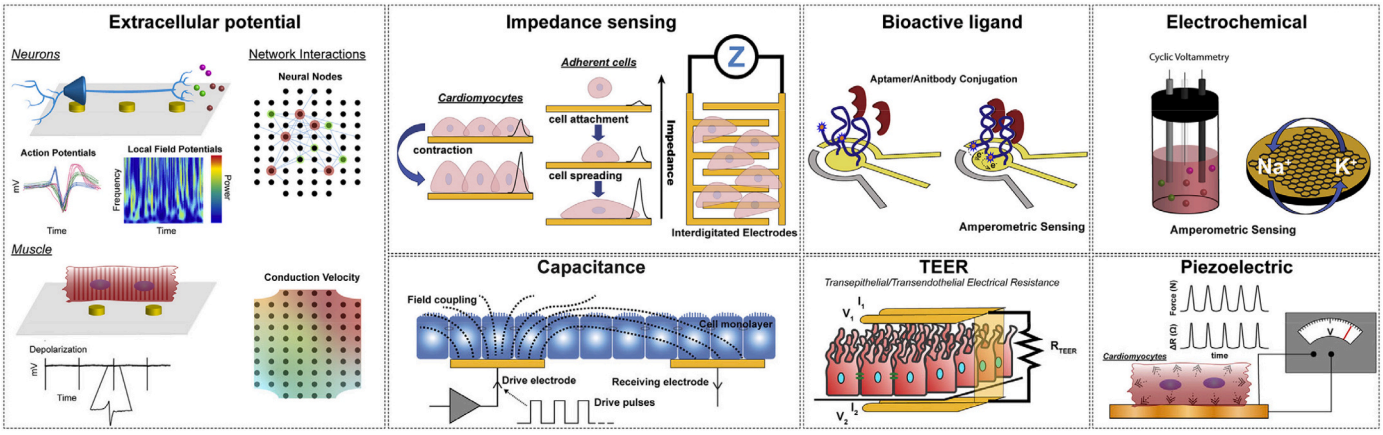

Fig. 8. Active and passive sensors that can be integrated into an OoC platform for a closed-loop feedback on the chemical and physical parameters related to culture system. The image is reproduced from [90], CC-BY-NC-ND 4.0.

processes of IECs proliferation and death are perfectly balanced to ensure the integrity of the intestinal epithelium and barrier function. It has been demonstrated that the excessive cellular death is associated with chronic inflammations, known as Inflammatory Bowel Disease (IBD) [92,93]. Even if the causes of IBD are not completely clear yet, the immune response, genetics, microbiome and environment factors concur to their development. In this condition, a peroxidation/anti-oxidation imbalance occurs, that consists in an excessive ROS generation (*i.e.* superoxide anion, hydrogen peroxide and hydroxyl radical) and consequent oxidative stress [94]. Oxidative stress is a pathological cellular state in which cells lack the ability to deploy an antioxidant defensive mechanism to prevent the cellular damage [95]. It showed an important role also in malnutrition, as it alters the intracellular redox status of the gut microbiota, which are unable to perform their regular functions for the host [96–98]. Intestinal cells are exposed to different oxygen concentrations, moving from the center of the lumen (with low O_2 concentration) to intestinal mucosa (well oxygenated by blood capillaries); a lack of this radial oxygen distribution is an indication of bacterial infection and gut inflammation [99].

Optical and electrochemical sensors are the most used devices to monitor oxygen inside organoids and organs-on-chip [100–102]. With optical sensors, remote and non-invasive measurements are possible, thanks to the intrinsic transparency of microfluidic materials, which can be simply coupled to optical sensing systems. An interesting application of optical sensors for luminal oxygen content monitoring integrated into a human pluripotent stem-cell-derived intestinal organoid can be found in [103]. A commercial miniaturized optical oxygen microsensor, equipped with a tiny probe with a tip size <50 μm, was attached to a microfiber optic oxygen meter. Luminal oxygen content data were collected by connecting the microsensor to a micromanipulator to guide

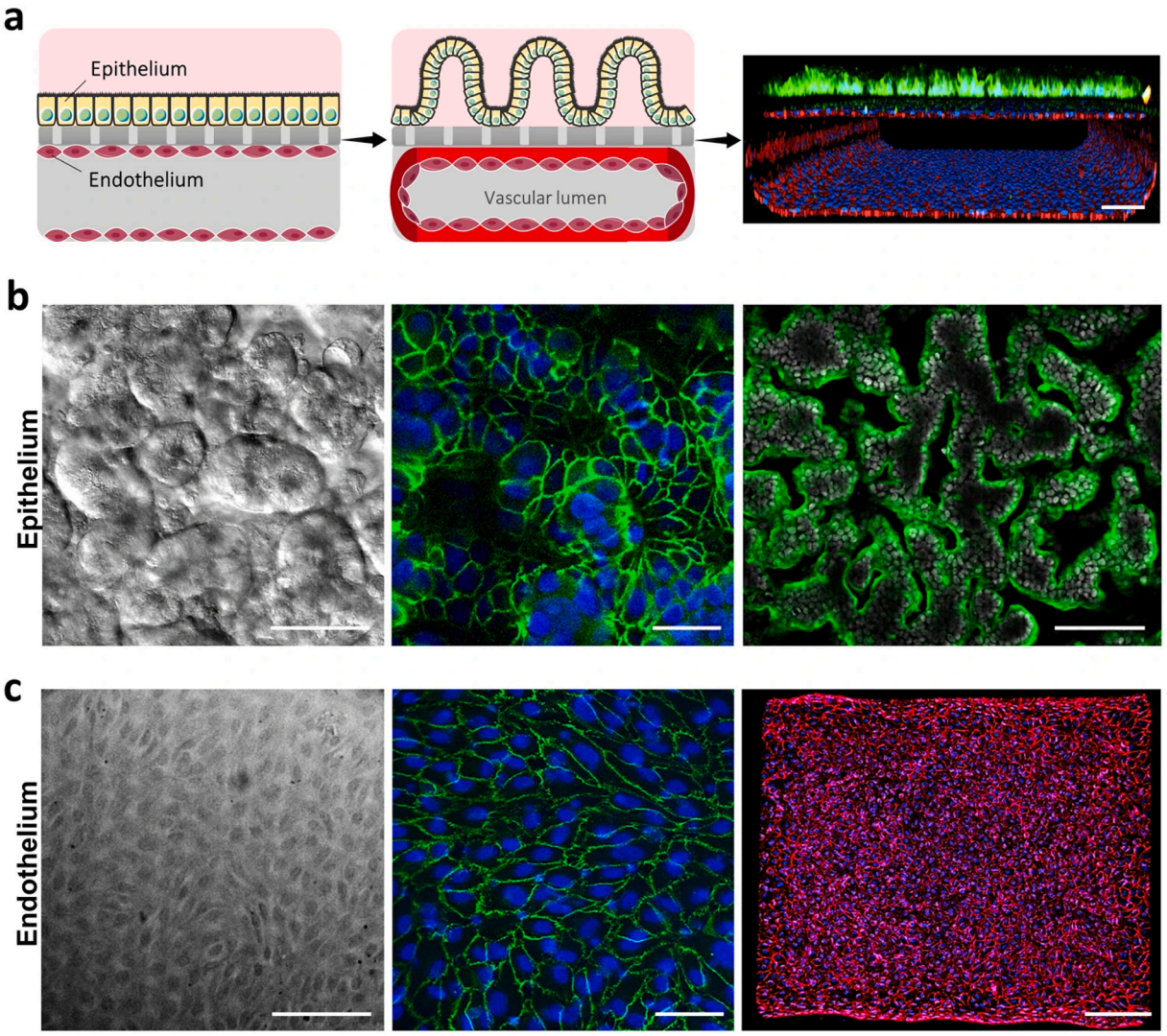

Fig. 9. Human Gut-on-Chip microfluidic culture device: (a) sketch of human intestinal epithelium and endothelium positions placed on opposite sides of the matrix-coated porous membrane within the two-channel microfluidic device (left); villus epithelium formation in the top channel interfaced with a planar endothelium that forms a lumen in the bottom channel (middle); immunofluorescence confocal micrograph showing a cross-section of the Gut-on-Chip device with the villus intestinal epithelium stained for villi (green) to visualize the apical brush border, and the planar endothelium stained for VE-cadherin (red) to visualize adherens junctions (right; bar: 100 μm). (b) Microscopic views showing the villus morphology of the human Caco-2 intestinal epithelium cultured for about 5 days in the Gut-on-Chip with flow (30 μl h − 1) and cyclic strain (10% at 0.15 Hz), viewed from above by DIC imaging (left; bar, 50 μm) or by immunofluorescence staining for the tight junction protein, ZO-1 (green, middle; bar: 50 μm) and villin (green, right; bar: 100 μm). (c) Microscopic views showing the planar morphology of the human endothelium cultured under identical conditions as in b, viewed from above by phase contrast imaging (left; bar: 50 μm) or immunofluorescence staining for the endothelial cell junction associated proteins PECAM-1 (green, middle; bar: 50 μm) and VE-cadherin (red, right; bar: 200 μm). Blue indicates DAPI-stained nuclei. The figure is reproduced from [106], CC-BY-4.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the sensor tip to the point of interest. Okkelman et al. [104] studied the redox metabolism of stem cells intestinal organoids using two emerging microscopy techniques based on luminescence lifetime measurements: fluorescence lifetime imaging microscopy for NAD(*P*)H and phosphorescence lifetime imaging microscopy for real-time oxygenation. They observed that the exposure of stem and differentiated cells at different concentrations of glucose leads to quantifiable shifts of oxygenation and redox status. This discrepancy in stem and

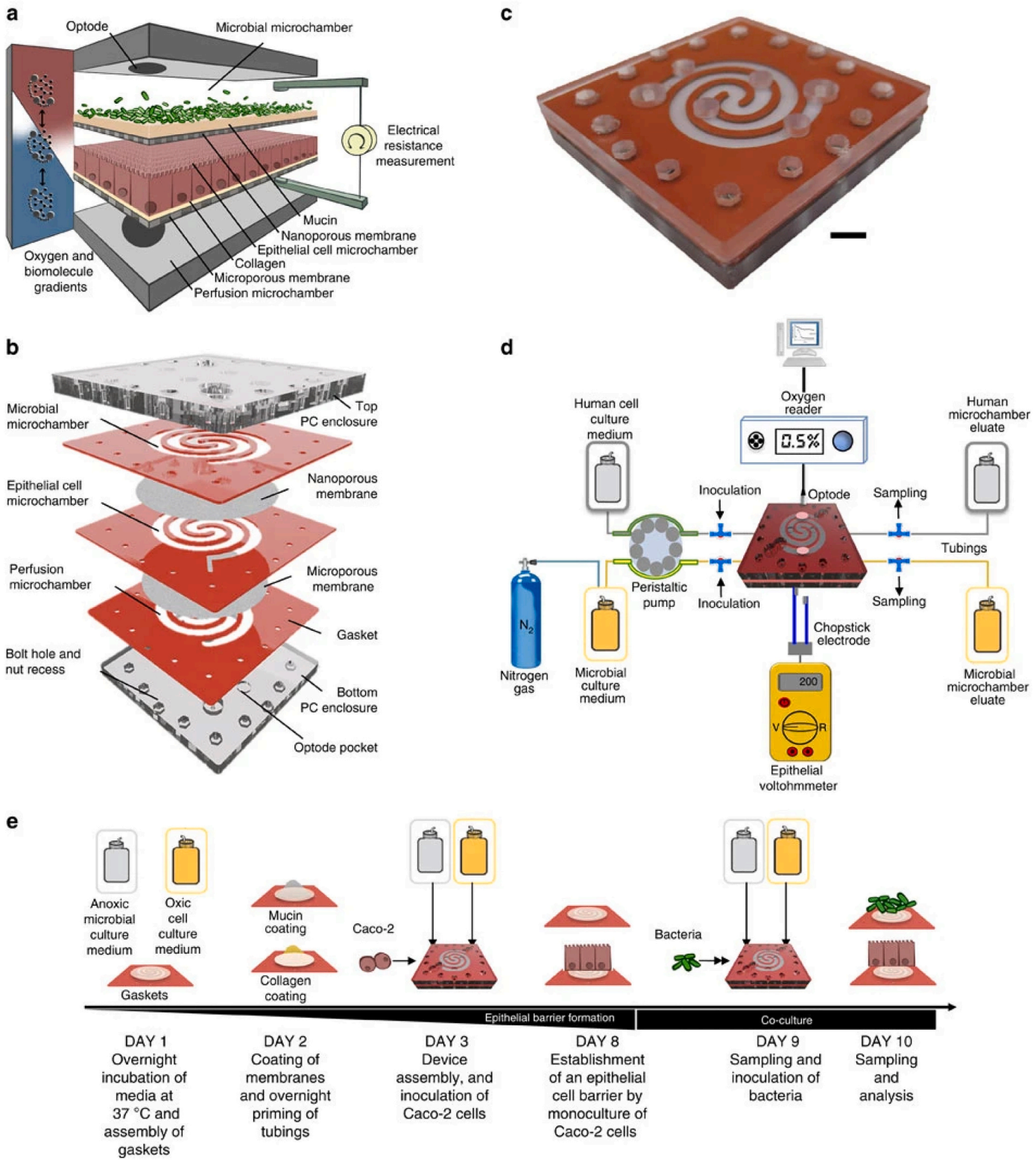

Fig. 10. Human Gut-on-Chip microfluidic culture device: (a) HuMiX model of gut. (b) Illustration of the modules composing the final device. (c) Picture of the assembled final device. (d) Schematic of the experimental set-up for the characterization of HuMiX model; the perfusion of oxic and anoxic culture media as well as the monitoring of the oxygen concentration and transepithelial electrical resistance are guaranteed. The oxygen concentration in the anoxic medium is kept constant at 0.1% by continuously bubbling the medium with N2 gas. (e) Representation of HUMIX protocol for the analysis. The figure is reproduced from [108], CC-BY-4.0.

differentiated cells response corresponds to crypt and villi compartments of gut epithelium. Live imaging microscopy may represent an innovative approach to study single cell metabolism at subcellular resolution, providing an important tool for OOC development. Perez et al. [105] presented a new class of optical sensors to evaluate the oxygen concentration in different 3D tissue culture microenvironments, by using phase fluorimetry on oxygen-sensor microbeads with remote imaging.

In the work of Jalili-Firoozinezhad et al. [106], a human GoC device, lined by human intestinal epithelial cells and vascular endothelial cells, was used to model human intestinal injury induced by acute exposure to γ-radiation. Cellular ROS generation due to radiation was quantified using CellROX Green Reagent fluorogenic probe. In the presence of ROS, the CellROX Green Reagent underwent oxidation, producing bright green photostable fluorescence. ROS level signal intensity was measured at excitation/emission wavelengths of 485 nm/520 nm (green), as shown in Fig. 9.

This test requires traditional fluorescence microscopy for analysis. The team of Ingber implemented the model of Intestine-on-Chip [107] co-culturing human intestinal cells with aerobic and anaerobic gut microbiota to study the interaction between intestinal tissue and microbiome. In this way, a controlled oxygen gradient is generated. Oxygen gradients are real-time monitored through six oxygen-quenched fluorescent particles embedded in the inlets, middles, and outlets of the top and bottom channels. Measurements are performed in real-time, with high sensitivity, in each spot localized in the microfluidic system. The capability to establish transluminal hypoxia gradient into GoC emphasizes the function of the intestinal barrier and ensures the physiological level of microbiota diversity. In [108], Shah et al. proposed the first approach to properly model the gastrointestinal human-microbe interface inside the microfluidic device (HuMiX). The GoC is made of three separated micro-chambers, including medium perfusion, human epithelial cell (Caco-2) culture and microbial culture, each equipped with an inlet and an outlet to permit single control of physiochemical parameters and downstream analysis. Oxic and anoxic media were simultaneously perfused through perfusion and microbial microchambers, respectively, to guarantee an oxygen gradient that better mimics the *in vivo* conditions. Oxygen levels in both chambers were real-time monitored by integrating optical sensors (optodes) while cell growth and differentiation were monitored by Trans-Epithelial_Electrical_Resistance (TEER) measurements, with the insertion of bulky chopstick electrodes. Optical fibers were employed to transmit oxygen measurements to the recording device (Fig. 10).

Electrochemical sensors are especially attractive because they directly convert the chemical information into an electrical one. Among the voltammetric techniques, amperometry is the most used for oxygen detection. A constant potential applied to an electrode immersed in solution induces the oxidation/reduction of an electroactive specie in a chemical reaction, which determines an electrical current proportional to the investigated analyte concentration [109]. Electrochemical sensors were employed by Baltsavias et al. [110], who performed for the first time integrated real-time Oxidation/Reduction Potential (ORP) measurements by using an implanted wireless sensor, powered and interrogated *via* ultrasonic waves, to monitor gastrointestinal tract redox states. In this way, the tendency of the chemical environment to lose or gain electrons was evaluated, considering that each microbiome generates a specific intestinal chemical environment, which can be subjected to alterations in presence of higher levels of oxidation. This represents an interesting approach to realize long-term experimental tests, for a deeper understanding of gut redox pathophysiology mechanisms and specific clinical treatments.

The above overview shows that optical and electrochemical sensors for oxygen monitoring do not represent a fully integrated solution. The commercially available solutions have large dimensions, incompatible with a direct and easy integration in OoC devices. Optical sensors are considered more reliable, and they are generally preferred for low oxygen concentration measurements since the electrochemical ones use up oxygen during analyses [111] and require a more expensive experimental set-up and more complex data analysis. Nevertheless, the direct transduction from chemical to electrical information, together with robustness and measurements reproducibility for on-line monitoring, make electrochemical sensors more suitable for miniaturization. One possible strategy to shrink their sizes could be the replacement of bulk electrodes with solid-state planar electrodes, by using a suitable combination of materials and microfabrication technologies [112]. Moreover, the required electrolyte solution and porous membrane can be implemented using solid polymers, making the sensors even more robust and enduring. Another important issue to face is represented by the long-term stability of the integrated pseudo-reference electrodes (*e.g.* Ag/AgCl), typically characterized by a limited stability in Cl^- containing medium.

3.2. pH monitoring in Gut-on-Chip

The important role played by pH during cell growth and differentiation *in vitro* is well known, since biological processes are extremely sensitive to acid-base chemistry [113]. pH is also a physical parameter that provides ample information about the health status of the gut. As a matter of fact, gut microbiota block bacteria invasion by preserving the intestine epithelium integrity through many processes such as pH modification [114]. Along the gastrointestinal tract, pH values span from 5 to 7 according to different processes, such as fermentation products or microbial metabolites absorption by host epithelial cells [115]. The deviation from normal values of colonic pH along with gut microorganisms is an indication of gastrointestinal diseases (for instance, colorectal cancer). Thus, monitoring pH is key in reproducing devices mimicking the gut.

Optical and chemical/electrochemical mechanisms are widely used for pH monitoring in OoC devices [116,117]. The first attempts to employ pH sensors in gut organoids/enteroids can be found in the works of Zachos et al. [118] and McCracken et al. [119] by using pH indicator dyes.

Zachos et al. used two-photon microscopy with SNARF4F pH-sensitive dye to measure intracellular pH in human small intestinal enteroids. An imaging software is required to obtain results from the regions of interest (Fig. 11). McCracken et al. differentiated human pluripotent stem cells into a gastric organoid to realize a suitable model for the study of human stomach fundus epithelium. SNARF5F pH-sensitive dye was used, and the real-time confocal microscopy was employed to observe the luminal pH variation in response to histamine. Luminal pH evaluation by optical techniques is challenging, due to optical interactions related to the presence of lumen between cells layer and extracellular matrix.

In OoC literature, two emergent classes of pH sensors can be found, *i.e.* electrochemical sensors employing either metal oxide-based electrodes [120] or gold interdigitated electrodes [121]. These devices are adapted for biological matrix and clinical applications where generally small sample volumes are involved. They have shown great potential in biosensors applications and chip integration as monitors for several biochemical and biophysical parameters.

3.3. Monitoring of intestinal barrier function in Gut-on-chip: cell integrity and membrane damage

Epithelia and endothelia surrounding the gut constitute a selective semipermeable barrier that regulates biomolecular transport between adjacent tissues. In gastrointestinal and in several related intestine diseases, an underlying pathogenic factor is the increased paracellular permeability of the intestinal epithelial barrier. Paracellular permeability increase is an indication of the breaking of TJ integrity caused by epithelial monolayer disruption. It is largely recognized that this disruption is at the base of many gastrointestinal and non-

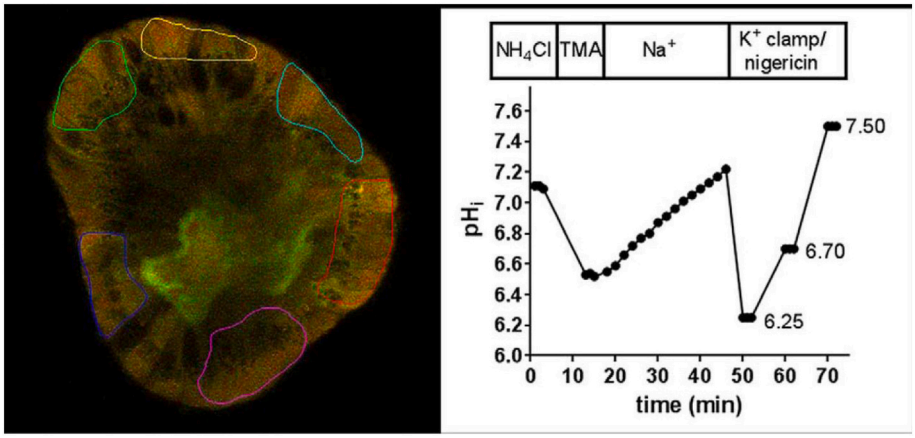

Fig. 11. NHE3 activity is studied in human enteroids as a model, where duodenal enteroid is stained with the pH-sensitive dye (SNARF-4F, left side) and imaged using a two-photo microscope (Olympus). The right panel describes the NHE3 activity after loading enteroids with acid and by pulsing NH4Cl followed by Na-free, tetramethylammonium (TMA) solution. This results in intracellular acidification and the pH values are calibrated after final exposure to K clamp solution containing nigericin at various pH values (*e.g.* 6.25, 6.70, 7.50). Measurements were recorded in the regions of interest indicated by multi-colored traces in left image by using MetaMorph Image Analysis software. The image is reproduced from [118], Copyright © 2016 by The American Society for Biochemistry and Molecular Biology, Inc.

gastrointestinal dysfunctions. As a matter of fact, many diseases of different origin - *i.e.* type I diabetes, multiple sclerosis, immunodeficiency, celiac disease, irritable bowel syndrome, food intolerances, *etc.*- could be considered strictly linked to the so-called "leaky gut syndrome" [122].

Commonly, the evaluation of paracellular permeability is mostly performed *in vitro* by cultivating cell monolayers in a Transwell system, in which a rigid porous membrane separates two fluidic chambers under static conditions. TEER and fluorescent techniques are the most used methods to monitor barrier integrity in 2D cell cultures [123,124]. TEER is a quick, conventional, non-destructive, and non-invasive conventional assay, which can be applied to monitor live cells during their various stages of growth and differentiation and allows for cell cultures to be re-used for additional studies. Many works report on the use of TEER analysis for 2D intestinal cells cultures [41,125–127], often performed together with fluorescent methods, such as Fluorescein IsoThioCyanate-DEXtran (FITC-DEX) assay [128–132].

With the advance in cells culture and microfluidic models, gastrointestinal barrier integrity was monitored mainly by FITC-DEX in 3D gut organoids [133,134] or microchip-based system mimicking the intestine [135]. In contrast, the use and integration of TEER analysis in OoC 3D design has resulted very difficult, mainly due to the small sizes of microfluidic channels used for cell growth and differentiation, which complicates reaching the epithelium to measure its electrical resistance [130]. The complete integration of TEER equipment into OoC is the most exciting challenge in this field and many attempts have been tried to pattern electrodes directly onto PDMS microfluidic devices [136]. The first approach to integrate TEER technique into a Multi-Organ-on-Chip (MOC) including a gut is found in [137]; the microfluidic system contains embedded electrodes to assess the formation and disruption of epithelial barriers both within a human Lung Arway Chip, lined by a fully differentiated mucociliary human airway epithelium, and in a human GoC, lined by intestinal epithelial cells. The gold electrodes were patterned onto polycarbonate substrates while PDMS layers and PET membranes have been irreversibly bonded by silane-based surface modification. This is a very simple layer-by-layer fabrication process for the integration of sensing electrodes into the microfluidic system. These electrodes were used to perform both TEER and cell layer capacitance measurements by using 4-points impedance analysis at varying frequencies, in real-time and non-invasive way (Fig. 12). However, the full electrodes-chip integration still remains an open challenge: size and geometries of the integrated electrodes should be further scaled down and optimized to guarantee more reliable electrical potential measurements across the tissue barrier, preventing measurements errors due to non-uniform current densities. To overcome the limitations of TEER measurements, a method was proposed in [138], based on the combination of impedance spectroscopy with electric stimulation to measure

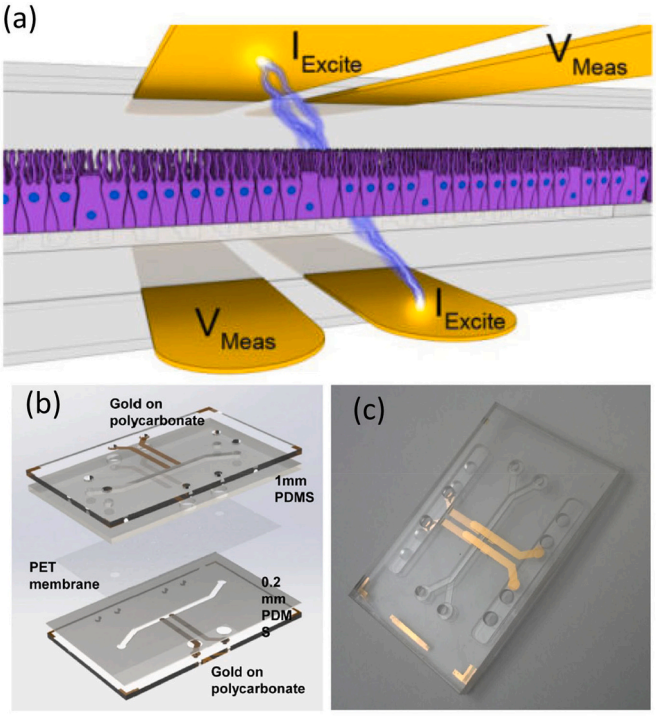

Fig. 12. (a) Schematic illustration of the TEER-chip with four points for impedance and capacitance measurements. A small current of 10 μA of varying frequency is applied between two electrodes marked with IExcite, placed on each side of the cell culture; the potential drop is measured between a second pair of electrodes marked with VMeas. (b) CAD model of the TEER-chip shows the gold electrodes patterned onto polycarbonate substrates. PDMS layers and PET membrane are laser-cut and assembled using silane-based surface modification to permanently bond together. (c) Photograph of the completed TEER-chip whose sizes are 25 × 40 mm. The overview image is adapted from [137]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the cell layer barrier function and to detect changes in villus differentiation within human intestinal epithelium cultured in a GoC (Fig. 13). Giampetruzzi et al. equipped the chambers with transparent Indium Tin Oxide (ITO) electrodes to realize a multi-integrated platform that monitored several microenvironment parameters during cell culture experiments [123], as shown in Fig. 14. The proposed low-cost transparent electrodes do not limit fluorescence investigation with standard laboratory equipment and keep a standard chip dimension similar to microscope glass slides. The technique allows for a continuous

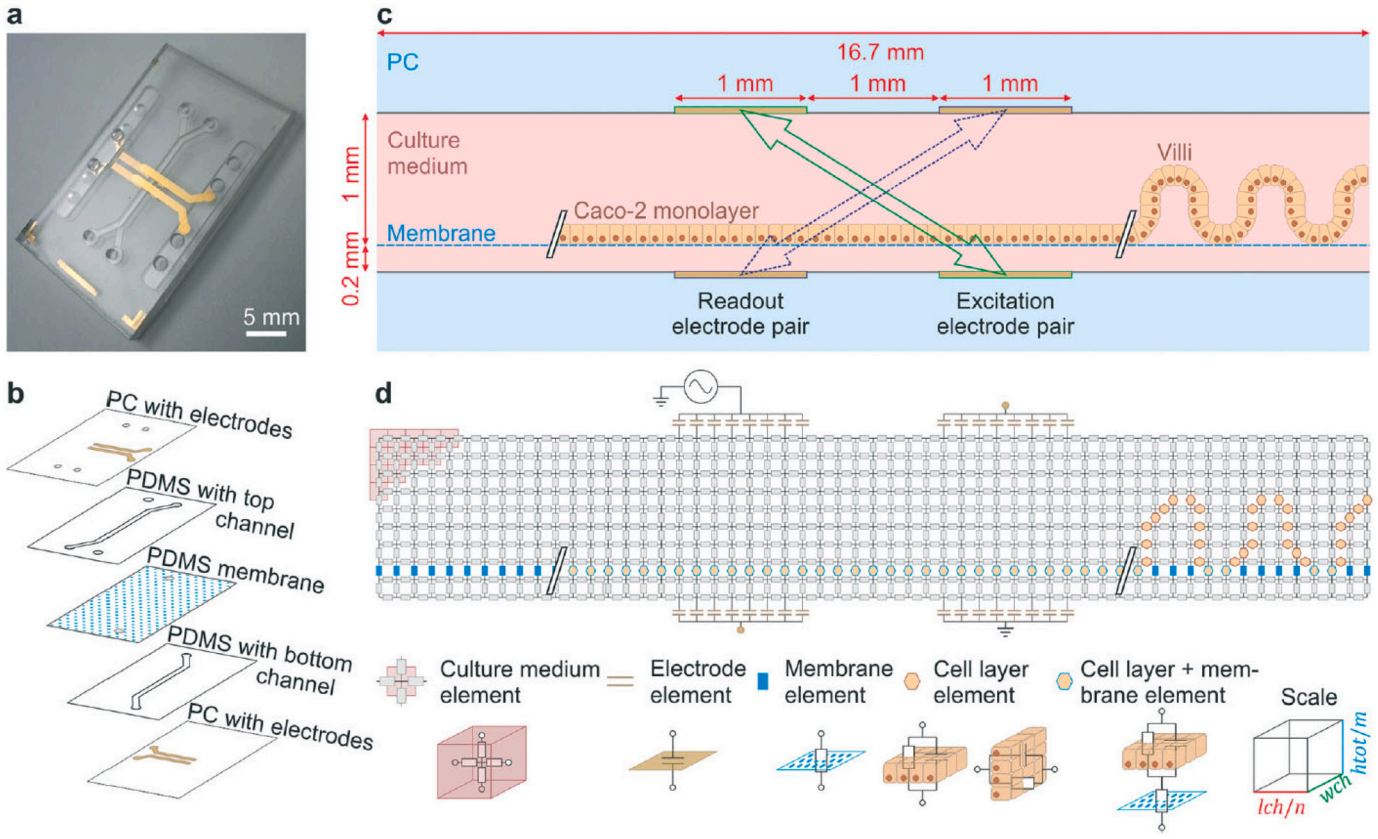

Fig. 13. Gut-on-chip design, working principles and the equivalent electrical circuit utilized for simulations. (a) Photograph of the fabricated Gut-on-chip device. (b) Schematic representation of the modules composing the final microfluidic device: two PDMS layers with laser-cut channels (1 mm wide) sandwich a porous PDMS membrane and are enclosed between two PC layers embedding semi-transparent gold electrodes. (c) A cross section picture of the main channel where the intestinal epithelial cells cultured on the membrane and the villus structure can be observed. During impedance measurements, current flows between the excitation electrodes (green, solid arrow) and through the bare membrane, flat monolayer or villi, and the output potential difference is measured between the readout electrodes (purple, dashed arrow). (d) The microfluidic chip is modelled by a 2D electrical circuit made of four resistive and/or capacitive elements representing the culture medium, the porous membrane, the cell layer and the electrodes. The figure is reproduced from [138], CC-BY-NC.3.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

automated *in situ* monitoring of cell layer formation and maturation. In addition, the proposed method suggests strategies to integrate more effectively multiple electrodes in the channels, thus avoiding risks of inhomogeneity in the potential distribution typically noted in TEER measurements. This could open the way to a direct cell culture on the electrode-integrated substrate for local probing.

4. Reproducing the interconnections of human gut in the body: Monitoring of ions and metabolites

Scientific and medical communities have accumulated evidence on the strong bidirectional connection between intestinal microflora (microbiota) and Central Nervous System (CNS) functions [139]. As a matter of fact, the perturbation of intestinal homeostasis compromises not only gastrointestinal tract functions but also other organs such as the brain. This is generally referred to as "Gut-Brain axis" to describe the hypothesis of gut influence on brain, not only limited to digestive functions and satiety sense, but also to behavior and cognitive abilities [140]. The Gut-Brain axis is characterized by two natural barriers: the intestinal barrier and the blood-brain barrier (BBB). Metabolites generated by microbiota activate CNS signaling mechanisms and some of them can cross both barriers, entering systemic circulation [141], sometimes causing the disruption of BBB, induced by pathological states. This is a clear evidence that gut microbes and their metabolites have a strong impact on brain functions and BBB integrity. For this reason, the monitoring of metabolites is generally studied in MOC platforms, which consider the interaction of different organs, where gut is inevitably included. This is valid for ions too, because, like metabolites, they are transported across tight membrane barriers (similar to the gastrointestinal and blood-brain barriers), to maintain the homeostatic environment and the brain-blood communication. In particular, a higher permeability of essential metals ions observed in the intestine for different reasons, such as IBDs or diet, could lead to non-essential and essential metal-dysregulations also in blood-brain, causing neurotoxicity and inducing neurological/neurodegenerative disorders.

4.1. Sensors for ions monitoring

Polarized epithelial cells, separating the basolateral and apical compartments of the gut internal cavity, carry out important physiological tasks, such as the transport of ions and water from a compartment to the other for human homeostasis preservation [142]. A complete study of organ requires the monitoring of extracellular ions concentration or ions gradient through the epithelial barrier, because hydro-electrolytic disorders can cause several diseases [143]. The determination of these disorders is extremely important, especially of those involving plasma sodium (Na^+) and potassium (K^+) ions dosages. Na^+ is mainly present in extracellular fluids and is important for organic functions, while K^+ is mainly found in intracellular environment [144]. The main methods to evaluate Na^+ and K^+ concentrations in biological fluids are flame photometry, Ion-Selective Electrodes (ISEs) and enzymatic colorimetric methods. Flame photometry is one of the oldest

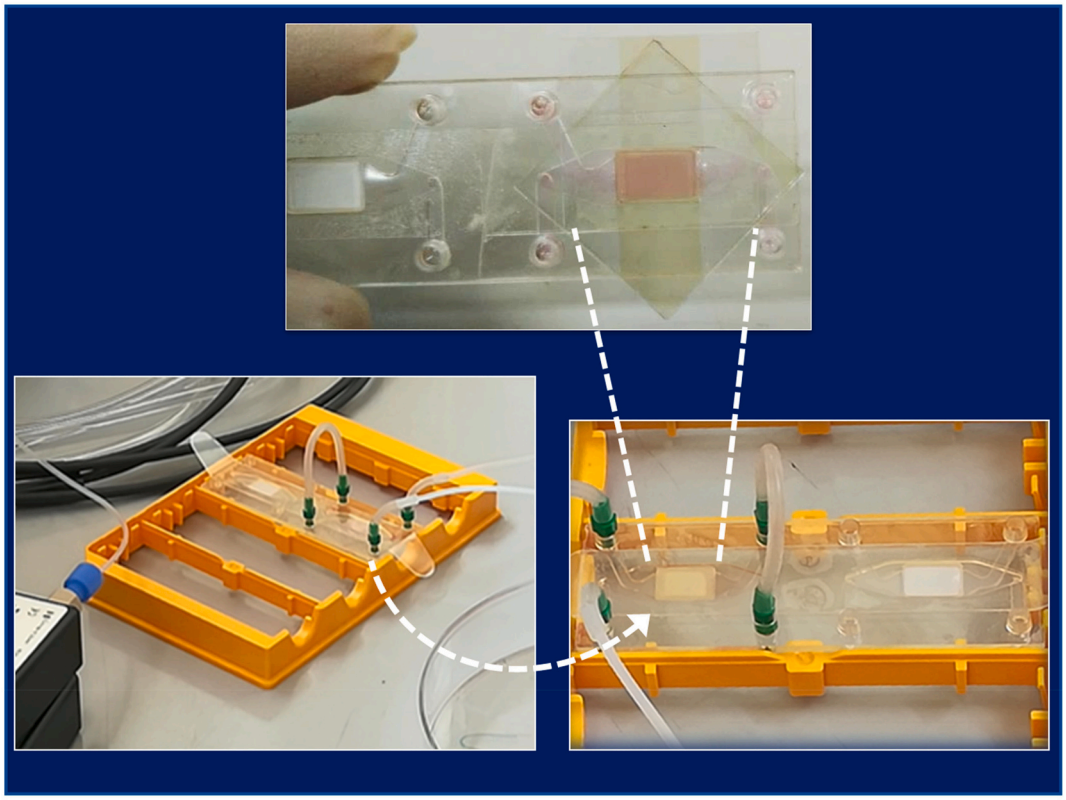

Fig. 14. Photograph of a commercial multifunctional platform for Gut-on-Chip Caco-2 cultures modified by embedding ITO electrodes. From [123] Copyright © 2019, Springer Nature Switzerland AG.

methods, consisting in the atomization of the solution sample into a flame and in the measurement of the emitted radiant energy from characteristic elements, which is proportional to their concentration [145]. It is considered a convenient laboratory method, due to the short analysis time and the use of small biofluid quantities (blood or urine), but it has a low throughput, and it needs complex and expensive equipment [146]. These limitations have motivated the use of electrochemical sensors equipped with ISEs, based on specific ion-selective polymeric membranes. The system consists of a reference electrode at constant potential and two "selective electrodes" between which a potential is developed as a function of the ions concentration on each side of the membrane. Ions concentration in solution is extracted from the potential difference measured between reference and selective electrodes, using the Nernst equation. The ISE method uses a small quantity of samples, and it is reliable, highly sensitive, selective, and fast responsive. Enzymatic colorimetry is a kinetic assay based on reactions that lead to chromophore species, which may be quantified by molecular spectroscopy [147].

In addition to Na^+ and K^+, other ions, such as iron (Fe^{2+}) and copper (Cu^{2+}), are important co-factors for a number of enzymes in the brain and they are often related to neurodegenerative processes [148]. The connection of Cu^{2+} and zinc ions (Zn^{2+}) intracellular transporters with these processes has been demonstrated in animal models of Alzheimer's disease, raising the possibility that higher levels of Fe^{2+}, Zn^{2+} and Cu^{2+} might be due to a disruption in the activity of transporters. Accordingly, exposure to toxic onsets that affect the activity of transporters could potentially contribute to the etiology/progression of many neurodegenerative diseases disrupting the CNS homeostasis. In this context, *in vitro* modeling of the gastrointestinal tract and BBB on chip could help to mimic the mechanisms of barriers physiological/pathophysiological modulation that determine iron and copper uptake and their bioavailability. This could also advance the study of the real-time impact of metal ions metabolic routes at the gastrointestinal level, and of the metals uptake and fluxes at the BBB level, to disclose the threshold between physiological balance and pathophysiological dysregulation that cause alteration of the metals concentrations delivered to the CNS. An impedimetric biosensor ultrasensitive to only essential metals, such as Cu^{2+}, Fe^{2+} or Zn^{2+} ions at physiological conditions, was also implemented, using proteins or peptides for sensors functionalization because of their high specificity to metal ions [149]. In fact, by engineering the amino acids sequences in proteins and peptides it is possible to create functional moieties that allow self-assembling on surface and creation of ion-chelating cavities [150]. The detection of Zn^{2+} and Cu^{2+} ion concentrations using traditional methods requires expensive instrumentation and complex sample preparation, yet it ensures a low limit of detection and high specificity [151]. With the perspective of developing portable point-of-care devices, the use of electrochemical biosensors has gained more attention thanks to their easy integration in more compact devices such as OoC [152]. The increasing miniaturization and flexibility of ISEs have extended their application field, from conventional potentiometric ion sensing to integrated sensing systems applied to wider scenarios, with the possibility to enlarge the monitored ions class, including those of interest for GoC.

4.2. Sensors for metabolites monitoring

In the past few years, there has been a significant increase of diseases related to the alteration of metabolism, causing pathologic conditions, such as obesity, type-2 diabetes, cardiovascular diseases, and metabolic syndrome. Numerous factors are responsible for this increase, such as age, diet, gender, lifestyle, use of antibiotics, genetic factors, and onset of inflammatory states [153]. Microbiota have an essential role in several metabolic functions, such as glycaemia modulation, lipid homeostasis, energy generation and vitamins production, and exert an important anti-inflammatory activity. Furthermore, microbiota contribute to the regulation of numerous biochemical and physiological

mechanisms, by producing metabolites and other substances capable to potentially influence the intimal and mutually beneficial relationship with its host. Metabolites consist of small molecules that represent intermediates or end-products deriving from microbial metabolism. Angus et al. reviewed the main classes of microbiota-derived metabolites and their alteration and role in pathogenesis of metabolic diseases [154]. It is well understood that a balanced interaction of microbes with the host exists and plays an important role in maintaining health. Alterations of this balance can contribute to the onset of several pathologies, such as cancers, immune-related diseases, metabolic diseases, inflammatory bowel disease, pulmonary conditions, oral diseases, as well as skin and neurological disorders, as summarized in Fig. 15 [155]. The identification and quantification of metabolites represent key steps in the understanding of the mutual effects of drug/food and intestinal microbial flora. It has been observed that, depending on diet composition, the microbiome has the faculty to produce an amount of volatile fatty acids that are important for the intestinal immune response and trigger response also in other organs and tissues. Thus, metabolites monitoring may be important to decode mechanisms and interactions occurring between gut-liver, gut-kidney or in the gut-brain axis, and to reproduce *in vitro* this important connection. Another reason for analyzing metabolites is their role as possible biomarkers, studying their variation in health or in pathological reactions or during drug metabolism [156]. This is corroborated by recent advances in the understanding of gut microbiota and in the capability to modulate them, which represents a novel strategy for the prevention and treatment of numerous metabolic disorders.

Long-term and continuous monitoring of metabolites allows

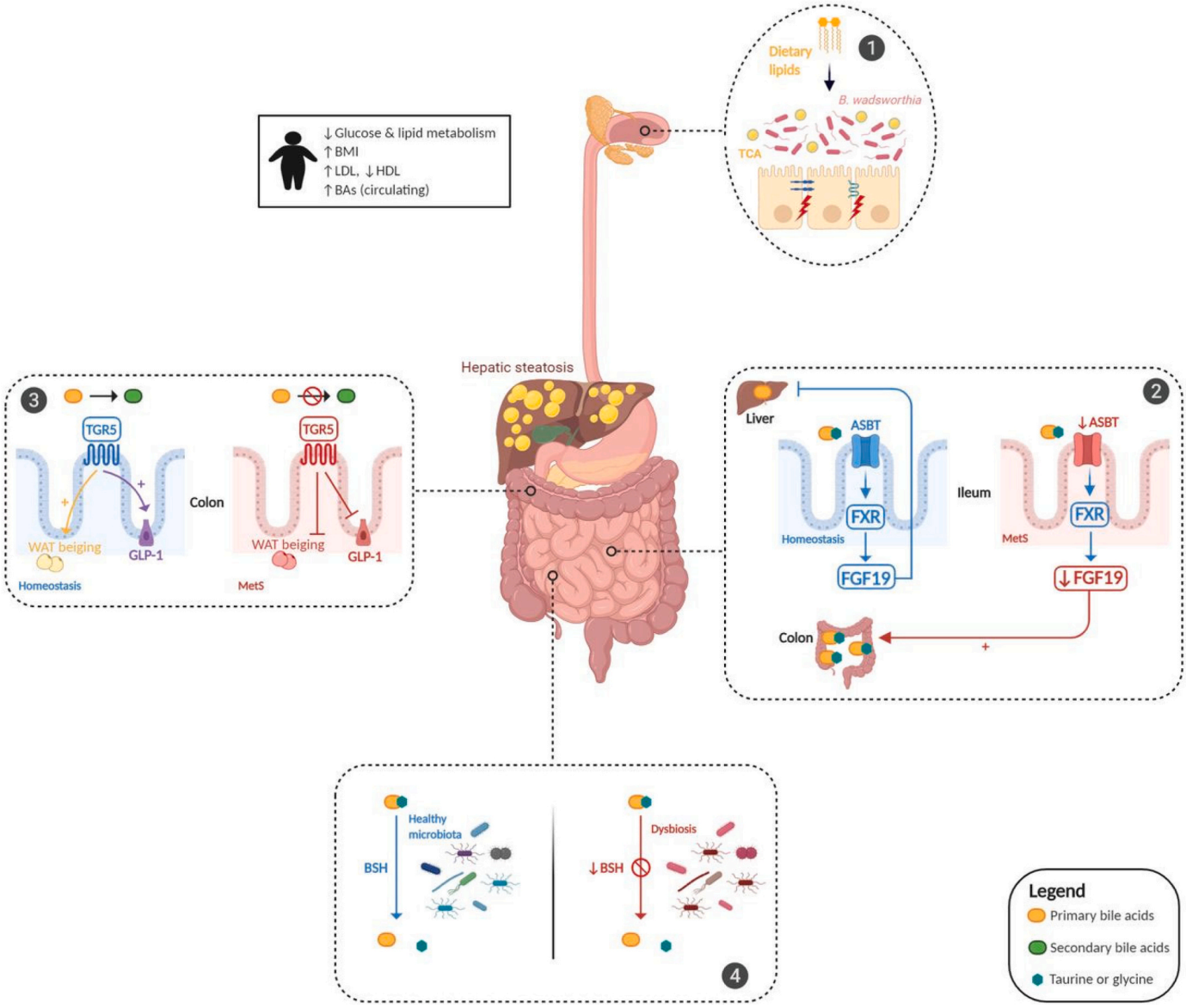

Fig. 15. Patients with metabolic syndrome (MetS) show an alteration of Bile Acid (BA) metabolism associated with hepatic steatosis and glucose and lipid dysmetabolism. (Panel 1) Dietary lipids consumption promotes the production of taurocholic acid (TCA), causing an increase in intestinal permeability and inflammation. (Panel 2) Gut microbiota is altered, inducing a deficiency in the ileal absorption of BAs, normally occurring *via* the apical-sodium BA transporter (ASBT). As a result, the expression of nuclear Farnesoid-X receptor (FXR), the fibroblast growth factor 19 (FGF19) in intestinal epithelial cells and the abundance of colonic primary conjugated Bas decrease. (Panel 3) Gut microbiota dysfunction inhibits the transformation of primary conjugated BAs to secondary BAs in the colon, causing the defective activation of Takeda-G-protein-receptor-5 (TGR5) which increases the glucagon-like peptide 1 (GLP-1), preventing white adipose tissue (WAT) browning. (Panel 4) Bile salt hydrolase (BSH) activity is compromised, leading to primary conjugated BA accumulation in the colon. BMI, body mass index; HDL, high density lipoprotein; LDL, low-density lipoprotein. The image was reproduced from [154], CC-BY-NC 4.0.

immediate feedback on the status and functionality of the investigated OoC, in response to environmental changes, either physiological or drug-induced. For this purpose, various approaches have been implemented, all aimed at combining sensing and nano/micro-fabrication technologies for the development of advanced miniaturized sensing devices to be integrated into *in vitro* microphysiological systems.

Despite the great work being done in this direction, sensors integration in OoC systems is currently still a challenge, because it needs to satisfied several criteria: i) the readout of the integrated sensing system need to be cost-effective and easy to use; ii) the fabrication methods used for the various components of the sensorized OoC platform must be compatible with each other; iii) cell viability and functionality must be preserved over the entire duration of the experiment; iv) the materials used for sensors fabrication/integration must be biocompatible; v) the integrated sensors must show high detection performance, including selectivity, sensitivity and reproducibility.

To date, very few works published in the scientific literature discuss the integration of sensors in GoC devices for the monitoring of metabolites, yet interesting attempts have been proposed for the integration of sensors in MOC platforms involving the human intestine. MOC systems, devoted to the understanding of gut interactions with other organs, consist of a series of compartments interconnected by microfluidics, according to modular approaches, in which the digestion system is emulated *in vitro* to mimic and study not only the effects of peristalsis, shear stress and enzymes on food/drug metabolism, but also the effect on gut microbiota. The applications of intestine insertion in different MOC span from drug or toxicity testing to disease modeling, drug screening, and cancer metastasis [157–159]. Maschmeyer et al. [160] realized a microphysiological platform for *in vitro* Adsorption-Distribution-Metabolism-Excretion (ADME) profiling and dose systemic drug toxicity testing of a MOC over 28 days. Within this time, the functionalities of four organs, liver-intestine-skin-kidney, were maintained in co-culture, with fluid-tissue ratios close to physiological values. The culture media was pumped through the four compartments by a peristaltic on-chip micropump, while the fluid excreted through the kidney epithelial cell layer was drained out through a second microfluidic circuit. Misun et al. [161] designed a microfluidic platform for *in situ* monitoring of glucose and lactate of 3D colon microtissues by integrating enzyme-based multi-analyte biosensors, made of four functionalized platinum working electrodes, a platinum counter electrode, and an Ag/AgCl reference electrode, directly placed into the ceiling substrate, as depicted in Fig. 16.

Most of recently proposed MOC systems are mainly focused on the fabrication and composition of the multi-organ modules, while the implementation of continuous, stable, and automated testing of these platforms still remains challenging. Current analytical investigations, based on the conventional bio-sensing technology, are mainly performed outside the chip and do not satisfy all the specific requirements completely. Conventional approaches, such as mass spectrometry (MS) and Enzyme-Linked ImmunoSorbent Assay (ELISA), do not allow for a continuum monitoring and their integration with low-volume bioreactors can hardly be achieved [162]. Miniaturized biosensors integrated into MOC devices may represent an effective tool to monitor the dynamic metabolic process of living cells with high selectivity and sensitivity [163]. Long-term biomedical investigations are performed using biosensors for metabolites analysis, firstly using low-cost optical solutions, generally adopted for pH and gas variations. However, they show several limitations, for instance when working in an automated and continuous manner for a long period of time. Long-term testing of MOCs is mandatory to realize a real-time monitoring of multi-organ interactions, especially aimed at evaluating the dynamic responses of multiple organs and potential chronic cellular reactions to drugs for a

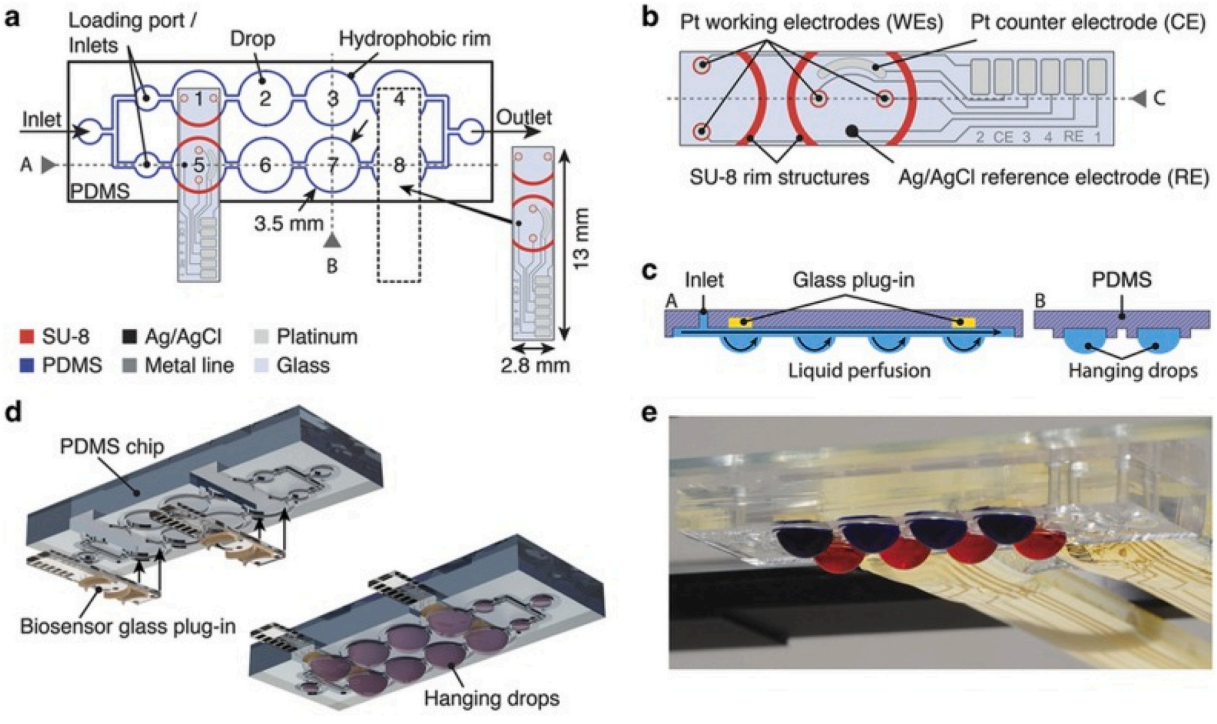

Fig. 16. Design and assembly of the microfluidic hanging-drop network and sensor glass plug-ins. (a) Picture of 2D-top view of the microfluidic chip; hydrophobic PDMS rim structures are blue-colored. Sensor plug-in is included in the PDMS recess indicated by dashed lines. The microfluidic platform is completed by SU-8 structures (red-colored) on the glass plug-in. (b) A schematic of the sensor unit containing four working electrodes (WEs), one counter electrode (CE), and one Ag/AgCl pseudo-reference electrode (RE); SU-8 rim structures are indicated in red. (c) Sketch of the cross-section view of the open microfluidic chip where the hanging drops, the inserted sensor glass plug-ins, and the perfusion flow can be observed. (d) 3D view of microfluidic device assembly. (e) Photograph of the final assembled microfluidic chip loaded with colored liquid for a better visualization of the two rows of drops. The image was reproduced from [161], CC-B-4.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

prolonged time period. Maintaining the functionality of multiple human tissues or organs in microphysiological systems for several days opens the possibility for their strategic employment in PBPK-PD aided drug development studies. However, a proper functional design of these platforms should be done, considering the various stages of drug development. For this purpose, Abaci and Shuler suggested the main design principles and parametric design equations that should be considered for the realization of two potential types of microphysiological platforms for PBPK/PD based drug studies [164]. Microfluidic optical pH and oxygen sensors demonstrated good stability in measuring microenvironment parameters for at least 5 days [165]. Zhang et al. developed a MOC system equipped with a microfluidic controlling breadboard for timed routing of fluids and a modular sensing platform operating continuously and automatically; the latter includes gold microelectrodes based-electrochemical immune-biosensors for biomarkers detection, optical biosensors for microenvironment monitoring and microscopes for the observation of organoid morphologies and behaviors [166].

Electrochemical sensors have been also proposed in literature to detect the metabolites/biomarkers molecules, despite the miniature-sized culture volume and the small number of cells in MOC systems. Table 1 provides an overview of the attempts of sensors integration in gut organoids or in GoC. Efforts should be devoted to integrate various analytical methods for the detection of biomarkers into the devices, such as equipping the chip with ELISA technology which still remains an off-chip technique. Actually, automated microfluidic platforms, such as bioreactors and OoC have been implemented with sensor systems for a long-term continual analysis of molecules of interest. Relevant and promising results have been obtained by microfluidic platform equipped with an electrochemical immunosensors system that allows for in-line measurement of cell-secreted biomarkers [12]. An example is given by an immunosensor integrated in a Liver-on-Chip and developed with disposable magnetic microbeads used to immobilize liver fibrosis biomarkers secreted from hepatocytes such as transferrin and albumin. For a continuous recognition of biomarker molecules, a system of microvalves has been implemented on the platform to provide automated and programmable operations of the immunoassay protocol: bead loading, washing steps, and electrochemical sensing [162]. Other types of proteins, microRNAs or exosomes secreted by cells, have been taken in consideration as important biomarkers [167]. For instance, Creatine kinase-MB (CK-MB) and troponins, acute cardiac toxicity biomarkers, have been detected using an aptamer-based microfluidic sensor in a Heart-on-Chip system. This has been fabricated from human embryonic stem cell-derived cardiomyocytes following exposure to a cardiotoxic drug, doxorubicin [168]. The same cytokines, as signaling proteins secreted from cells in stress conditions (immune system activation, during cancer progression, or in tissue damage) were designated to develop sensors in OoC devices. Cytokines sensors were fabricated using techniques based on traditional immunoassay principles such as bead-based or aptamer-based assays (Sandwich ELISA), experimented in different Liver-on-Chip and microfluidic hepatic platforms [169,170]. Hence, considering the lowered detection limits and the increased sensitivities recently acquired by immunosensors, they could allow for a long-term *in vitro* detection of biomarkers, using low volumes and obtaining important information on cell-tissue- and organ-functions reconstructed into OoCs. However, few and limited results are provided in literature regarding sensorized Intestine-on-Chip platforms.

5. Advances in Gut-on-Chip: application on global diseases and pandemic infections

The possibility to recreate complex pathophysiological features of human pathologies made OoC-based disease models an important research tool, aimed to predict drug pharmacokinetic responses in patients affected by chronic, tumor or neurodegenerative diseases, reducing the number of failed animal models or clinical trials [171]. OoC has proved to be also a very mature technology for the investigation of severe and unknown bacterial-viral infections, reproducing underlying mechanisms of different microbes-host interactions in real-time and with high resolution [172,173]. These results, together with the reproduction of tissue-tissue interfaces, mechanical signals, biofluid flows and organ physiology, encouraged the development of OoC as a valid alternative to conventional *in vitro* culture systems and animal models. Microfluidic human GoC models, specialized to recapitulate the correct human pathophysiology, were adopted to study and understand the infection pathway of bacterial, parasitic, or viral pathogens that generally affect the gastrointestinal tract, leading to the onset of gastroenteritis and inflammation, with the aim to develop effective and faster therapies.

Recently, research has been focused on the study of emerging pathogens, especially those connected with viral infections of the respiratory tract, and belonging to four main groups: influenza viruses, enteroviruses, coronaviruses and adenoviruses [174]. Even if these pathogens generally attack the upper respiratory tract/lung, they have triggered several infections regarding other body districts or organs. An example is given by enteroviruses that cause severe diseases, including respiratory illness, myocarditis, chronic dilated cardiomyopathy, and aseptic meningitis, with no currently available vaccines. Recently, intestinal infection from an enterovirus, the Coxsackievirus B serotype 1, was tested *in vitro* using an OoC supporting the culture of highly

Table 1
Summary of integrated sensors solutions into gut organoids or GoCs.

	Parameter	Model	Sensor	References
Oxygen	Luminal O_2 content	Organoid	Miniaturized optical O_2 microsensor	*Hill* et al. [103]
	Redox metabolism	Organoid	Fluorescence and phosphorescence microscopy-coupled with O_2 probe	*Okkelman* et al. [104]
	O_2 content in gut tissue	Organoid	phase fluorimetry coupled with O_2-sensor microbeads	*Lesher-Pérez* et al. [105]
	ROS	Gut-on-Chip	CellROX Green Reagent fluorogenic prob	*Jalili-Firoozinezhad* et al. [107]
	O_2 levels	Gut-on-Chip	Integrated O_2 sensors (optodes)	*Shah* et al. [108]
	Gastrointestinal tract redox states	*In Vivo* Gut Microbiome	Electrochemical (redox potentials measurement)	*Baltsavias* et al. [110]
pH	Intracellular pH	Enteroid	Electrochemical	*Zachos* et al. [118]
	Luminal pH	Organoid	Electrochemical	*McCracken* et al. [100]
Intestinal barrier integrity	Para-cellular permeability	Organoid	No sensors	*Leslie* et al. [133]
			Fluorescence quantification	*Hill* et al. [134]
	Para-cellular permeability	Microchip-based system	No sensors	*Imura* et al. [135]
			Permeation tests	
	Epithelial barrier integrity TEER	MOC	Gold Impedance sensor	*Henry* et al. [137]
	Epithelial barrier integrity	Gut-on-chip	impedance spectroscopy	*van Der Helm* et al. [138]
	Epithelial barrier formation and integrity TEER	Intestinal barrier model on chip	ITO impedance sensors	*Giampetruzzi* et al. [123]
Metabolites	Glucose and lactate	3D colon microtissues	Electrochemical	*Misun* et al. [161]

differentiated human villus intestinal epithelium under conditions of continuous perfusion and cyclic mechanical strain [175]. The study demonstrated the dependence of the infection evolution on the route of virus entry. When the infection takes place apically through the intestinal lumen, the villi morphology is damaged within 24 h from the infection, while a complete loss of epithelial integrity and barrier function occurs after 48 h. An active intracellular replication was confirmed, with a polarized release of new virions from the apical microchannel, which is more numerous in case of apical infection. When the virus was inoculated from the basolateral side *via* the vascular compartment, separated from the epithelial channel, it migrated through the intestinal lumen, and the release of new virions was again polarized towards the apical side of the gut epithelium. However, a lower number of virions was released from the basal surface of the epithelium, while significantly high levels of cytokines were released to the luminal side of the epithelium, independently from the route of virus entry. The study demonstrated the capability of GoC technology to mimic secondary infections starting from the disease propagation in *in vitro* models. Thanks to a more realistic and complex host–pathogen interaction for human enteric infection, GoC models show great potential in gaining knowledge of a broad range of enteroviruses, representing a valid support to deepen the understanding of gut-related infections, as well as a powerful tool for the development of vaccines and therapies.

Nowadays, the SARS-CoV-2 virus is causing a major pandemic. This virus belongs to the Coronaviruses family that primary causes lung infections through binding of ACE2 receptors present on the alveolar epithelial cells [176]. Airway Chips were developed [177] together with common *in vitro* models to better understand the pathway of virus entry, its replication, the strain-dependent virulence, the host cytokine production, and recruitment of circulating immune cells in response to infection by SARS-CoV-2. These are microfluidic devices which emulate the epithelium of human bronchial airways. The study adopted cells that express high levels of ACE2 and TMPRSS2, consequently used to assess the inhibitory activities of 7 clinically approved drugs. Two of these drugs, the amodiaquine and toremifene, significantly inhibited entry of the pseudo-typed SARS-CoV-2 virus in the human Airway Chips,

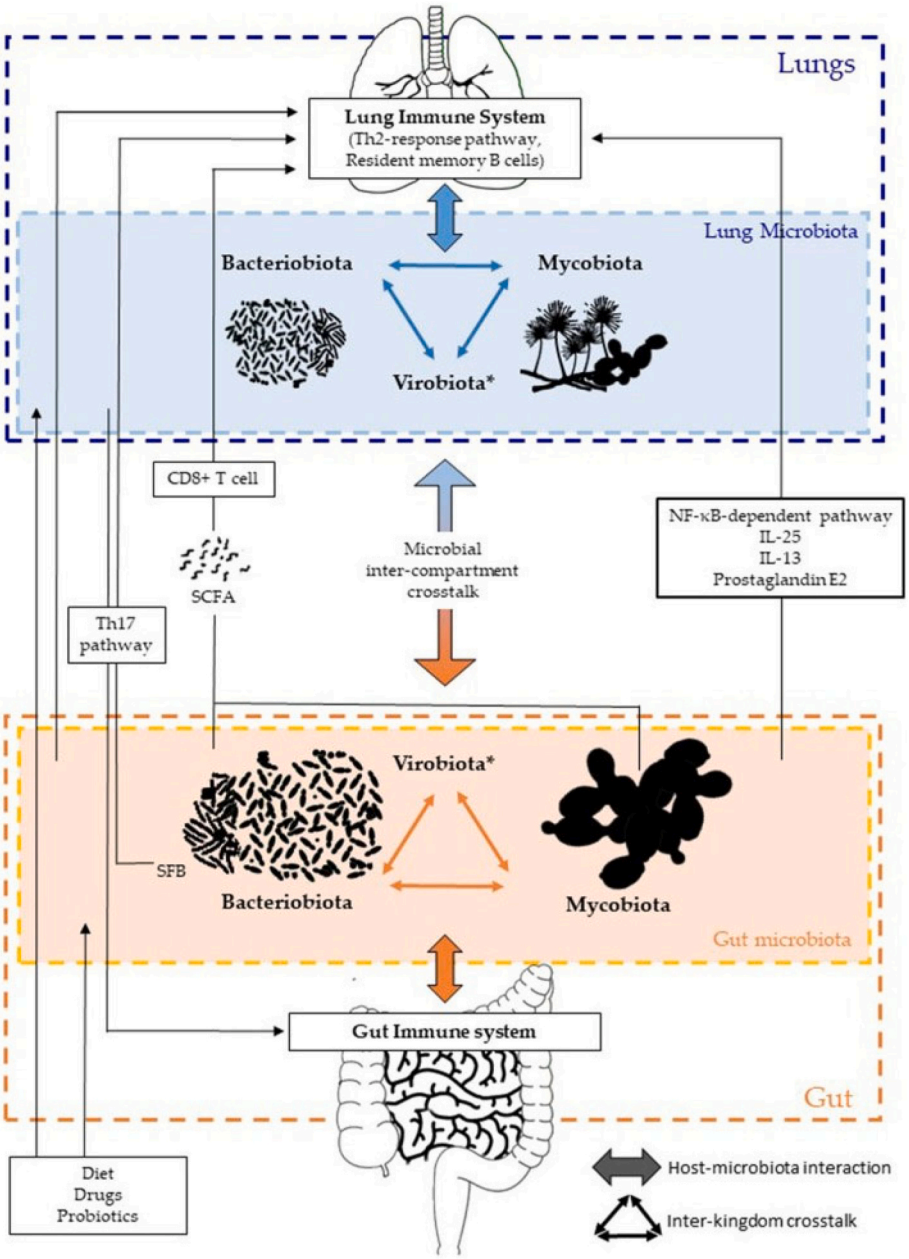

Fig. 17. Sketch of gut–lung axis inter-compartment crosstalk. Bacteriobiota, mycobiota, and virobiota strictly interact within each organ. Gut microbiota influences both immune systems of gut and lung, through different interactions involving either CD8+ T cell, Th17, IL-25, IL-13, prostaglandin E2, and/or NF-kB–dependent pathways. Lung microbiota favor immune tolerance, through neutrophil recruitment, production of pro-inflammatory cytokines mediated by receptor 2 (TLR2), and the release of antimicrobial peptides, such as b-defensin 2 stimulated by T helper 17 (Th17) cells. At the same time, they impact the gut immune system too, since cases of intestinal microbial disruption have been attributed to Th17 cell mediation after lung infection due to virus. Nevertheless, all the involved mechanisms are not completely known. The image is reproduced from [180], CC-BY-4.0.

anticipating clinical tests, suggesting rapid cell-based screening assays in expedite drug repurposing during a viral pandemic. However, it is now known that SARS-CoV-2 affects the gut, too. In fact, SARS-CoV-2 RNA was also found in the feces of infected patients, concluding that the intestinal epithelial cells also express ACE2 receptors [178]. Furthermore, it was discovered that the gut microbiota influence lung diseases and are in turn perturbed by the respiratory virus infection [179]. This potential communication between gut and lungs has reinforced the hypothesis of the existence of a gut-lung axis, which interferes with the immune system and shapes the progression of respiratory diseases [180], as clearly described in Fig. 17.

In this complex scenario, researchers were also projected to test the effect of SARS-CoV-2 infection on the intestine, too. In [181] a micro-engineered Intestine-on-Chip device was used to study a human intestinal epithelium (co-cultured human intestinal epithelial Caco-2 cells and mucin secreting HT29 cells) that was infected by SARSCoV-2. It was observed that after 3 days, the viral infection destroyed the intestinal villus, and reduced the expression of tight junction (E-cadherin), which is an index of severe damage of intestinal barrier integrity caused by virus. The presence of up-regulated cytokine genes, TNF signaling, and NF-kappa B signaling-related genes revealed an abnormal activated immune response, as well as an atypical RNA and protein metabolism in both epithelial and endothelial cells. This Intestine-on-Chip infection model provides an accurate duplicate of the intestinal pathophysiology and human response to SARS-CoV-2 infection, which is difficult to achieve by existing *in vitro* culture models. Moreover, it represents an innovative bioengineered *in vitro* model system and a very promising tool to accelerate our understanding of COVID-19 and devise novel therapies (Fig. 18).

Future studies could involve the connection of multiple organs infected by COVID-19, to facilitate the understanding of the underlying mechanisms, as well as to speed up testing of possible therapies and vaccines able to adapt to the variations of the virus. The search for promising tools to acquire more information in less time and at lower costs is key to address recent challenges such as pandemic influences and diseases. In the light of the presented discussion, the importance of microfabrication technologies emerges as an effective instrument to provide low-cost miniaturized sensors, characterized by high selectivity and quick response time, to be integrated into GoC for a reliable control of crucial parameters to diagnose COVID-19 rapidly. The integration of sensors for the monitoring of epithelial barrier integrity is fundamental to assess the production of antiviral compounds that are hostile to

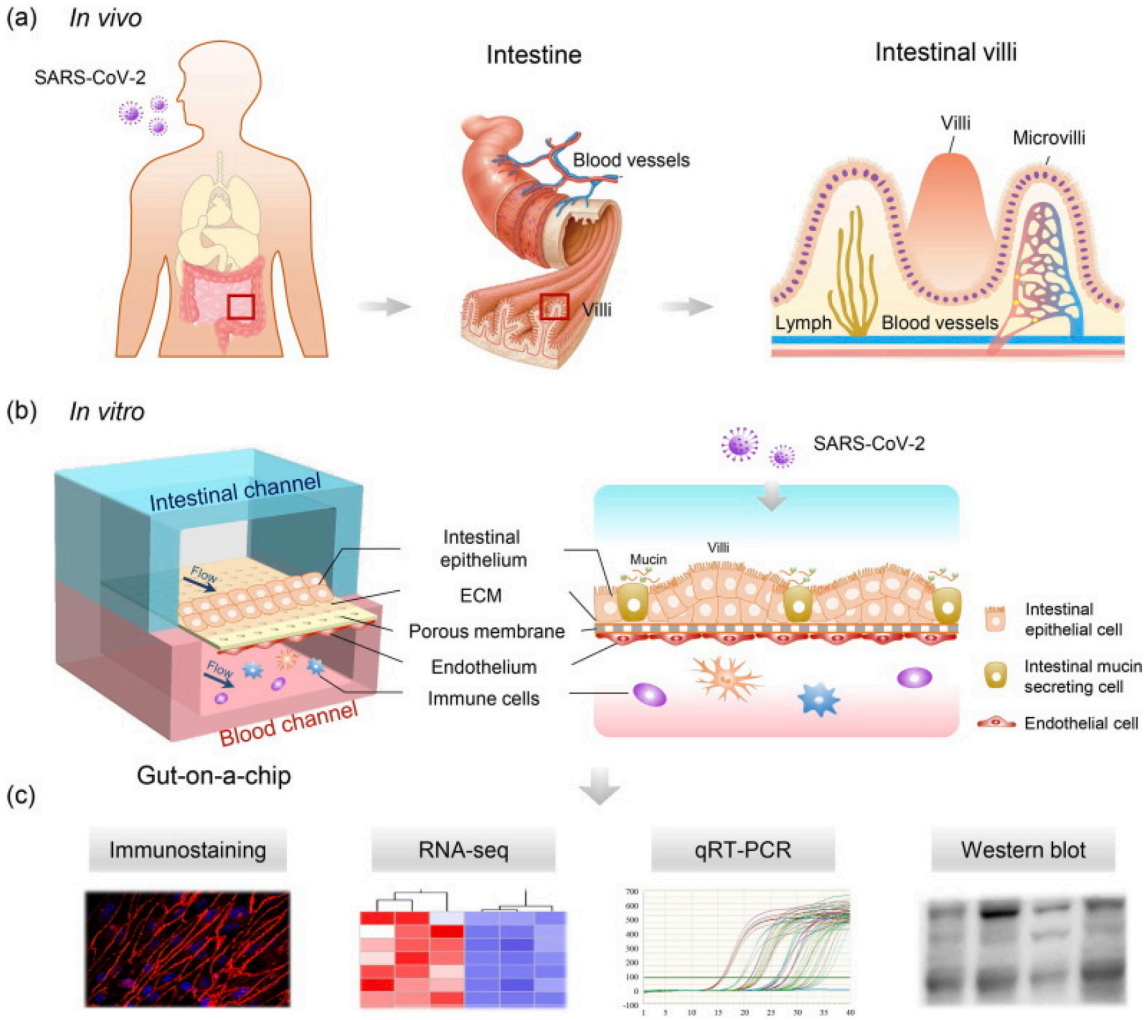

Fig. 18. Schematic of SARS-CoV-2-induced intestinal infection model on chip. (A) Representation of an *in vivo* infection of the small intestine induced by SARS-CoV-2 (B) illustration of *in vitro* gut infection caused by SARS-CoV-2. The Gut-on-Chip is fabricated using a PDMS porous membrane which separates an upper intestinal epithelial channel (blue) and a lower microvascular endothelial channel (red). The intestinal barrier was created by intestinal epithelial Caco-2 cells and intestinal mucin secreting HT29 cells co-culture on the top channel. The progression of virus infection was mimicked by introducing human peripheral blood mononuclear cells (PBMC) to the bottom vascular channel. (C) The response of the intestinal chip to SARS-CoV2 infection is analyzed by using different methods. The figure is reproduced from [181], CC-BY-NC-ND 4.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

viruses. Integrated pH-sensors are mandatory, too, because a low pH (1.5–3.0) of gastric acid should be guaranteed to protect against infection by ingested pathogens [182]. COVID-19 virus may not survive at normal gastric acid pH levels of 1.5–3, but there is evidence that it can survive at hypochlorhydria (low stomach acid) conditions, where the pH level rises up to 3 or above, with higher risk to contract virus disease [183]. In this context a human GoC, mimicking the physiology of a human gut as best as possible – *i.e.* microbioma as center for immunity, neuronal cells as secondary brain, a site responsible for drug absorption – and equipped with suitable sensors to monitor physical/chemical parameters, is an even more indispensable device. This will first allow to identify the interaction of SARS-CoV-2 virus with one or many of the 1500 microbiota species in the gut, and then develop early diagnosis for a better control of the virus spread, especially in asymptomatic carriers [184].

6. How to overcome limitations of sensors integration in Gut on Chip: adaptation of sensing solutions coming from other organ-on-chip models

A full integration of sensors into OoC systems could offer the possibility to perform *in situ*, real-time and long-term monitoring and testing of microphysiological platforms, exploiting multiple functionalities on chip. This approach allows for a better understanding and control of the investigated system, for example by exploring the dynamics of drug effects that may allow a more accurate prediction of the human organ reactions. Previous studies devoted to sensors integration in OoC may guide adaptation to GoC devices for a more accurate and detailed knowledge of this organ. An attempt towards full integration of sensors in Kidney-on-Chip was proposed in [167] to study drug absorption and metabolism. The microfluidic platform was equipped with integrated transparent conductive electrodes for TEER measurements, a pH sensor and a microscope for the real-time monitoring of the tissue, aiming at evaluating cell adhesion and changes in culture medium pH under pathological conditions and drug treatment.

Another interesting result was obtained on a 3D printed electrochemical sensor-integrated Transwell system, in which electrochemical sensors and gold electrodes for impedance measurements were successfully integrated into the system [185]. A flexible porous membrane was used as substrate for cells culture. Gold electrodes were realized on the top side of the membrane to monitor cells growth and attachment by impedimetric measurements, whereas a three-electrode system (Au working, Au counter, and Ag/AgCl reference electrodes) was fabricated on the bottom side of the membrane to monitor molecular release at the culture site by Capacitance-Voltage measurements, avoiding the use of downstream fluidics. A Caco-2 epithelial cells line was cultured and ferrocene dimethanol was injected on the upper side of the membrane to diffuse towards the sensors placed at the bottom, thus simulating a metabolite release event from the basolateral (bottom) side of a cultured cell layer. The impedance electrodes, directly integrated on the cell culture membrane, supported the evaluation of cells growth and barrier integrity through a facile and non-invasive method, bypassing the conventional TEER technique. Even though the analysis system is integrated into 2D-type cells culture, it could be transferred into a GoC design for real-time and non-invasive monitoring of metabolites and epithelial barrier integrity.

The complete integration of ISEs in a GoC model may help studying the mechanism regulating the transport of the essential Cu^{2+} and Zn^{2+} metal species across tight membrane barriers along the gastrointestinal tract. To this aim, the same approach used in [186] to monitor K^{+} and Na^{+} by microsensors equipped with micro ISEs could be implemented into a GoC for Cu^{2+} and Zn^{2+} ions monitoring.

Fluorescence is an optical detection method largely used for many biological and chemical assays. Its full integration in OoC systems remains a challenge, mainly for the critical optical paths needed for alignment and coupling across different materials and for the low efficiency of the fluorescence signal collection. Berner et al. [187] implemented a laser-induced fluorescence platform to measure in direct optical test formats, thus opening a path towards powerful point-of-care testing devices based on fluorescence-labeled immunoassays. The strategy proposed by Berner may lead to easier, more versatile, and scalable fabrication of microfluidic channels on these chips.

Oxygen control in OoC devices is very important for cells viability, activity, and differentiation. Bossink et al. [188] proposed a method to fabricate optical oxygen sensors integrated into an OoC. They exhibit a patch geometry with design freedom in terms of size and shape, and they were integrated in different sites of a conventional two-channel OoC device. This is an interesting step forward in the field of sensors integration in OoC, especially for organs with intrinsic oxygen concentration gradients, such as the gut.

7. Challenges, community needs and conclusions

The combination of cell biology, engineering, and biomaterial technology has enabled huge progress in the development of GoC models. This is mainly due to the important advances in terms of organ microenvironment simulation, tissue interfaces and mechanical stimulation. However, further development is still needed to validate individual and interconnected organ models and many technical challenges remain to be addressed, before GoC, and more generally OoC technology, can be successfully accepted and adopted in broader contexts. From a technological point of view, to date one of the most discussed considerations derives from the absence of standardized universal design rules and materials for the fabrication of OoC devices. Regarding the materials choice, PDMS is commonly used in OoC devices. However, this polymer shows some critical drawbacks, such as its tendency to adsorb small hydrophobic molecules. This represents an obstacle for reliable cytotoxicity drug tests [189]. Recently, materials alternative to PDMS are being tested, such as thermoplastic elastomers with a Young modulus close to that of PDMS (for instance Styrene-Ethylene-Butylene-Styrene (SEBS), or polyurethane and polymethyl-methacrylate (PMMA)) or other sustainable materials [190–192].

Looking from a wider and more visionary perspective towards the development of OoC platforms, the so-called high-throughput screenings (HTS) has not been implemented and standardized yet. Modern drug discovery approaches are characterized by the mandatory need to test compound libraries against potential targets (genes or molecular mechanisms) which play a key role in the pathogenesis of the investigated diseases. HTS assays with high-density well plates can test between 10 k - 100 k compounds within 24 h to investigate the potential effects against the defined targets. Major pharmaceutical companies annually perform 25–35 HTS campaigns with a library of about 100 k - 500 k compounds [193]. At present, a high degree of experiment parallelization with multiple single tissues can be performed using the state-of-the art OrganoPlates™ by MIMETAS Dutch company; the OrganoPlates™ are 384-well plates enabling loading of cell-laden hydrogels, taking advantage of PhaseGuides™ in-channels structures for gel meniscus pinning. Different versions are available, with culture chips combined with 2- and 3-adjacent channels, which allow from 40 to 96 parallel cultures or co-cultures in multiple configurations. Compounds and stimuli can be directly added from the apical and basolateral sides of the culture, enabling perfusion and supports for various barrier integrity-, transport-, and migration assays [194]. Gumuscu et al. [195] introduced a higher density platform with 500 cell-laden hydrogel microarrays in closed microfluidic systems using non-fluorescent capillary barriers. This chip enables the fabrication of picoliter-volume compartments of photopolymerized and thermo-gelling hydrogels; however, the single tissues are not independently addressable with fluids, which limits independent high-throughput screenings and experiments.

In addition to the limitations related to alternative materials availability and high- throughput screenings achievement, many challenges

still remain open, such as:

- a complete integration of TEER measurements devices into GoC, made difficult by the small size of microfluidic channels in which cells grow and differentiate;
- TEER electrodes materials and cells cytotoxicity behavior;
- further optimization of size, geometries and positioning of integrated electrodes for TEER measurements, for a more reliable measurement of electrical potentials across the tissue barrier, reducing measurement errors related to non-uniform current densities;
- improvement of imaging techniques for a more effective monitoring of the 3D tissues structure development into the chip, with potential additional information about multi-cellular systems;
- integration of mass-spectroscopy analysis for the investigation of metabolites and proteomic on chip without collecting medium, as a new road to identify biomarkers as also to better reproduce normal or disease gut models;
- a more complete study of systemic pathology, pharmacology and pathogen invasion of the gut organ as an element of MOC systems, which require the integration of long-term testing devices for real-time monitoring of multi-organ interactions and chronic cellular reactions in a more accurate and steady way;
- advances in electrochemical sensors technology to evaluate Cu^{2+} and Zn^{2+} species in a GoC model for a better understanding of the mechanisms controlling the transport routes of these essential metals across tight membrane barriers along the gastrointestinal tract;
- incorporation of several addressable electrodes in an array design for multiplex assays and electrochemical imaging of cell cultures;
- passing of the fouling limit of electrodes materials which prevents long-term assays, investigating metal nanoparticles electrochemical sensors to enzymatic ones;
- improvement of low-cost reading sensors, implementing additive manufacturing techniques and avoiding the noble metals materials;
- development of high-throughput strategies and solutions for a reliable interfacing of these chips to laboratory instrumentations and automation of cells and liquids injections, avoiding manual loading of platforms;
- availability of highly parallelized systems to monitor simultaneously the physiological state of a large number of analyzed samples.

This work critically reviews the state-of-the-art of Gut-on-Chip systems and the importance of on-chip integration of sensors with multiple functionalities, for a reliable, *in situ*, real-time, fast, reproducible and long-term monitoring and testing of microphysiological systems. The limitations together with the open challenges to achieve a complete devices integration are widely discussed, in terms of materials availability, sensors sizes optimization, 3D tissues structure imaging techniques improvement and high throughput multiplex assays configuration. This review proposes also possible solutions to overcome some of the discussed weaknesses about full sensors integration, through the adaptation of strategies already adopted for other Organ-on-Chip systems. All the discussed topics evidence also the high-priority of the synergic cooperation between engineers and biologists to provide a versatile workforce, capable to tackle the difficulties and the challenges faced by Organs-on-Chip sector to achieve effective sensors integration. This could represent an interesting step towards the strengthening of personalized medicine.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank A. Campa, E. Melissano, F. Casino, M.C. Martucci and P. Cretì for the support to perform this study.

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