Establishment and evaluation of on-chip intestinal barrier biosystems based on microfluidic techniques

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ABSTRACT

As a booming engineering technology, the microfluidic chip has been widely applied for replicating the complexity of human intestinal micro-physiological ecosystems *in vitro*. Biosensors, 3D imaging, and multi-omics have been applied to engineer more sophisticated intestinal barrier-on-chip platforms, allowing the improved monitoring of physiological processes and enhancing chip performance. In this review, we report cutting-edge advances in the microfluidic techniques applied for the establishment and evaluation of intestinal barrier platforms. We discuss different design principles and microfabrication strategies for the establishment of microfluidic gut barrier models *in vitro*. Further, we comprehensively cover the complex cell types (e.g., epithelium, intestinal organoids, endothelium, microbes, and immune cells) and controllable extracellular microenvironment parameters (e.g., oxygen gradient, peristalsis, bioflow, and gut-organ axis) used to recapitulate the main structural and functional complexity of gut barriers. We also present the current multidisciplinary technologies and indicators used for evaluating the morphological structure and barrier integrity of established gut barrier models *in vitro*. Finally, we highlight the challenges and future perspectives for accelerating the broader applications of these platforms in disease simulation, drug development, and personalized medicine. Hence, this review provides a comprehensive guide for the development and evaluation of microfluidic-based gut barrier platforms.

1. Introduction

A dysfunctional gut barrier can induce a range of diseases, including inflammatory bowel disease (IBD), metabolic syndrome, and even cancer [1,2]. *In vivo*, the intestinal barrier not only acts as a physical barrier that prevents harmful substances (such as bacteria and toxins) from entering the human body, but also acts as a functional carrier, allowing nutrients and drugs to be absorbed, metabolized, and transported across the body [3]. Multiple cell types (e.g., intestinal epithelium, Paneth cells, goblet cells, endothelium, and immune cells) and symbiotic microorganisms maintain the specificity, integrity, and complexity of the

intestinal barrier [2,4]. In addition, the unique anaerobic-aerobic interface, physiological peristalsis, and bioflow — in combination with the functionally coupled gut-organ axis — enable homeostasis and communication between the gut barrier and other organs [5,6]. Although animal models are widely utilized to study intestinal physiology and pathology, they fail to accurately reflect human physiological responses because of species and microbiota differences [7,8]. Thus, *in vitro* models that mimic the human gut barrier are extremely valuable for nutritional evaluation, drug screening, and research on intestinal mechanisms.

Traditional *in vitro* intestinal barrier models prepared by culturing

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human enterocytes derived from tissue biopsies can partially mimic intestinal ecosystems, simulate enteric diseases, and be used for drug studies [9,10]. However, traditional plate cultures do not provide information regarding the permeability of gut barriers. To overcome these aforesaid limitations, Transwell models in which the intestinal epithelium, aerobic bacteria, and endothelium are cocultured in a compartmentalized manner on different sides of a porous membrane have been proposed. But, uncontrollable oxygen gradients and unstable biochemical signals are generated in these static cultures. Moreover, Transwell-based *in vitro* gut barrier models cannot recapitulate *in vivo* gut barriers and the related controlled *in vivo* microenvironments in a high-fidelity manner [11]. Hence, there is an urgent need to develop and evaluate novel and reliable *in vitro* platforms that simulate the human gut barrier more accurately.

Recently, advances in nanofabrication and microfluidics have led to the development of *in vitro* modular gut barrier-on-a-chip models. These engineered gut barrier-on-a-chip models enable the reconstruction of the proliferative and differentiated regions of the intestinal epithelium, long-term perfusion of the organoids-derived monolayer, as well as the *in situ* coculture and real-time monitoring of commensals [12,13]. Pivotal hallmarks of the intestinal microenvironment — such as fluid dynamics, peristalsis, the anaerobic-aerobic interface, and the gut-organ axis — can also be simulated in these systems. The highly controlled ecosystem allows the formation of villi, mucus and polarized villi-crypt

architecture, as well as a functional gut barrier on a microchip. Furthermore, owing to the versatile potential and integration abilities of microfluidic chips, multidisciplinary technologies such as oxygen sensors, electrochemical sensors, 3D imaging, and multi-omics have been applied to engineer intestinal barrier-on-a-chip platforms [12,14–17]. In the context of interdisciplinary cooperation, newly developed microfluidic gut barrier models have shown great potential in establishing human-like intestinal systems, evaluating drug metabolism and toxicity, and discovering the mechanisms of nutrient absorption and regulation. Hence, a substantial and general review of these technologies can have far-reaching influences on multiple fields (e.g., life science, biomedicine, and clinical pharmacy). Meanwhile, an up-to-date review can also inspire researchers with different professional backgrounds to explore the development of microfluidic gut barriers for basic research and practical application.

Therefore, this paper reviews the recent progress in microfluidic techniques for the construction and evaluation of intestinal barriers from a logistics perspective. First, it provides a brief snapshot of intestinal barriers *in vivo* (Fig. 1). Second, it systemically introduces microfluidic strategies for the construction of intestinal barrier models *in vitro*, including fabrication materials, sterilization techniques, microfabrication procedures, design principles, cell sources, and extracellular microenvironment parameters (e.g., oxygen gradient, peristalsis, bioflow, and gut-organ axis). Third, it highlights state-of-the-art
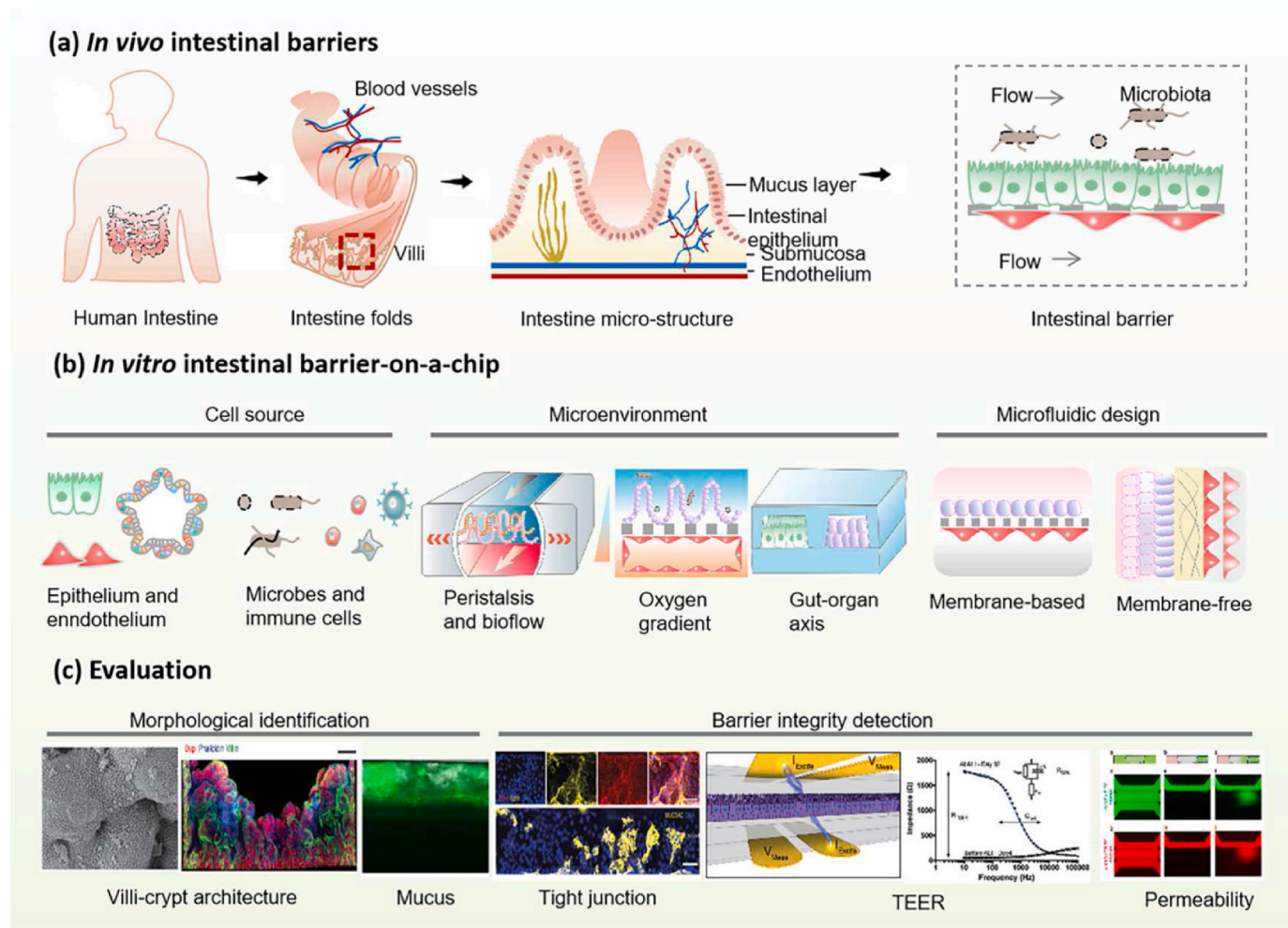
Fig. 1. Microfluidic techniques for on-chip intestinal barrier establishment and evaluation. (a) *In vivo* intestinal barriers. (b) Microfluidic techniques for integrating the cell source, microenvironment, and microfluidic design into a gut-on-a-chip, which can be used to establish a functional intestinal barrier-on-a-chip platform *in vitro* in a physiologically relevant manner. (c) SEM, 3D imaging setups, and biosensors facilitate morphological identification (e.g., villi-crypt architecture and mucus) and barrier integrity detection (e.g., tight junction, TEER, and permeability). The images in Fig. 1c have obtained reprint permission [34,37,41,52].

multidisciplinary techniques (e.g., biosensors, 3D imaging, multi-omics, and artificial intelligence) for establishing and monitoring *in vitro* intestinal barriers. Finally, it presents the current indicators used for evaluating microfluidic intestinal barrier platforms through morphological identification and barrier integrity detection, including polarized villi-crypt architecture, mucus distribution, tight junction proteins, transepithelial electrical resistance (TEER), and permeability.

2. Snapshot of *in vivo* intestinal barrier

In order to effectively design and prepare gut-barrier-on-a-chip models *in vitro*, it is necessary to elucidate the morphological and functional characteristics of the intestinal microecosystem. Intestinal folds are organ-specific structures and are generated by the proliferation and differentiation of the intestinal epithelium [18]. The adjacent microvilli, together with basal proliferative crypts, form a polarized crypt-villus axis. Interestingly, the reconstitution of the folded intestine involves a directional regeneration process in which basal proliferative crypts replenish the villi along the crypt-villus axis [19]. In addition, the mucus layer is deposited on the surface of the intestinal epithelium. Under normal conditions, the gut barrier is intact and homeostasis is maintained. The villi-like protrusions have a large surface area and rich networks of capillaries, enhancing the versatility of the intestinal barrier [5]. The main functions of the gut barrier are to absorb, metabolize, and transport nutrients, drugs, and other materials from the digestive system to the vascular system, allowing their distribution throughout the body [20]. Additionally, the intestinal epithelium contains a complex microbial community, and the associated gut immune system maintains the functions of the gut barrier, enabling immune regulation and providing protection against pathogens [21,22]. The intestine's unique aerobic-anaerobic environment and continuous fluid movement are crucial for the long-term survival of microorganisms and intestinal cells [23]. Periodic peristalsis is a fundamental component of the intestinal microenvironment and involves the rhythmic muscular contraction and relaxation of the intestines, pushing intestinal contents along the gastrointestinal tract [4].

Notably, the human gut barrier is a semipermeable and selective interface that connects the gastrointestinal tract with other organs (e.g., the liver, kidneys, pancreas, and heart) through blood circulation. Indeed, the human body is a complex physiological system in which multiple organs act in a coordinated manner and influence each other's functions [7]. Gut barrier dysfunction can induce a range of diseases, including IBD, metabolic syndrome, and even cancer [24,25]. Therefore, it is essential to develop an in vivo-like gut barrier and conduct a comprehensive assessment of the structure and function of established *in vitro* gut barriers to gain a clear understanding of intestinal homeostasis and disease pathogenesis.

3. Microfluidic strategies for the construction of intestinal barrier models *in vitro*

Building human-relevant gut barrier models is the cornerstone of subsequent research. To construct near-physiological gut barriers *in vitro*, important units such as cell sources and microenvironment parameters can be integrated into microfluidic chips in a controllable manner -using microfabrication techniques and design principles. In this section, fabrication materials, sterilization techniques, microfabrication techniques, design principles, cell sources, and key microenvironment parameters are systematically highlighted and introduced. We also summarize key indicators of microfluidic technique-based intestinal barrier establishment and evaluation (Table 1).

3.1. Fabrication materials

Materials have many influences on the fabrication technologies, monitoring and even the experimental results studied on the chip. In this

case, there are some key considerations in selecting certain materials used to manufacture chips, such as biocompatibility, optical transparency, gas permeability, cost and preparation technology.

3.1.1. Inorganic materials

Early, the first-generation of microfluidic chips were made of inorganic materials such as silicon or glass. They are both resistant to organic solvents, thermostability, and chemical inertia, which make them ideal for in-situ fabrication of microstructures [63,64]. Unfortunately, the optically opaque nature of silicon makes optical imaging and analysis difficult. In contrast, glass exhibits excellent optical transparency, lower cost, higher resistance to high temperature and greater elasticity to high pressures [63,65]. The main concern is that the use of amorphous glass results in anisotropic structures with a high-aspect-ratio [66]. In many cases, inorganic materials are always used to prepare the molds needed for intestinal chips [67], rather than directly for intestinal cell culture.

3.1.2. Elastomers

Lagging behind silicon/glass materials, polymer materials were also included in the preparation of microfluidic chips in the following years. Compared with inorganic materials, various polymers with specific properties provide great flexibility for the preparation of chips due to their easy availability, low cost, and easy integration [68,69]. According to their physical traits, polymers can be classified as elastomers, thermosets and thermoplastics [63].

Elastomers consist of cross-linked polymer chains that can be stretched or compressed when an external force is applied and can be restored when the force is withdrawn. In general, such materials are usually reproduced by soft lithography to reproduce the pattern of the mold, so as to achieve the microstructure of the chip. For such fabrication or replication, a mold is needed. Depending on the material and size required, molds can be manufactured by milling, lithography, deep-ultraviolet (DUV) lithography, electron beam, etc., or in combination with other processes such as wet etching or reactive ion etching (RIE) [66,70]. The most common elastomer used in microfluidic chips is polydimethylsiloxane (PDMS), which was introduced as early as 1998 by M. Whitesides [71,72]. Generally, liquid PDMS prepolymer obtained by mixing PDMS monomer with curing agent in a certain proportion can be cured by heating. Therefore, we can adjust Young's coefficient and thermosetting through the mixture of monomer and curing agent [73]. Due to its low surface tension, PDMS can be easily peeled off the mold after curing. It can also be irreversibly bonded with other materials such as PDMS, glass and silicon by surface modification to form a sealed microfluidic chip [74,75]. In particular, complex chips with multi-layer structures can also be expediently made by assembling many PDMS pieces through holes to connect every layer [76]. For instance, Guo et al. established an intestinal chip consisting of an upper and lower layer of PMDS separated by a through-hole PDMS membrane, formed by oxygen plasma bonding [16]. PDMS is well suited for long-term cell culture due to its good gas permeability. By seeding intestinal cells, researchers can easily reproduce the intestinal barrier exhibited tissue polarization, mucin production, and transporter expression, physiological immune tolerance [33,36,40,42,49]. High elasticity is another advantage of PDMS. By exerting cyclic strain on a PDMS-based gut chip, researchers can easily mimics in vivo-like physiological peristaltic motions [19,33, 47]. Qin's team developed an on-chip valve in which the on-off state of the channel is controlled by the elastic deformation of the PDMS film [77–79]. In addition, gut chips fabricated via PDMS are compatible with other components, including electrochemical sensors, optical imaging devices, and biomaterials, due to their high repeatability and flexibility.

Every coin has two sides. Due to the hydrophobicity of PDMS itself, it will absorb hydrophobic small molecules, which may affect the intestinal absorption, metabolism and transport of drugs [80]. Therefore, the stability and bioavailability of drugs in the system, as well as the accuracy and precision of drug detection, are not optimistic [81]. To

Table 1
Summary of the key indicators of microfluidic technique-based for intestinal barrier establishment and evaluation.

Organ types	Intestinal cells	Co-cultured cells	Microbes	Materials	Preparation technology	Peristalsis	Bioflow/μL min	Electrodes	TEER/Ω cm2	Findings
Gut [17–19] [26–38],	Caco-2 cells [17–19,26–38]	N.A.	LGG [33]; *B. bifidum* [17]; *B.scentis, E.hallii* [36]; Shigella flexneri [37]; Coxsackievirus B1 [35]	PDMS [17–19,27,29,30,33–38]; PMMA [17,29,32]; PC [17,27,30,32]; COP [26]; PET [26,28,38]; Si [17,26,36]; PEVA, PLGA [31]; Collagen [17,19,26,30,31,33–38]; Glass [18,38]; NC [18]; Organopalte [34]; TOPAS [28]	Photolithography [17–19,29,30,32,33,35,38]; Milling [26]; 3D printing [27,31,36]; Wet etching [28]; Laser cutting [30]	10 % strain, 0.15 Hz [17, 19,33,35–37]	0.5–20 [17, 18,26,28, 30,32,35, 37]; 100 [38]; 500 [33]; Rocker [34]	Pt [32], Gold [27, 30]; PEDOT: PSS [26]; ITO [28]; Platinum [29]; Sliver [17, 31,36]; Chopstick [33]	~600–1000 [17,26,28, 30]; ~1500 [32]; ~4000 [33]; ~8000 [7336]; 9300-13000 [29]	1.Monitoring tissue differentiation and barrier function by on-chip electrodes [26–31] 2.Exploring drug metabolism and transport [18,19,32] 3. Reproducing the *in vivo*-like microenvironment [33,36,38] 4.Modeling tissue polarization, pathogen infection, microbe-mediated barrier repair [17,34,35,37]
Gut [15, 39–41]	HT-29 cells [39]; Caco-2 cells [15, 40,41]	MDCK cells [39]; PBMCs, HUVECs [40]; HIMECs [15]; hAECs [41]; Macrophages [40]	*L.rhamnosus, C. albicans* [40]; *B. fragilis* [15]	COC [39]; PDMS [15,41]; Collagen [15]; PS,PET [40]; PC [41]; Si [15]	Injection modeling [39,40]; Photolithography [15]; Laser cutting [41]	10 % strain, 0.15Hz [15]	1 [15,41]; 10-100 [39, 40]	Gold [39, 41]	50-80 [39]; ~4000 [41]	1.Sustaining a physiologically relevant level of microbial diversity [15] 2.Displaying the physiological immune tolerance of the gut to microbial-associated molecular patterns [40]
Gut [2,16, 42–47]	Caco-2 cells [2, 16,42–47] HT-29 cells [16, 44]	HUVECs [16,42, 43,45]; HMVECs [47]; PBMCs [2,16,42, 46,47]; THP-1, MUTZ-3 [44]	LGG [42]; VSL#3 [46,47]; SARS-CoV [16; *L.plantarum, B.Lactis* [43]; *L.casei L5 BGB* [45]; *L,rhamnosus, B.longum* [2]; E.coli [2,45]	PDMS [2,16,42,43,45–47]; PMMA [2,45]; PC [42]; Si [42,43,45]; Organoplate [44]; Collagen [2,43–46]	Photolithography [2, 16,42,43,45–47]; Milling [2]	15 % strain, 0.15Hz [45–47]	0.5–6.5 [16, 42,43, 45–47]; 30 [2]; Rocker [44]	Chopstick [2]; Gold [43, 44]	~60–300 [2, 43,44]; ~1200 [46]; ~3500 [47]	1.Identifying the contributions of mechanical motions and microbiome to intestinal inflammation and bacterial overgrowth [47] 2. Reproducing stromal reshaping and probiotics translocation and viral infection [2,16,46] 3.The probiotics or drugs resulted in a substantial responded recovery of barrier function [42–45]
Gut [48–53]	HiPSCs [50]; Biopsy-derived organoids [48–53]	HIMECs [48,49]	*F.Prausnitzii, E.rectale, B. thetaiotaomicron* [51]; Intestine bacteria [48]	PDMS [49,53]; Collegen [48–50,52,53]; Organoplate [50,51]	Photolithography [53]	10 % strain, 0.2 Hz [48, 49]; 10 % strain, 0.15Hz [52]	0.5–10 [48, 49,51–53]; Rocker [50]	N.A.	~30 [50]; ~1800 [51]	1.Reproducing the gut phenotype, mucus accumulation and barrier crosstalk [50,51,53] 2.Intestinal organoid-on-chip more closely simulated whole human duodenum *in vivo* [49] 3.Modeling environmental enteric dysfunction and enterocolitis [48,52]
Gut-liver [54–57]	Caco2 cells [54, 55,57]; Colon organoids [56]	HepG2 cells [54, 55,57]; Macrophages PBMCs, Kupsser cells,T cells,Th17 cells, Dendritic cells [56]	N.A.	PET [54]; Organoplate [56]; Collegen [56,57]; PDMS [54,55,57]	Photolithography [55,57]; Laser cutting [56]	N.A.	15 [57]	N.A.	N.A.	1.Paradoxical modulation of IBD-related inflammation by short chain fatty acids [56] 2.Reproducing the initiation and progression of hepatic steatosis [55,57] 3.Simulating drug absorption and metabolism behaviors [54]
Gut-kidney [58,59]	Caco-2 cells	Proximal tubule epithelium [58]; GECs [59]	N.A.	Organoplate [58]; PDMS [59]; Collagen [58,59]	Photolithography [59]	0.02 dyne/cm2 [59]	Rocker [58]	Chopstick [58]	~588 [58]; ~1300 [59]	1.Allowing TEER studies in a perfused chip without interference by artificial filter

(continued on next page)

Table 1 (*continued*)

Organ types	Intestinal cells	Co-cultured cells	Microbes	Materials	Preparation technology	Peristalsis	Bioflow/μL min	Electrodes	TEER/Ω cm2	Findings
										membranes [58] 2. Assessing drug absorption related nephrotoxicity [59]
Gut-liver-kidney [60]	Caco-2 cells	HUVECs; HepG2 cells; HK-2 cells	N.A.	PDMS,PC,Collagen	Photolithography	N.A.	16	N.A.	N.A	Investigation of absorption, metabolism and toxicity of ginsenosides compound K
Gut-liver-skin-kidney [61]	Primary intestinal epithelial cells	HepaRG cells; HHSteCs; Proximal tubule cells	N.A.	PDMS,PET,Glass	Etching	N.A.	0.51–3.26	Volt-Ohm meter	~100	ADME profiling analysis and repeated dose systemic toxicity testing of drug candidates
Gut-liver-kidney-vascular [62]	Primary colon cells	HUVECs; Kidney cells; Hepatocytes	N.A.	COP,Collagen	Etching	N.A.	48–600	Chopstick	~234	Integrating bioflow and real-time sensing in drug development workflows

Human induced pluripotent stem cells (HiPSCs), Human umbilical vascular endothelial cells (HUVECs), Human intestinal microvascular endothelial cells(HMVECs), Human primary hepatic stellate cells (HHSteCs), Peripheral blood mononuclear cells (PBMCs), Human kidney cells (HK-2), Glomerular endothelial cells (GECs), Fetal Rhesus Kidney-4 (FRKH-4), T helper cell (TH17), Lactobacillus rhamnosus GG (LGG), VSL#3 (L.acidophilus; L.plantarum; L.paracasei; Lactobacillus delbrueckii subsp.bulgaricus; B.breve; B.longum; B.infantis; Streptococcus thermophilus; E.coli). Scanning electron microscope (SEM), Fluorescence microscope (FM), Confocal laser scanning microscope (CLSM), Transmission electron microscopy (TEM), Zonula occludens-1 (ZO-1), Fluorescein isothiocyanate (FITC), Tetraethyl rhodamine isothiocyanate (TRITC), poly-lactic-glycolic acid(PLGA), polyethylene-vinyl-acetate (PEVA), cyclo-olefin polymer (COP), cyclic olefin copolymer (COC). *N.A. indicates that the specified feature has not been measured or reported.

overcome these limitations, various modification methods such as UV radiation, O2 plasma treatment and hydrophilic material coatings have been considered to clean the surface and reduce the surface energy to delay the hydrophobic recovery of PDMS [82].

3.1.3. Thermosets and thermoplastics

Thermoset materials such as SU-8 photoresist and polyimide have been used to manufacture microchannel structures. When heated or irradiated, these materials allow curing to form rigid molds with patterned microchannels. In general, these materials are resistant to high temperatures, most solvents, and are optically transparent. Their high strength makes it possible to manufacture high-aspect ratios structures. Unlike thermoset plastics, thermoplastics soften at glass transition temperature and can be reshaped, molded and bonded. Common thermoplastics for the fabrication of microfluidic chips are polymethyl methacrylate (PMMA), polystyrene (PS), polycarbonate (PC), polyethylene terephthalate (PET), copolymers (COC), and fluorinated ethylene propylene (FEP), etc. Thermoforming can produce thousands of replicas at high throughput and low cost, but it requires metal or silicon templates to allow the plastic to flow. There are some limitations to the use of thermoplastic polymers. First, not all polymers are transparent (e.g., polyether ether ketone and polypropylene), which makes microscopy or imaging difficult [83,84]. Second, some materials have strong self-fluorescence characteristics and are not suitable for analytical detection [85]. Third, poor gas permeability has a negative impact on long-term cell culture [86]. Notably, Wu's team designed an FEP microfluidic chip that allowed a variety of biological cells to adhere and proliferate [86]. Despite its high melting temperature, the material does not adsorb biomolecules, is optically transparent, and is soft enough, which is suitable for imaging and processing. In addition, some thermoplastics are used in chips in the form of thin membranes. For instance, Guo et al. seeded the mixture of Caco-2 and HT-29 cells on a PET membrane-based gut chip to assess the absorption of seven drugs, showing a high correlation between their permeability values and human fraction absorbed [87]. Similarly, PC/PDMS membranes can also serve as substrates to facilitate the formation of monolayer barriers [41, 88,89].

3.1.4. Hydrogels

Hydrogels can simulate the natural ECM environment through spatio-temporal control of biochemical and physical signals, due to their high biocompatibility and tunable traits, such as permeability, elasticity and chemical reactivity [90]. Significant advances in hydrogels offer a huge opportunity to generate intestinal barrier models with crypt-villi structures in a high-fidelity manner [81]. Sung and his colleagues made a 3D collagen scaffold using a sacrificial mold method and inoculated Caco-2 cells onto the scaffold to form a 3D topography of the small intestine [91]. Notably, the 3D villi model displayed highly enhanced physiological activities, including transmembrane mucin expression, permeability coefficient of FITC-dextran and resistance to bacterial infection, compared to those of the 2D culture mode. Costello et al. reconstructed a 3D intestinal barrier on a synthetic hydrogel scaffold using polylact-glycolic acid (PLGA) [92]. The system enabled the differentiation of the intestinal epithelium as they moved along the recession-villus axis like to the natural gut. The mechanical deformation of hydrogels can also affect the behavior characteristics of cells. Although natural hydrogels have low cytotoxicity, good biocompatibility and biodegradability, they lack controllable mechanical traits, which is why they are often used in combination with synthetic hydrogels. For example, Zhao's team simulated the gastrointestinal folding process by attaching cell-laden gelatin methacrylate to a pre-stretched matrix that undergoes mechanical instability and evolves into a morphological pattern [93]. This principle is also applicable to the simulation of the respiratory tract and urogenital tract. Nikolaev et al. pioneered skillfully guiding stem cell-derived organoid morphogenesis on a composite hydrogel scaffold to create perfusable intestinal tubes

[94]. The proposed concept of hydrogel-guided stem cell self-assembly into similar physiological organs from form, size and function has wide applicability and will enable more research to be carried out. In a word, a variety of natural and synthetic hydrogels fabricate a 3D intestinal barrier by directing a range of cellular behaviors, such as cell adhesion, proliferation, and differentiation.

3.2. Sterilization techniques

Most intestinal chips are still produced as prototypes in the lab. In this case, we need to consider the compatibility of fabrication materials with the sterilization techniques. The properties of the materials determine which sterilization protocols can be used, which can sometimes be a limiting factor in the selection of fabrication materials. Common sterilization methods include autoclaving, ultraviolet radiation, ethylene oxide, low-temperature hydrogen peroxide, chlorogenic acid disinfection, etc [95]. Improper sterilization methods may affect the mechanical properties of the materials, resulting in chip deformation or leakage, thus affecting the normal use of the chip [96, [97]. For example, PMMA and PC cannot be subjected to traditional autoclaving methods because they require exposure to high temperatures, which can result in the melting of both materials [96,98]. The chemical properties of PDMS make it absorbent to certain organic compounds, drugs, and biomolecules, which can be a concern for sterilization and experimental outcomes. Notably, prolonged immersion of PDMS in ethanol can cause the material to dissolve and even absorb chemicals, affecting the tightness of the chip [99]. Generally, PDMS materials are sterilized by autoclaving and ultraviolet irradiation [100]. For natural polymers, because they are often derived from animals or plants, which may contain harmful chemicals or microorganisms that may affect the results, sterilization is also essential. On the one hand, autoclaving not only reduces the physical properties of gelatin, but also has no significant inactivation effect on endotoxin [98]. On the other hand, autoclaving can also cause the destruction and denaturation of the active ingredients in the hydrogels (e.g., hyaluronic acid and gelatin). So, hydrogel materials can be sterilized by lyophilization after filtration, ethylene oxide and gamma-radiation to ensure the stability of composition and structure [95]. Each material has unique properties in terms of bio-inertness, temperature sensitivity, and chemical reactivity, which influence their selection for specific applications and sterilization methods. Overall, when selecting defined materials, it is essential to evaluate the feasibility of available resources, material properties and sterilization protocols.

3.3. Microfabrication techniques

Over the past few decades, multiple microfabrication technologies, including photolithography, milling and laser cutting, molding [65,101, 102] and 3D printing technology, have been successfully applied for the construction of *in vitro* intestinal barrier models. In this part, we will introduce these established technologies in detail.

3.3.1. Photolithography

Photolithography involves shining light onto light-sensitive materials (e.g., photoresist) coating a hard substrate (silicon or glass) to create patterned mold surface [103]. A typical photolithography process consists of several steps [104,105]. First, a photomask with elaborate geometric patterns is drawn using AI software. Second, the photomask is placed on a substrate (e.g., glass and silicon) coated with the photoresist, which is selectively solidified under light exposure. Third, after rinsing with a developer, the substrate is broken down, and its geometry becomes similar to that of the photomask. Zhao et al. used photolithography to produce an embedded gut chip and study the treatment of barrier function injury and inflammation caused by drug-resistant bacteria using probiotics and antibiotics [55]. Moreover, Andresen et al. established a tight functional gut barrier with P-glycoprotein 1

transporters and mucus secretion, effectively assessing drug transport through the monolayer barrier in a microfluidic gut chip [32]. Yoshimura's team developed a micro total bioassay chip manufactured by photolithography that integrates intestinal, liver, and breast organs to effectively assess the biological activity of anticancer drugs and endocrine disruptors [106]. With the improvement of chip integration, higher requirements are put forward for the photolithography process. Notably, more fine lithography techniques such as deep-ultraviolet (DUV) lithography, extreme ultraviolet lithography and electron beam lithography have been developed to improve processing precision and achieve smaller dimensions (<10 nm) [107].

Typically, wet etching and reactive ion etching (RIE) are also combined with photolithography to make a silicon/glass structural substrate that is used as a mold or microfluidic component for the fabrication of intestinal barriers [108]. Essentially, wet etching refers to the removal of excessive substrate materials (silicon dioxide and aluminum metallic) following exposure to strong corrosive reagents (e.g., hydrofluoric acid, phosphoric acid, potassium hydroxide, and potassium hydroxide) in order to obtain a defined microstructure [107]. RIE is a dry etching technology that utilizes high-energy ions and free radicals in plasma to chemically react with the materials on the surface of the sample to achieve material removal [108]. Thus, wet etching and RIE are classical isotropic etching methods. Inherently, they enable high-throughput manufacturing owing to their simple and rapid process, low equipment dependence, and inexpensive nature. On the one hand, with wet etching and RIE, microarray structures with a size of less than 10 μm can be easily prepared to fabricate the basement membrane of gut chips [12]. Workman et al. fabricated a 7 μm through-hole PDMS membrane that was cast onto a silicon mold that was fabricated using photolithography and reactive ion etching [109]. On the other hand, wet etching is required to obtain well-defined gas chambers in microfluidic gut chips to reflect *in vivo*-like intestinal writhing. However, researchers have also adopted manual procedures such as avulsion to avoid the use of the etching technique due to irreversible corrosion and uncontrollable damage to substrates during the etching processes. For example, Ingber et al. generated a classical gut chip with peristalsis-like motions and flow using photolithography, wet etching, and artificial avulsion [33]. Notably, the combination of photolithography and wet etching or RIE extends beyond the gut, highlighting their potential in the *in vitro* remodeling of organs such as the peristaltic gut, breathing lung, and rhythmic heart [33,107,110,111].

3.3.2. Milling and laser cutting

Milling and laser cutting are also two rapid prototyping techniques commonly used to manufacture microfluidic gut chips. Milling is a process of machining that uses miniature cutting tools to remove materials from a substrate in order to create a desired shape in thermoplastics [112]. Specifically, this process involves the movement of the cutting tool in a variety of directions, typically controlled by a computer numerical control system, which allows for the creation of complex shapes and contours with high precision. Netti's team used a micro-milling machine to create a 3D intestinal stromal equivalent on a PMMA substrate, demonstrating the formation of crypt-villi polarization and mucus deposition [113]. Baldwin et al. recapitulated the gastrointestinal tract-brain axis on a milled micfrofluidic chip [89]. Bacterial extracellular vesicles produced by *Bacteroides thetaiotaomicron* migrated from the apical surface of the intestinal epithelium to the differentiated neuronal chamber. It is worth mentioning that the surface characteristics of the milled microfluidic model, particularly surface roughness, are critical parameters that can significantly influence the device's operational efficiency and precision [89]. On the one hand, these limitations can be overcome by fine adjustment of the milling cutter spindle speed and feed speed [112]. On the other hand, treating micromachined substrates with plasticizers such as cyclohexane, chloroform and acetone can effectively reduce their surface roughness [114,115]. Ahmed et al. found that steam treatment with chloroform for 30 s

reduced the surface roughness of PMMA-based chips machined by micro-milling and smoothed them to a roughness of about 39 nm [116].

Laser cutting is a micromachining process that uses a high-energy laser beam to cut materials such as metals and plastics [117]. That is, the material absorbed by the laser energy is ejected from the substrate surface to produce 3D channels with micron/submicron accuracy [118]. Sidar et al. used laser-cut acrylic sheets and silicone gaskets as cell culture chambers and chip-sealing gaskets respectively for *in situ* organoid culture [119]. This method subjected the liquid in the chip to slight pressure, effectively inhibiting bubble formation and simulated the flow of fluid through the lumen of the intestinal organoids. To reproduce the topography of the gut, Costello et al. micro-molded a porous poly-ethylene-co-vinyl-acetate (PEVA) scaffold by laser cutting and observed the extent to which the scaffold villi shifted from their position, similar to actual villi [31]. In addition, the laser-cut 3D substrates can be reversed-molded to form biodegradable hydrogel scaffolds that replace bioinert matrix materials [120,121]. March's team formed a complex hydrogel scaffold made of collagen and polyethylene glycol by reverse-shaping a laser-cut plastic substrate [120]. Notably, compared with Transwells, intestinal epithelium cultured on villus-like scaffolds showed decreased TEER and improved enzyme activity of the brush edge. Similarly, Costello et al. designed a porous PLGA scaffold by replicating a laser-cut PMMA mold, mimicking the actual density and size of human intestinal villi [121]. Consequently, by precisely mimicking the intestinal villi topography, researchers can facilitate the differentiation of cells along the villous axis, akin to that observed in native intestinal tissue.

3.3.3. Molding

The molding processing technique can be divided into hot embossing and injection molding according to process principles [65]. Hot embossing is a relatively simple manufacturing process for replicating microchannels on a mold by applying force and temperature onto substrates such as silicon, glass, and polymer materials. The mold with suitable materials and precision is the prerequisite for hot embossing [122]. Tovar et al. proposed a height-changing glass mold with submicron resolution that can quickly generate microfluidic chips with high aspect ratios [123]. Among of polymer materials, the PMMA is a classical thermoplastic material, which is suitable for generating microfluidic chips by hot embossing [124]. Hot embossing is also embodied in directly embossing parallel microchannels on metal materials, but embossing metals typically require higher temperatures or pressures than polymers, which have lower melting points and better fluidity [125]. This technique is characterized by its cost-effectiveness and its capability to fabricate microstructures featuring a high aspect ratio along with intricate micro-pin lamellae. Nonetheless, achieving an optimal surface finish necessitates meticulous management of temperature and other critical parameters that impact the process [126,127].

Injection molding constitutes a process wherein the thermoplastic material is initially subjected to uniform plasticization within a heated barrel, followed by its forced introduction into the confines of a pre-closed mold cavity via a plunger or a reciprocating screw mechanism [65]. Subsequently, the material is allowed to solidify and take the shape of the mold through the application of cooling mechanisms, culminating in the formation of the desired molded component. A key benefit of the technique is its cost-efficiency, akin to photolithography, where the expense for high-precision microfabrication is primarily associated with the production of a master template. To guarantee high-quality in the fabrication, it is imperative to exercise precise control over critical process parameters, including temperature, pressure, and the rate of injection [128,129]. Maurer et al. manufactured a polystyrol-based gut chip by injection molding, displaying the physiological immune tolerance of the intestinal lumen to microbial-associated molecular patterns [40]. Overall, molding technology offers outstanding advantages in greatly simplifying the production timeline and process intricacy, which is amenable to large-scale production.

3.3.4. 3D printing

3D printing is an emerging technique that allows direct fabrication of objects layer-by-layer through computer assistance [130]. It can control the spatial distribution of thermoplastic materials, and can also 3D print cells on a substrate along with the extracellular matrix such as hydrogels. For instance, Mazrouei et al. 3D printed pluronic molds and PDMS well arrays to enable *in situ* loading of gelatin methacryloyl and cells [131]. Kim et al. adopted collagen and decellularized small intestine submucosa as a cell-laden bioink for forming small intestinal epithelium with a finger-like microscale villus architecture [132]. By flexibly adjusting the hydrogel component, crosslinking degree printing speed and printing time of the bioink, a more realistic physiological 3D villus model could be obtained. *In vivo*, tissue formation is largely dependent on the regulation of morphogenetic programs that allow different kinds of cells to interact and self-assemble in space and time. In the co-culture system of Caco-2 cells and human umbilical vein endothelial cells prepared by Kim et al., 3D intestinal villi showed high glucan permeability and glucose uptake capacity [133]. Lutolf's team deposited organoid-forming stem cells directly into the extracellular matrix to reproduce the self-organization of the macro-scale tissue [134]. By manipulating the cell density and geometry, the researchers were able to construct native tissue constructs with the intestinal tube, branched vascular, and crypt-villus regions. This approach allowed for regulating organogenesis through the deposition of supporting cells and mimicked the organ boundaries present in the gut by printing different epithelium.

3.4. Design principles

In order to remodel *in vivo*-like gut functional barriers using microfluidic organ chips *in vitro*, different design principles have been developed and validated. The physiological structure of gut barrier models can be established using two strategies: a membrane-based design and a membrane-free design.

3.4.1. Membrane-based design

Initially, microfluidic-based gut barrier systems with a membrane-based design were inspired by conventional Transwell platforms with a sandwiched construction. A typical feature of this design involves the use of a porous membrane between the upper and lowers layers, which have microchannels. The cells can be flexibly attached and cultured on the surface of the upper and lower channels as well as on the membrane. This structure allows us to introduce the culture medium into the channels of gut chips in a controlled and perfused manner. For instance, Hwan's team implanted a collagen scaffold that mimicked the intestinal villi into a membrane-based microfluidic chip [38], where the combined effect of the 3D structure and fluid shear provided sufficient stimulation to induce cell differentiation in a physiologically relevant mode. Shin et al. recreated the 3D epithelial barriers from organoids-derived intestinal epithelium on a membrane-based chip, showing spontaneous 3D morphogenesis and demonstrating enhanced physiological function and biomechanical properties [135]. To mimic peristalsis-like motions, cyclic stretching can be applied through gas chambers located on either side of the gut chip via a vacuum controller. For instance, Wang et al. investigated the transport and absorption of Hg (II) by intestinal epithelial cells under peristalsis [136]. Notably, when the tensile strain is increased from 1 % to 5 %, the absorption rate of Hg(II) is significantly increased by 23.59 % and the expression of Piezo1 and DMT1 on the cell surface was upregulated [136]. This research confirmed that the complex microenvironment is extremely important for the structure and physiological function of the intestinal epithelium. Generally, the formation of the epithelial monolayer barrier only needs several days, which is far beyond traditional culture paradigms (e.g., Transwell and well-plate mode) in terms of both morphology and function. As shown in Fig. 2a, human intestinal cells (Caco-2) and human umbilical vascular endothelial cells (HUVECs) can spontaneously form a gut barrier containing folded villi and an intact vascular lumen in separate channels
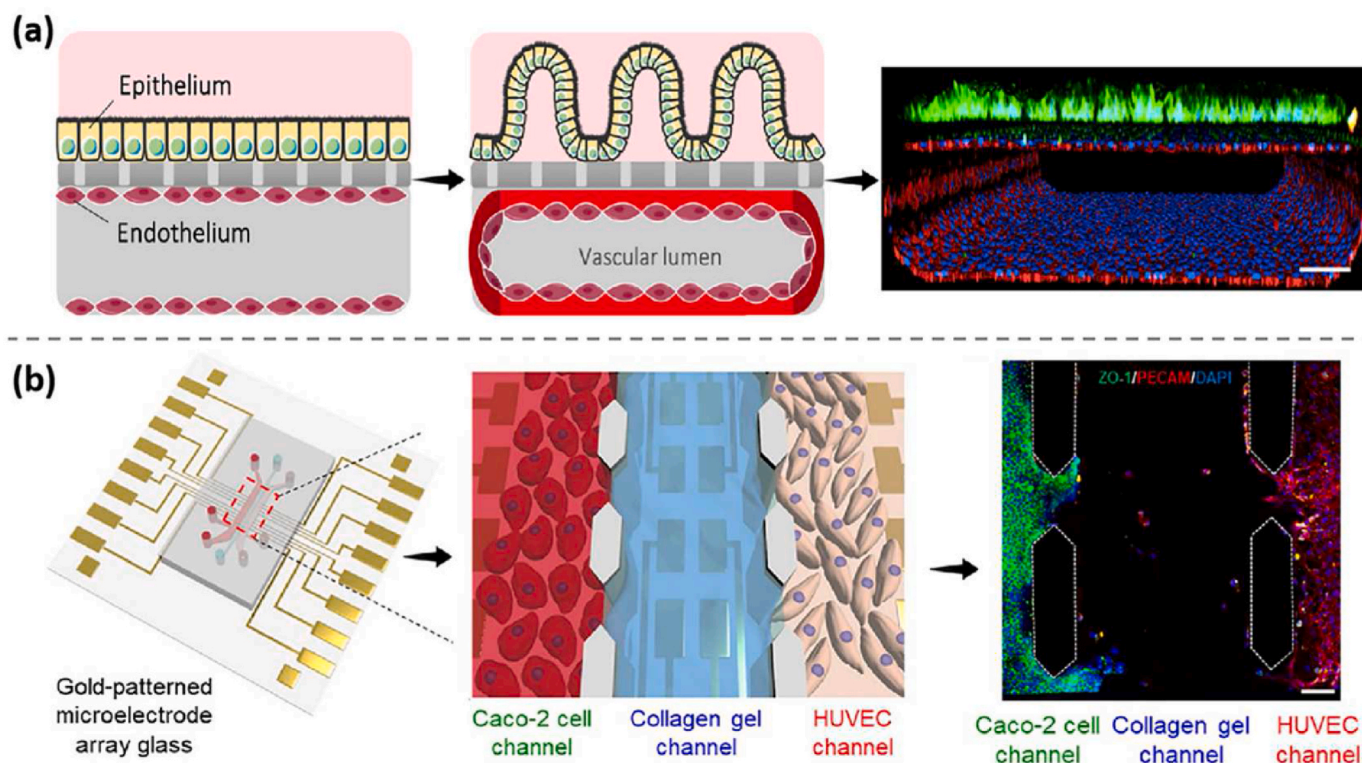
Fig. 2. Design principles. (a) *In vitro* intestinal barrier models can be established using microfluidic strategies based on a membrane design [137]. Copyright 2019, Springer Nature. (b) *In vitro,* intestinal barrier models can also be established using microfluidic strategies based on a membrane-free design [43]. Copyright 2022, Springer Open.

after culturing for 5 days [137]. Using this model, radiation-induced abnormal barrier functions — including cell apoptosis, cytotoxicity, lipid peroxidation, DNA breakage, villus weakening, tight junction damage, and integrity impairment — have been explored. Markedly, pretreatment with dimethyloxaloylglycine has been found to markedly inhibit these effects, demonstrating the potential of the microfluidic gut barrier system for screening therapeutic drugs. Overall, the membrane-based design has been widely used to fabricate physiological gut barriers and further explore gut-microbe communication, disease models, and drug efficacy [33,48,49,135,138]. However, one drawback of this design is that it only allows low-resolution imaging due to the low membrane transparency. Nevertheless, this disadvantage can be addressed by using membranes with higher transparency, such as polydimethylsiloxane (PDMS) and polytetrafluoroethylene porous membranes, and avoiding the use of a polyester membrane [139].

3.4.2. Membrane-free design

Another commonly used strategy for developing a microfluidic-based gut barrier model involves the membrane-free design, in which two chambers are separated horizontally by a middle channel containing microstructures [34,39,43,58] Generally, the intestinal epithelium and endothelium are seeded on the bilateral channels, while the extracellular matrix is loaded in the middle channel to imitate the *in vivo* intestinal basement membrane. In one study, Jeon et al. simulated the *in vivo* gut barrier by coculturing Caco-2 cells and HUVECs in a gut chip, achieving a highly polarized epithelial morphology (Fig. 2b) [43]. A collagen type 1 gel with good permeability, defined protein components, and known thickness and stiffness ensures communication between the epithelium and endothelium and is valuable for developing *in vivo*-like gut barriers. In these models, it can effectively adhere to the apical surface of villi and introduce bacterial cells into the gut lumen for 2 h. Observably, probiotics can enhance gut barrier integrity, promote recovery following lipopolysaccharide (LPS)-induced gut barrier injury,

and attenuate the inflammatory response [43]. In addition, other specific cell types can also be integrated into the gut barrier system. Nowadays, gut-liver chips, gut-kidney chips, and gut-brain-microbe chips have been developed to investigate the effects of additional stimuli (e.g., drugs and their metabolites) on the target organ (e.g., organ physiology and pathology, drug toxicity, pharmacokinetic, and drug efficacy) due to intestinal transportation, absorption, and metabolism [54,55,59,60,140,141]. Haan et al. designed three consecutive reactors to dynamically explore enzymatic digestion in a mouth-stomach-intestine chip [142]. Studies showed that omeprazole was broken down when treated with stomach acid, but when introduced into the system in a manner that mimicked enteric-soluble preparations, omeprazole reached the cellular barrier unharmed. Verapamil, in contrast, is not affected by digestion. Kamei's team integrated the gut-liver axis on a chip based on a membrane-free design and simulated the initiation and progression of non-alcoholic fatty liver disease (NAFLD) under free fatty acids (FFAs) exposure [57]. The study found that cell DNA was damaged after 1 day of exposure to FFAs. After 7 days of exposure to FFAs, there was a large accumulation of fat droplets in both the intestine and liver, increased DNA damage, and eventually cell death. These pathological features are similar to those seen in patients with severe NAFLD. Compared to the membrane-dependent design, the membrane-free design is simple and easy to operate, has a low technology threshold, and involves very little complex assembly. Moreover, this design is more suitable for exploring cell-matrix interaction and cell behaviors, including cell adhesion, cell invasion, and cell migration via an optical microscope. For instance, Hoyos-Vega et al. simulated gut neuro-epithelial connections by developing epithelial and neuronal chambers connected by on-chip microgrooves [143]. Significantly, the presence of epithelial cells enhanced the density and directivity of neuronal processes. In another membrane-free chip, Morelli et al. observed enterotoxin-induced damage to the human intestinal epithelium, showing a dose-dependent reduction in barrier permeability

[144]. Overall, membrane-free chips can visually dissect the neuro-epithelial connections, injury and recovery of functional barriers between the gut and other organs.

3.5. Cell sources

The gut barrier not only participates in the transportation, absorption, digestion, and metabolism of foods and drugs, but also protects the body from bacteria, viruses, and other hazardous substances [145]. The selection of cell sources largely determines the complexity and physiological relevance of gut barriers and affects their functional characteristics. The most fundamental cell types for gut barrier models are the intestinal epithelium and endothelium. Microbes and immune cells can also be selectively incorporated into the ecosystem as needed. In the following sections, we will discuss the representative cell types used for the construction of artificial gut barriers.

3.5.1. Epithelial cells

Currently, cancer cell lines and primary intestinal cells derived from human tissue have been validated and used extensively in *vivo*-like gut barrier models. Initially, cell lines (e.g., Caco-2, HT-29, and H84) received great attention because of their ease of culture, strong ability to expand, and inexpensive cost [146]. Among them, Caco-2 cells — which possess features of small intestine cells — have remained the most popular and standard cell line for gut barrier remodeling for decades [5]. After 5 days of culture, Caco-2 cells can spontaneously form *in vivo*-like morphological structures such as microvilli on the apical side of differentiated epithelial cells and well-distributed cupped crypts everywhere [19,42]. However, Caco-2 cells are limited by their lack of a significant mucus layer, which is an important barrier that not only physically isolates the intestinal tissue, immune system, and microbial populations, but also provides defense against pathogen invasion [147]. Therefore, mucus-secreting HT-29 cells are usually used to compensate for the drawbacks of Caco-2 cells [16,44]. For instance, to actually reflect the response of the intestinal epithelium-vascular endothelium barrier to virus infection, Guo et al. cocultured Caco-2 cells and HT-29 cells on the upper channel of a gut chip [16]. In contrast, Boulant et al. confirmed that the infection of Caco-2 cells with SARS-CoV-2 results in weaker innate immunity than the infection of T84 cells [148]. Additionally, T84 cells with their differentiated crypt structure are more suitable for studying the mechanisms related to the transport and absorption of

amino acids, proteins, and peptides [149].

Unlike regular cell lines, biopsy-derived intestinal organoids maintain several hallmarks of native tissues in terms of both genotype and phenotype. They contain more abundant cell types (e.g., intestinal epithelial cells, goblet cells, endocrine cells, intestinal stem cells, etc.) and are capable of more mature functions (e.g., absorption, metabolism, transport, and infection) [109]. Intestinal organoid-derived barriers are more sensitive to the secretion of inflammatory factors than Caco-2 cell-derived gut barriers [50]. Typically, intestinal organoids are extremely suitable for high-fidelity organ reconstruction, target identification, and preclinical efficacy evaluation [150–152]. For instance, Jalili-Firoozinezhad et al. digested tissue-derived small intestinal organoids into single cells or small clumps and seeded them on the surface of a porous membrane, generating a gut barrier-on-a-chip with a dense monolayer (Fig. 3a) [109]. Transcriptomic results indicated that the organoid-derived gut barrier more closely resembled the human duodenum *in vivo* than the Caco-2 cell and 3D duodenal organoids. Karalis's team deciphered the mechanisms that drive human intestinal permeability on a human biopsy-derived intestinal organoid chip [153]. Recombination of the colonic epithelial apical junction complex and induction of apoptosis mediated the effect of interferon on the intestinal barrier. Contrary to the protective effect described in animal models of colitis, interleukin-22 induced on-chip intestinal barrier breakdown. Certainly, human-induced pluripotent stem cells can also differentiate into intestinal organoids and further be used to prepare a monolayer barrier-on-a-chip to evaluate the responses of tumor necrosis factors (Fig. 3b) [49]. Besides, patient-derived organoids with disease genotypes and phenotypes can provide a reliable source of cells for *in vitro* studies of disease occurrence and progression [154]. Notably, Shin et al. reconstructed *in vitro* intestinal barriers, reflecting disease-specific differentiation characteristics based on intestinal organoids derived from patients with colorectal cancer, Crohn's disease and ulcerative colitis [155]. Emerging organoids can also be applied for drug testing on microfluidic chips. Stresser's team characterized the enzymology of prodrug biotransformation in patient-derived jejunal and duodenal organoid chips [156].The pharmacokinetic results showed that fosamprenavir was hydrolyzed to amprenavir by phosphatase, while Dabigatran etexilate exhibited the epithelial enzyme carboxyl esterase 2-mediated de-esterification. In follow-up studies, I believe that the connected gut-liver-vascular chip can simulate the ingestion process of oral drugs, and can better predict the biotransformation, bioavailability
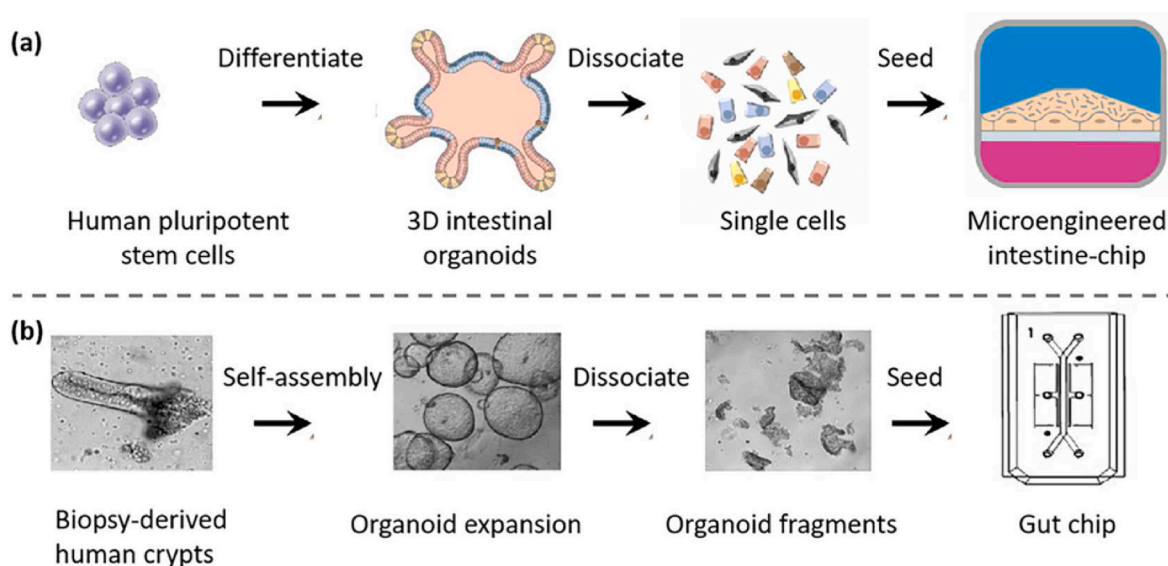

Fig. 3. Emerging intestinal organoids can serve as sources of epithelial cells for the construction of the gut barrier on a microfluidic chip. (a) Stem cell-derived intestinal organoids [109]. Copyright 2018, American Gastroenterological Association. (b) Biopsy-derived intestinal organoids [49]. Copyright 2018, Springer Nature.

and metabolites of drugs. Ai et al. developed an intestinal organoid chip to achieve fast and high-throughput drug screening for colorectal cancer therapy [157]. Chemotherapeutic drug-sensitivity testing on the organoid chip generalized the heterogeneous responses of individual patients and showed a high relevance to clinical findings [157]. Overall, the aforementioned microfluidic organoid models have enhanced the utilization of microfluidic gut chips and intestinal organoids. We believe that the use of organoids as a source of epithelial cells to construct gut barriers and mimic intestinal microphysiological systems on a microfluidic chip will be a prevailing trend in biomedical research.

3.5.2. Endothelial cells and immune cells

The vasculature is essential for modeling the intestinal epithelium-vascular endothelium barrier. HUVECs and human intestinal microvascular endothelial cells (HIMECs) are the most commonly used vascular cells. Usually, endothelial cells are introduced into the lower layer of the gut chip, forming a classical vessel lumen under continuous fluid movement and *in vivo*-like shear stress. Compared with Caco-2 cells alone, HUVECs contribute to the organization of proliferative and differentiated pools of the intestinal epithelium on the chip, providing a higher apparent permeability coefficient, intestinal alkaline phosphatase activity, and superior absorption function [42]. Flow conditions enable the removal of metabolic waste from the overall system and guarantee the timely supply of oxygen and nutrients, ensuring the maintenance of good cell viability and robust function [158]. Also, the continuous movement of fluid from the vascular compartment can enhance the maturation, permeability, and stability of the gut barrier-on-a-chip [158]. In addition, coculture of the intestinal epithelium and the vascular endothelium can maintain homeostasis and dramatically enhance resistance to hazardous substances.

In vivo, human blood contains several immune cells, such as peripheral blood mononuclear cells, which participate in immune responses and sustain the balance of physiological systems [159,160]. Given the superior integration ability and flexibility of microfluidic chips, gut barriers can synergistically integrate multiple cell types and the extracellular matrix in the same culture system in a more bionic manner. For example, Ingber's group cocultured enterocytes, vascular endothelial cells, microbes, macrophages, and probiotics in a membrane-based gut barrier chip to explore the intestinal injury and inflammatory responses induced by *Escherichia coli*, and study host-microbial communication and anti-inflammatory effects [45].

3.5.3. Microbes

The human gut contains diverse microbes (e.g., anaerobic, aerobic, facultative anaerobic, and facultative aerobic microorganisms), which constitute an enormous microbial community. There is strong evidence that the gut community plays a pivotal role in modulating human health and disease [161]. Typically, commensal microbiomes participate in nutrient metabolism, immune regulation, and gastrointestinal homeostasis. Hence, an in-depth analysis of the relationships between the gut, microbes, and exogenous materials is important for discovering novel therapeutics against gut disease and gut disorder-induced pathologies in other organs. Emerging microengineered chip models have facilitated the exploration of these key aforementioned events.

Gut microbes are involved in regulating a variety of host metabolic responses; producing metabolites that are critical to gut health, such as short-chain fatty acids; and metabolizing bile acids, steroids, bile acids, choline, and other molecules [104,162]. In fact, some complex carbohydrates, such as dietary fiber, are digested by gut microbes and fermented at the proximal end of the colon to produce short-chain fatty acids, such as butyric acid, acetic acid, and propionic acid, which are known to have important neural activity [51,163]. In turn, these fatty acids affect the growth of bacteria in the colon by affecting water absorption and lowering fecal pH. Griffith and colleagues surveyed fermentation by the bacterium *F. prausnitzii*, which exhibits high oxygen sensitivity, on a primary human colonic mucosal barrier [51]. Acetate,

propionate, and butyrate were detected on both the apical side and basolateral side of the gut barrier. The high concentration of butyrate in the basal compartment indicated that in the presence of *F. prausnitzii*, butyrate was actively absorbed by the intestinal epithelium and transported to the basal chamber via the gut barrier.

Intestinal microorganisms also play a crucial role in the biological processing (e.g., absorption, metabolism, transformation, and bioactivity alteration) of many compounds [161,164,165]. This has also been observed in animal models. For instance, Ginsenosides compound K (CK) is generally metabolized by gut microbes via beta-glycosidases and not in the liver [166]. Using a single chip model, Du's group found that only 20 % of ginsenosides CK was absorbed by the gut barrier and about 40 % was metabolized by the liver [60]. However, in a gut-liver-kidney chip model, CK content gradually decreased along the gut, vascular lumen, liver and kidney, eventually reaching 70 %, 3.4 %, and 0 % in the gut, liver, and kidney, respectively. That is, most CK stayed in the gut and rarely traveled through the vascular lumen to reach the liver and kidneys. Therefore, this multi-organ platform can more truly reflect the actual distribution of drugs or compounds in the human body. Therefore, in order to truly simulate the pharmacokinetics and efficacy of specific compounds and drugs, existing evaluation systems must be improved by incorporating gut microbes.

Recently, there has been a great focus on the importance of microbes in human health and disease. *In vitro,* human-relevant gut barrier systems are indispensable for exploring microbial pathogenicity. Kim et al. demonstrated that an intact gut barrier can effectively constrain inflammatory host-microbiome cross-talk by suppressing inflammatory factor secretion and oxidative stress following LPS simulation or *E. coli* infection [46]. Moreover, microfluidic gut barriers can also be used for modeling infection and managing known and emerging pandemics, complementing animal models. Guo et al. found scattered mucin distribution, villus damage, attenuated tight junction expression, and abnormal protein and RNA metabolism following SARS-CoV-2 exposure, indicating that the virus damages barrier integrity [16]. Villenave et al. explored human enterovirus (coxsackievirus B1) infection and replication in a membrane-based gut chip with bioflow and peristalsis-like motions [35]. The cytopathic effects produced by the enterovirus, as well as inflammatory cytokine release and caspase activation in the polarized epithelium were detectable in the apical and basal effluents of the chip. Maurer and colleagues fabricated an immunocompetent gut barrier by coculturing innate immune cells, living *Lactobacillus rhamnosus*, Caco-2 cells, and HUVECs in an organotypic intestinal chip [40].

Gut barrier chips can elucidate the microbiome's contribution to pathogen-related intestinal pathophysiology and offer reliable treatment options in a clinically relevant manner, potentially replacing and surpassing traditional models. For instance, Kim et al. confirmed that probiotic and antibiotic treatment can limit the immune cell-associated barrier damage caused by pathogenic bacteria, consistent with findings from animal and clinical models [47]. Pre-colonization by microorganisms can weaken opportunistic pathogen-evoked barrier damage, limit the growth of pathogens in the intestinal compartment, and reduce the translocation of the fungi into the vascular system. *Bifidobacterium bifidum* was found to effectively reverse the gut barrier dysfunction caused by TNF-α and LPS [17]. In summary, advanced microfluidic gut barrier systems provide the capacity to parse host-microbe interactions and microbe-triggered barrier malfunction, uncover interaction mechanisms between probiotics and pathogens, and enable the development of microbe-based therapeutic regimens.

3.6. Microenvironment parameters

Microenvironment parameters are extremely important for *in vitro* gut barrier establishment, and they determine the physiological correlation of on-chip systems with the real human intestinal barrier. Typically, oxygen gradient, peristalsis, blood flow, and the gut-organ axis serve as the key biophysical and biochemical cues in the ecological

niche. In this section, we will discuss how microfluidic techniques can be used to bioengineer and mimic the extracellular environment *in vitro*.

3.6.1. Oxygen gradient

The *in vivo* intestinal epithelium has a mucosal oxic-anoxic interface, wherein the oxygen concentration rapidly declines, maintaining good symbiotic relationships between the host and the microbiome [12]. An *in vitro* gut barrier model must accurately simulate the oxygen distribution observed *in vivo*, as it affects genotype, phenotype, metabolism, and gut-microbiome communication. To create the interface, Kim et al. prepared an anaerobic medium by dissolving an anoxic gas mixture (5 % O_2, 5 % H_2, and 90 % N_2) in the culture medium and then introduced the anoxic and oxic medium into the upper and lower lumen, respectively (Fig. 4a) [36]. In this system, commensal obligate anaerobic bacteria (e. g., *Bifidobacterium adolescentis* and *Eubacterium hallii*) were cocultured with the gut epithelium for up to 7 days without compromising cell viability. *In vivo*, the generation of a steady-state oxygen gradient is driven by spontaneous breathing, convection, and diffusion. Inspired by these mechanisms, Grant and colleagues attempted to naturally reduce the oxygen level in the epithelial chamber via aerobic respiration, using a gut chip coated with a gas-impermeable film [167]. Notably, the proposed strategy was validated by both theoretical data from computer simulations and practical findings from experiments, showing that an oxygen gradient was achieved in a gut barrier chip. Villus morphology and a continuous endothelium could be formed; meanwhile, gut barrier integrity was maintained for 3 days under the hypoxia gradient [167]. In principle, the hypoxia gradient established was more physiologically relevant than the gradient achieved using premixed gases. Compared to the use of premixed gases, this approach has unique advantages, including the more rapid balancing of oxygen levels before the experiments, no requirement for low-oxygen incubators, and low interference, which may improve the practical application and scalability of the gut barrier models. To more easily detect oxygen levels, Ingber et al. developed a novel strategy to integrate oxygen sensors within the gut barrier chip [15]. So far, the sensor-based chip technology has been extensively expanded to the real-time monitoring of oxygen levels and/or TEER in organ-specific barriers such as the gut barrier, gas-liquid interface, and blood-brain barrier.

3.6.2. Peristalsis and bioflow

Currently, two strategies are used for mimicking *in vivo*-like peristaltic motions in a gut chip. One involves regulating the application of air pressure off the chip to the fluid medium on the chip, resulting in cyclic changes in fluid pressure within the epithelium and endothelium channels and accompanying periodical peristalsis in the basement membrane (Fig. 4b) [45]. The other involves directly applying air pressure to the vacuum chamber of the chip, leading to the contraction and relaxation of the vacuum chamber wall, driving the mechanical deformation of adjacent cell culture chambers [33]. Both these strategies require the incorporation of computers into microfluidic devices to manipulate the power output of the gas, which is a prerequisite for biomimetic intestinal peristalsis. As a proof of concept, microfluidic gut chips serve as powerful platforms for gaining new insights into gut pathophysiology. For example, the cessation of peristalsis-like mechanical movements can induce bacterial overgrowth in the intestines, reflecting the hallmark of IBD and ileus [47]. Sauvonnet's group introduced *Shigella* into the gut lumen in a mechanically active chip, discovering high rates of *Shigella* invasion across the gut barrier. The results confirmed that the crypt-like invagination of the gut is beneficial for the early adhesion and colonization of *Shigella*, and intestinal peristalsis maximizes the infection and invasion of *Shigella* [37]. Jin's group skillfully created a human colon organoid-on-a-chip platform. In this model, the rhythmic peristalsis of a pressure channel facilitated the peristaltic cultivation of organoids across a sequence of microarrays within the medium channel [168]. After treatment with polymeric micelles, peristaltic organoids showed a weakened uptake, shedding light on the importance of the microenvironment in the evaluation of anti-tumor efficacy.

In addition to constant mechanical forces, intestinal cells also experience shear stress due to the biofluid on the apical surface. The mechanically sensitive microvilli of the apical intestinal epithelial monolayer can conduct the shear stress of fluids. Studies have
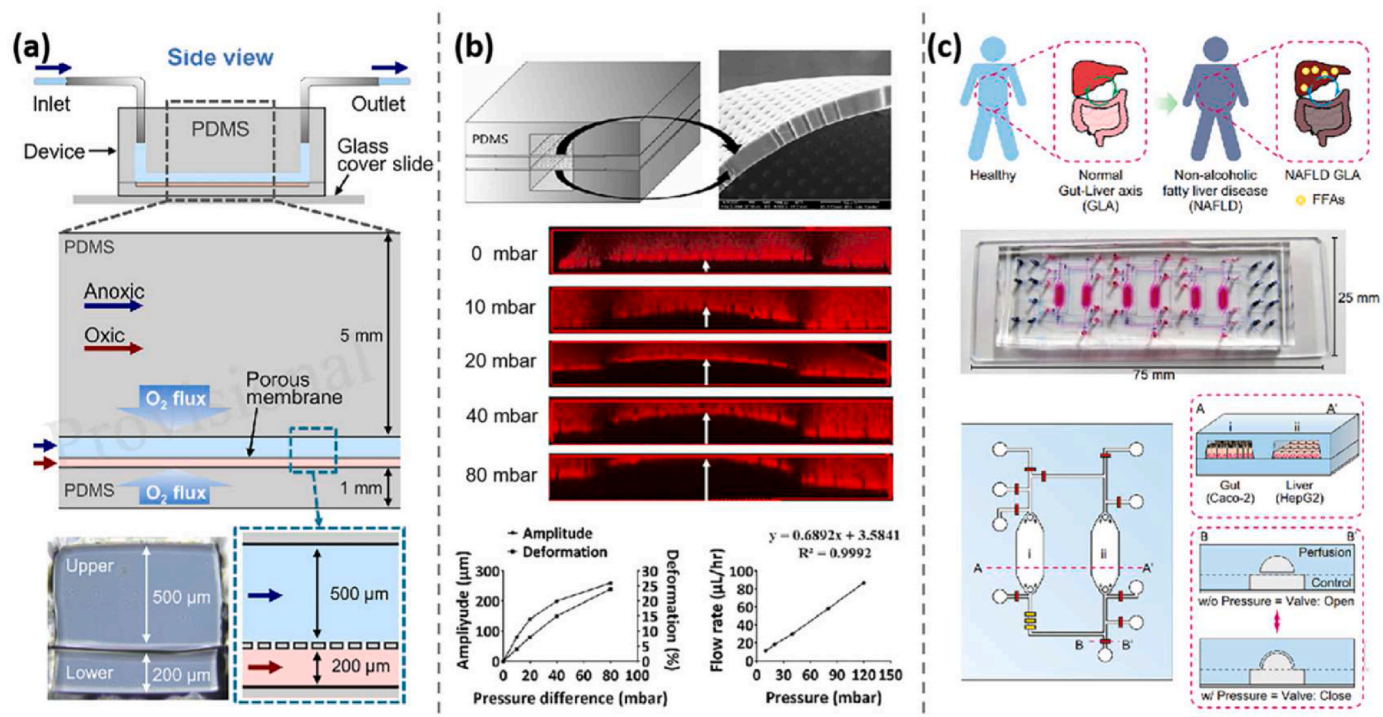

Fig. 4. Microenvironment parameters. (a) Oxygen gradient [36]. Copyright 2019, Frontiers. (b) Peristalsis [45]. Copyright 2020, Frontiers. (c) Gut-liver axis [57]. Copyright 2023, Springer Nature.

increasingly highlighted the potential of microfluidic technologies for simulating the fluidic environment via constant laminar bioflow to the epithelial channel. For instance, Delon et al. systematically investigated the effects of biofluid on a Caco-2 cell-derived monolayer barrier in a perfused chip within a physiologically relevant range of shear stress (0–0.03 dyn/cm^2) [169]. High shear stress (0.03–0.026 dyn/cm^2) promoted microvilli formation, CYP450 enzyme expression, F-actin levels, mucus production, and mitochondrial activity. However, medium shear stress (0.026–0.013 dyn/cm^2) obviously enhanced the expression of tight junctions in the gut barrier. Another study showed that relatively low shear stress can promote the formation of a gut-specific mucus layer [51]. Flow can facilitate the establishment of a transepithelial Wnt signal gradient on the basolateral side and modulate the expression of the flow-dependent Frizzled-9 receptor, promoting stem cell differentiation and 3D villus-like morphogenesis [170]. Therefore, a detailed understanding of the role of biofluids will enable the development of *in vitro* gut barrier models with customized designs according to a specific purpose.

3.6.3. Gut-organ axis

The human body is a complex system in which multiple organs within the same physiological environment cooperate and influence each other. Inspired by this fact, chips with various gut-organ axes have been developed, including the gut-liver axis, gut-kidney axis, gut-brain-microbiome axis, and gut-liver-skin-kidney axis [61,171]. Meanwhile, the gut-organ axis concept has advanced the application of gut chip technology, enabling the modeling of complex organ physiology and the systematic exploration of disease etiology. Lee et al. found that intestinal barrier impairment exacerbates the adverse effects of fatty acids on the skin, resulting in decreased viability, increased secretion of inflammatory factors, and enhanced expression of specific inflammatory dermal disease markers [172]. Chung's group found microbe-derived metabolites could effectively promote the growth, differentiation and mature of neurites on a gut-brain axis chip [165]. Interestingly, microbe-derived exosomes and metabolites could enhance axonal growth and synaptic activity in Alzheimer's disease models. As a downstream organ, the liver is usually linked to the gut barrier via bioflow and defined channels in a single microfluidic chip to establish a gut-liver axis. So far, gut-liver chips have been widely applied for replicating human physiomimetics, modeling pharmacokinetics, and evaluating the efficacy (e.g., anti-inflammatory and anticancer) and negative effects of orally administered or biologically active drugs [54,56,173,174]. For instance, Wilmes's team explored the microbe-mediated bidirectional crosstalk on a gut-liver axis chip [175]. The study showed that intestinal bacteria E. coli altered the metabolism of irinotecan by converting its inactive metabolite SN-38G to the toxic metabolite SN-38. The system is a powerful tool to uncouple microbe-drug interactions and is expected to provide an *in vivo*-like equivalent replacement for new drug development. Trapecar et al. built a gut-liver microphysiological system that could reveal the mechanisms and signaling pathways involved in IBD-related innate inflammation and microbiome-derived short-chain fatty acid-mediated hepatic metabolism [56]. As shown in Fig. 4c, Kamei et al. designed a planar gut-liver chip with a closed circulation loop for modeling nonalcoholic fatty liver disease [57]. Single-cell profiling revealed some characteristics of the disease, including lipid droplet accumulation as well as the increased expression of genes related to cellular responses to copper ions and endoplasmic reticulum stress. Notably, the coculture mode dramatically protected both Caco-2 cells and HepG2 cells from free fatty acid-induced apoptosis. Further, the evaluation of drug toxicity following intestinal absorption has been extensively explored using gut-kidney and gut-liver-kidney chips [59, 60]. In the future, more complex and representative *in vitro* human gut-organ axis models (e.g., body-on-a-chip) will be developed and serve as miniature body equivalents, providing a promising alternative to animal models.
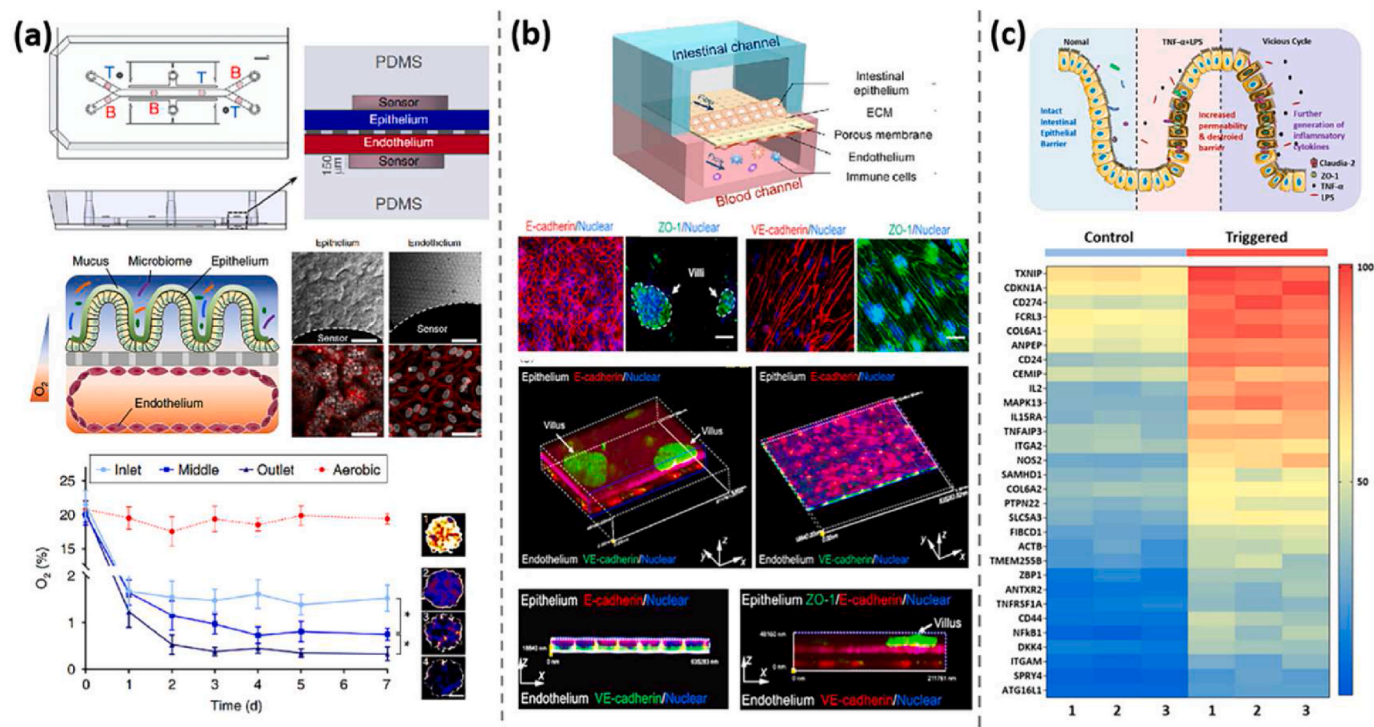
4. Multidisciplinary strategies for engineering intestinal barrier-on-chip platforms

By combining microfluidic chips with multidisciplinary technologies, the real-time monitoring of the intestinal barrier can be achieved, enabling the optimization of the gut barrier model to meet research needs. Several evolving multidisciplinary techniques have been explored to assess organ-specific structure and function, including biosensors, 3D imaging, and multi-omics. In this section, we will introduce the principle, performance, and application of these technologies in gut barrier chips.

4.1. Biosensors

Electrochemical sensors are the most frequently used biosensors in *vitro* gut barrier models. They are embedded within microfluidic chips in a harmless manner and enable the real-time monitoring of oxygen levels and TEER, respectively. Given the high gas permeability of PDMS materials, oxygen sensors can rapidly respond to changes in oxygen concentrations (within 30 s). Ingber et al. strategically placed six sensor spots containing oxygen-quenched fluorescent particles in the inlets, middle regions, and outlets of the dual channels within a gut barrier chip (Fig. 5a) [15]. Changes in oxygen tension could easily be detected based on the changes in fluorescence signal intensities monitored using a fluorescence readout system. The developed microdevice could measure oxygen concentrations as low as 0.3 %, providing a strict anaerobic environment (<0.5 % O$_2$) for obligate anaerobes. In this study, the complex microbiota isolated from human stool specimens could be stably cocultured with the intestinal epithelium-endothelium interface on a microfluidic chip under a physiological transluminal hypoxia gradient. The proportions of *Firmicutes* and *Bacteroidetes* in the system were similar to those in human feces, highlighting the feasibility of this biosensor-based gut chip for complex gut microbiome culture [15]. Azizgolshani et al. could quantitatively evaluate renal active transport and oxygen consumption via integrated oxygen sensors on an array chip [62]. This platform could also integrated electronic sensors for quantifying colon barrier function in real-time. Oxygen biosensor-based gut barrier models not only serve as tools for fundamental research but also as discovery platforms for the development of microbiome-related therapies, probiotics, and nutritional products [15,51].

Electrochemical sensors integrated into the gut chip can also measure TEER in real time, reflecting gut barrier integrity and function [15, 41,158]. Usually, at least two electrodes are patterned and integrated into microfluidic gut chips. The electrical impedance from the readouts quantitatively reflects the integrity of the epithelium-endothelium interface. Nair et al. rapidly evaluated the impedance across the cellular barrier by depositing thin gold on the electrodes, expediently monitoring tumor-tissue barrier interactions [39]. Additionally, the electrode structure may create an uneven field that leads to unreliable impedance data, so sensor materials and sensor configuration are also important factors in accurately evaluating TEER values. Gold and platinum electrodes are the often-used electrode materials in gut chips due to their inertia and good biocompatibility [29,39,176]. Bossink et al. monitored the formation, disruption and recovery of the intestinal barrier by integrating platinum electrodes [29]. However, due to their polarization, gold and platinum electrodes can be affected by high electrode-electrolyte interface impedance, which may not be able to sense small changes in the TEER [177,178]. Silver electrodes are also common due to their non-polarization and low interface impedance [31]. Unfortunately, studies have shown that silver ions are cytotoxic, which is why silver electrodes are unsatisfying for long-term monitoring. At present, semitransparent electrodes and indium tin oxide (ITO) electrodes are widely used in gut chips because of their transparency and high electrical conductivity, which allow visual inspection of cultured cells. Marrero et al. developed semitransparent electrodes by doping semiconductor polymer poly (3,4-ethylenedioxythiophene) with

Fig. 5. Multidisciplinary strategies for engineering intestinal barrier-on-a-chip platforms. (a) O$_2$ biosensors can rapidly respond to changes in oxygen concentrations within the microfluidic system [15]. Copyright 2019, Springer Nature. (b) Real-time imaging can be used to monitor and examine intestinal physiopathology [16]. Copyright 2020, Science China Press. (c) Omics analysis, such as RNA sequencing, can provide information on a range of important genes and potential signaling pathways [17]. Copyright 2023, The Royal Society of Chemistry.

polystyrene sulfonate [26]. The electrode material can cover the entire cell culture area, providing a uniform electric field and filtering out signals below the electrode cutoff frequency. Giampetruzzi et al. introduced ITO electrodes into the gut barrier or real-time analysis of TEER levels [28]. Interestingly, the proposed strategy is microbubbles tolerant during the measurement, thus adapting to long-term cell culture. Therefore, the optimal electrodes should have high sensitivity, stability, minimal polarization, fast response time, and compatibility with the chip device. Currently, electrochemical sensors are regarded as auxiliary tools for the functional analysis of various barriers, including the blood-brain barrier, gut barrier, and gas-liquid interface. In the future, more diversified biosensors (e.g., enzyme sensor, microbial sensor, and immunosensor) will be integrated into gut barrier models to model the *in vivo*-like complex gut ecosystem.

4.2. 3D imaging

Confocal laser scanning microscopes are often used as 3D imaging systems and analytical equipment. Compared to traditional optical microscopes, these state-of-the-art microscopes offer more comprehensive information along the x, y, and z axes. Their key advantages include their high resolution and fast scanning speed. At present, this sophisticated technology is widely used to detect the 3D phenotypes (e.g., microvillus and crypt) and fine outlines (e.g., cytoskeleton and tight junction) of gut barriers. As shown in Fig. 5b, Guo et al. achieve the 3D reconstruction of a polarized intestinal barrier, with the intact distribution of junction proteins and villus-like structures, via a full-sized confocal scan [16]. 3D imaging enabled the clear visualization of whole and large-scale Caco-2 tubules and short and long cytokine-triggered barrier injury, including fragmented E-cadherin morphology with short major axes and weak compactness [179]. Stresser's team found that tight junction protein ZO-1 localized on the villi structures of the epithelial cells [156]. The intestinal alkaline phosphatase signal was co-localized with the brush-border membrane

protein villin. Such 3D confocal images also could identify the colocalization of Bifidobacterium bifidum with tight junction proteins [17]. Lim's group observed that tumorigenic *Bacteroides fragilis* (ETBF) disrupted disruption of the 3D intestinal barrier and initiation of colorectal tumor signals [180]. The pre-colonization of beneficial intestinal microbe Lactobacillus could effectively diminish the colonization of ETBF, prevent ETBF-mediated pathogenic behaviors, and maintain the healthy state of the intestinal barrier.

4.3. Multi-omics

Omics is a global approach for studying biological processes in living organisms and involves comprehensive explorations of organisms, cells, and tissues from a holistic perspective. Multi-omics includes transcriptomics, metabolomics, and proteomics, which involve investigations at the genetic, molecular, and protein levels, respectively. Among of them, the transcriptome is the necessary link of biological function between genetic information and the proteome. Transcriptional regulation is the most studied and the most important mode of regulation in the gut barrier. For instance, RNA sequencing can be used to uncover a range of important genes and potential signaling pathways, enabling researchers to more comprehensively explore biological information in the context of gene sets. Tian and coworkers treated an intestinal epithelial monolayer with LPS and TNF-α to simulate IBD in a gut chip (Fig. 5c) [17]. The heatmap from RNA sequencing revealed prominent variations in key genes involved in inflammatory signaling, immune responses, and autophagy. KEGG pathway enrichment analysis revealed that the toll-like receptor pathway was activated and overexpressed in the pathological model. Beaurivage et al. compared gene expression differences in an organoid-derived gut model using a microfluidic chip with a traditional static culture. Interestingly, the identified genes were involved in intestinal digestion, transport, and metabolism and included those encoding intestinal absorptive enzymes (SI and ALPI) [181].

Microorganisms mainly perform catabolism and have very rich biological activity. Earlier, the identities of intestinal symbiotic bacteria were confirmed using Sanger sequencing [51]. Biological information at a species level (e.g., metabolic groups and genetic background) enables a better understanding of microbial diversity. Thus, 16S rRNA gene sequencing has become an important means to study the composition and distribution of microbial communities in gut barrier systems [15]. Metagenomic sequencing from animal trials remains the primary strategy for inferring systemic communication between the human gut and microorganisms [7]. The metabolon global metabolomics platform has been used to measure biochemicals in cell and media samples [52,182]. For instance, using untargeted metabolomic analysis, Duan et al. found 364 metabolites dysregulated in liver cells following $PM_{2.5}$ exposure [183]. Additionally, classical label-free analytical methods, such as liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy, are also exceptionally sensitive off-chip detection techniques capable of simultaneously assessing numerous analytes from limited sample quantities. Zhang et al. detected the fermentation products of *Faecalibacterium prausnitzii*, including short chain fatty acids, butyrate, propionate, and acetate, using LC-MS [51]. Martinez et al. revealed the composition and content of short-chain fatty acids in gut chip systems using ^{1}H NMR spectroscopy, requiring only a small amount of media [184]. Indeed, the development of multi-omics provides a more comprehensive perspective to gain deeper insights into pathophysiology. In the future, multi-omics datasets from immune cells and all cultured cells in the chip system could be evaluated. Meanwhile, machine learning and bioinformatics may be applied to organize and analyze multi-omics databases.

4.4. Artificial intelligence

In recent years, artificial intelligence (AI) has gained significant attention within the field of biomedicine due to its remarkable learning capabilities and high accuracy in applications such as drug discovery and disease diagnosis. It enables the exploration of human-gut microbiome datasets to identify functional molecules, drugs and microbiome-based therapeutics [183,185]. For instance, Wang and colleagues applied a technique called "Long Short-term Memory, Bidirectional Encoder Representations from Transformers and Attention" to generate a comprehensive pipeline for identifying candidate antimicrobial peptides from human gut microbiome data. The antibacterial activity of 181 candidate antimicrobial peptides was higher than 83 % [186]. In addition, dysbiosis or invasion by exogenous pathogens can induce a variety of diseases, especially gastrointestinal diseases. Hence, disease diagnosis and prediction can be markedly improved by detecting the changes in microbial profiles in affected individuals [187]. However, understanding the extensive biological information associated with gut microorganisms is a formidable challenge. In this context, AI offers a promising tool for analyzing large-volumes of data related to microorganisms, enabling disease classification and status determination [188].

Notably, machine learning has been used to identify specific biomarkers of enteric diseases (e.g., colorectal cancer and Crohn's disease) based on global explanation methods [189,190]. For instance, Rynazal et al. employed explainable AI for gut microbiome-based colorectal cancer classification, successfully classifying patients into subgroups with distinct bacterial biomarkers and probabilities of colorectal cancer [190]. Additionally, clinical data can also be mined to build auxiliary clinical models for improving the analysis as well as diagnosis, differentiation, prognosis and outcome of diseases [191]. Although research combining AI technology with gut chips is quite limited, this field shows immense potential for advancements. The ultimate goal of integrating these interdisciplinary technologies is to enable accurate disease diagnosis and treatment and provide efficient and non-invasive strategies for clinical decision making.

5. Indicators used to evaluate microfluidic intestinal barrier models

Once microfluidic-based gut barrier models have been established, appropriate detection tools with sufficient sensitivity are needed to identify the topographic characteristics of barriers as well as the absorptive efficiency, transport ability, and metabolism of the target compounds across the barrier.

5.1. Morphological identification

A polarized villi-crypt architecture and mucus distribution are representative morphological characteristics of the gut barrier. In the following section, we will highlight evaluation methods for these morphology indexes and the related progress in research.

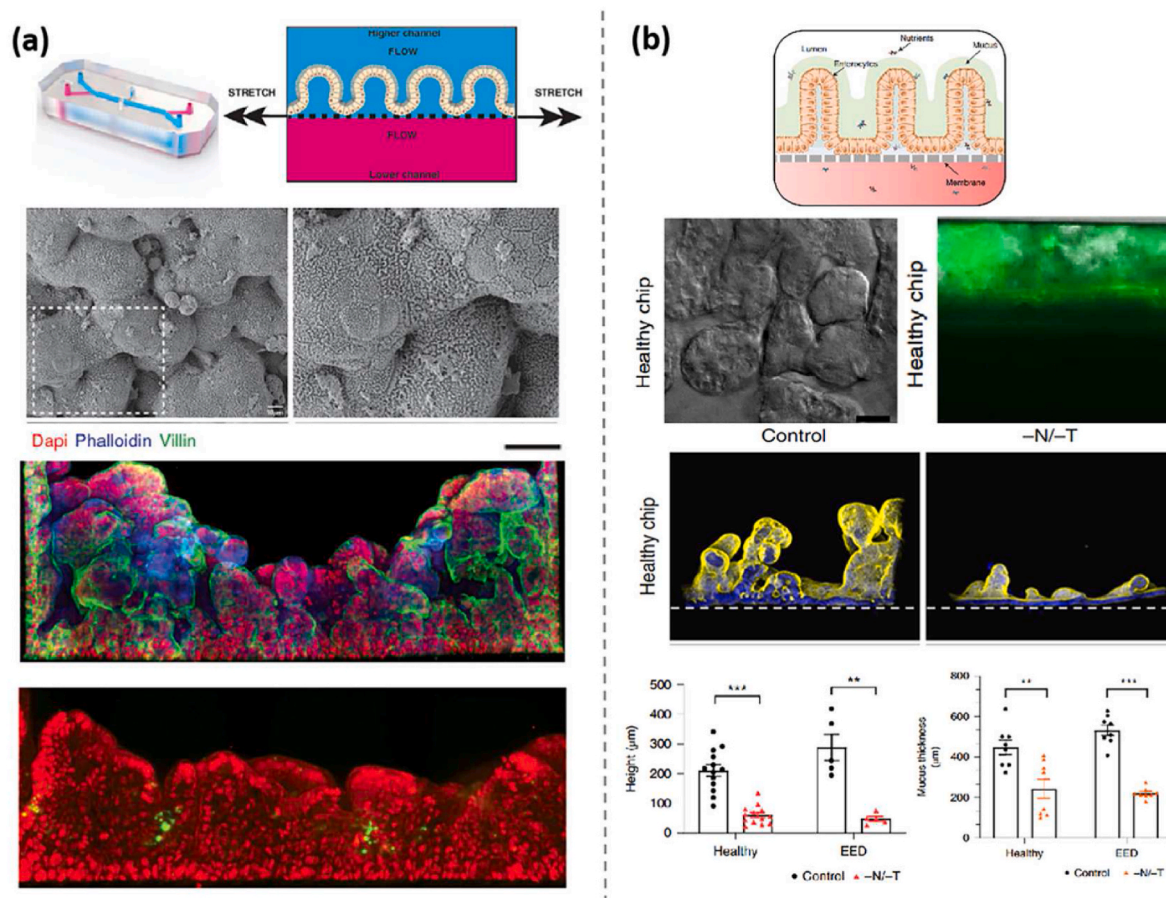#### 5.1.1. Polarized villi-crypt architecture

In vivo, adjacent microvilli together with the basal proliferative crypts form a polarized crypt-villus axis. Bioengineered microfluidic chips are effective at mimicking the human gut *in vitro* [12,38]. When human intestinal Caco-2 cells are cultured in microfluidic gut chip devices under controlled microenvironment conditions such as bioflow and peristaltic movement, they spontaneously regenerate into intestinal villi containing basal proliferative crypts, differentiate into multiple intestinal cell lineages, and recreate several hallmarks of small intestine physiology [19]. Basal proliferative crypts can restore this polarized morphology through a regenerative process, reconstructing themselves and replenishing the villi along the crypt-villus axis [19]. Notably, proliferative cells can migrate 50 μm upwards along the villi from the anchoring substrate within 20 h. In addition, using representative SEM images, Grassart et al. found that about 70 % of *Shigella* cells are enriched and located within crypt-like invaginations (Fig. 6a) [37]. To evaluate the generation of gut epithelium by Caco-2 cells, the nucleus, cytoskeleton, and intestinal villi of the gut were co-stained *in situ* with DAPI, Phalloidin, and Villin, respectively. Subsequently, a basal crypt-like architecture between adjacent villi and folding morphology was clearly observed [37]. The patient-derived monolayers displayed 3D epithelial differentiation, villi-crypt morphology and the expression of disease-specific characteristics (e.g., CEA and CD133) [155]. Overall, quantitative and qualitative results can be used to identify the polarized villi-crypt architecture, and microscopic observations can reveal finer cellular behaviors.

5.1.2. Mucus distribution

In vitro, gut barrier chips can support intestinal cell differentiation and mucus bilayer accumulation and produce *in vivo*-like mucus thickness [53]. The mucosal layer serves as a significant barrier during drug delivery. For instance, increasing the intestinal mucosal adhesion of oral drugs can increase drug absorption and permeability and thus boost therapeutic effects [192]. As shown in Fig. 6b, in the presence of moderate nicotinamide and tryptophan deficiency, the thickness of the mucus layer covering the epithelium and the height of the villus-like structures in gut barrier models can decrease by about 1-fold and 3-fold, respectively, under both healthy and disease conditions [52]. In addition, the formation of intestinal mucus in the chip can also be detected using specific dyes. For instance, Zhao and coworkers directly stained and visualized carbohydrates such as glycocalyx and acidic mucopolysaccharides within the mucus using wheat germ agglutinin and alcian blue staining, respectively [42]. In general, consistent quantitative and qualitative data can better reflect the structural characteristics of an intestinal microphysiological system.

5.2. Barrier integrity detection

An intact gut barrier can maintain the microenvironment of the intestinal lumen and prevent invasion by foreign pathogens. Therefore,

Fig. 6. Indicators used for evaluating the morphological structure and mucus secretion in microfluidic intestinal barrier models. (a) A polarized villi-crypt architecture is one of the representative morphological indexes of microfluidic intestinal barrier models [37]. Copyright 2019, Elsevier Inc. (b) In the presence of moderate nicotinamide and tryptophan deficiency, the thickness of the mucus layer covering the epithelium is observably decreased in both healthy and intestinal disorder models [52]. Copyright 2022, Springer Nature.

accurately assessing gut barrier integrity, evaluating pathogen-induced injury, and studying the therapeutic effects of probiotics are meaningful for shedding light on human health and disease. The three common indicators for real-time, noninvasive assessment of gut barrier integrity are tight junction proteins, TEER, and permeability.
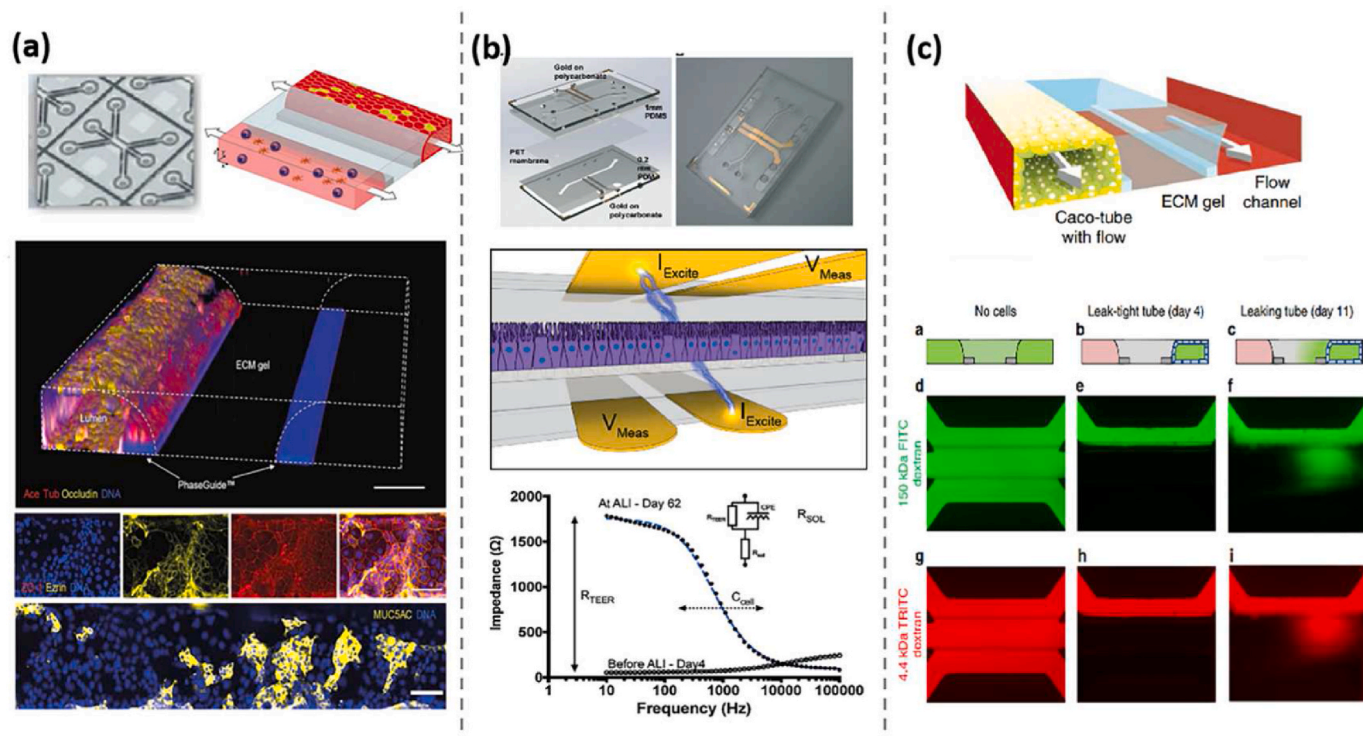
5.2.1. Tight junction

Tight junctions are formed by interacting peripheral proteins and transmembrane proteins. They are the primary structures connecting adjacent epithelial cells and play a vital role in maintaining intestine-specific cell polarity. In the gut barrier, the tight junction protein zonula occludens 1 and the transmembrane protein occludin constitute the core of the tight junction. Gijzen et al. assessed the intestinal tube based on *in situ* staining for occludin, ezrin, and ZO-1 localized at junctional sites, which serve as functional fences (Fig. 7a) [44]. Acetylated tubulin and mucin 5AC showed clear local expression in the coculture tubules. Moreover, specific epithelial and endothelial junction markers such as E-cadherin, VE-cadherin, F-action, and G-actin, were often identified in the gut barrier models [137,181]. Cytochrome P450, which regulates drug metabolism, was also stably expressed in the intestinal epithelial monolayer [38,169]. In addition to immunostaining, real-time quantitative PCR, bead-based immunoassays, and western blotting can be performed to analyze the expression levels of junction markers [48,50]. Therefore, appropriate strategies for characterizing the tight junctions of the gut barrier should be selected based on experimental requirements.

5.2.2. TEER measurement

Transepithelial electrical resistance (TEER) serves as a reliable

indicator reflecting the degree of differentiation and integrity due to its strong correlation with the electrical impedance across the epithelium-endothelium interface and the tight junctions connecting neighboring cells. Ohm's law method and electrochemical impedance spectroscopy (EIS) are two main methods used to determine the TEER values of on-chip gut barriers. They all rely on the same principle: apply a current signal to the barrier and then measure the resulting voltage drop [176]. The Ohm's law method has advantages that only a single frequency needs to be evaluated, and no data fitting is required to obtain the TEER value [193]. However, Ohm's law method cannot collect information about the battery layer capacitance contained in the spectrum. In contrast, EIS is a non-invasive technique used to measure the impedance of a cell layer at different frequencies [194]. It applies a small alternating current voltage across the cell layer and measures the resulting current to obtain the impedance spectrum. EIS can obtain not only TEER, but also capacitance-related information of the cell layer [30]. Through equivalent circuit analysis of the measured impedance spectrum, electrical parameters can be obtained to characterize the mechanical properties of the cell barrier. For instance, Fig. 7b illustrates the design by Ingber et al. featuring a two-channel organ chip enabling real-time TEER detection, applicable to any monolayer barrier established within the compact chip [41]. Maoz's team incorporated mobile electrodes on a gut chip to dynamically monitor the growth dynamics of intestinal cells in real-time throughout the channel based on the EIS [27]. Van der Helm et al. found that the formation of villi led to a decrease in epithelial resistance and an increase in capacitance by performing electrical simulations [30]. Indeed, full impedance spectroscopy can provide a straightforward quality control tool for assessing

Fig. 7. Indicators used for evaluating barrier integrity. (a) Tight junction proteins localized at junctional sites, serving as functional fences [44]. Copyright 2020, Society for Laboratory Automation and Screening. (b) Two-channel chips enable the real-time detection of TEER across the monolayer gut barrier [41]. Copyright 2017, The Royal Society of Chemistry. (c) The permeability of unknown or candidate compounds throughout the gut barrier can be explored based on the diffusivity of fluorescent tracers [34]. Copyright 2017, Springer Nature.

epithelial growth and differentiation, and also reflect the destructive response of harmful substances to the integrity of the intestinal barrier [27,41,58]. However, it is worth noting that TEER values often exhibit significant measurement variability influenced by factors such as cell type heterogeneity, cell density, medium composition, and electrode positioning [3,145,195]. For example, gut barriers derived from human induced pluripotent stem cells demonstrated TEER levels within the physiological range (40–100 Ω cm^2), akin to primary intestinal tissues *in vivo* [196]. In contrast, a Caco-2 derived gut barrier on a chip exhibited considerably higher TEER values (4000 Ω cm^2) after 3 days of inoculation [47]. Furthermore, microfluidic-based gut barriers exhibited substantially elevated TEER levels compared to conventional Transwell cultures, often exceeding threefold. Collectively, TEER values across existing gut barrier chip models span a range from 20 Ω cm^2 to 4000 Ω cm^2, underscoring the necessity of considering multiple indicators for assessing overall system viability [47,50].

5.2.3. Permeability

The fundamental purpose of establishing *in vitro* gut barriers is to investigate the permeability of unknown or candidate compounds. Permeability is a key evaluation metric for organ-specific barriers and is primarily reliant on the diffusion characteristics of tracers. Tracers, such as fluorescent or electroactive probes, are introduced into the targeted channel of the gut chip and subsequently examined in the opposing channel.

Fluorescent tracer-based permeability assays are very commonly used. Trietsch et al. evaluated barrier permeability by introducing a culture medium infused with fluorescent probes into the Caco-2 lumen (Fig. 7c) [34]. An intact and dense gut barrier effectively prevented the leakage of the fluorescent dye. However, drug-induced barrier damage allowed the partial transport of FITC-dextran toward the basal side. Notably, staurosporine-induced loss of barrier integrity was observed in a concentration-dependent manner. Furthermore, gut barrier integrity

can also be quantitatively examined based on the fluorescence intensity of fluorescent probes on the opposite side. Kimura et al. monitored the polarized transport activity of the intestinal barrier by online fluorescent measurement [105]. As diffusion capacity is influenced by various factors, including the properties (e.g., molecular weight, polarity, and concentration) of tracer agents and inherent barrier features (e.g., tightness and saturation), understanding permeability for distinct molecules is crucial. Common fluorescent tracers employed for evaluating gut barrier functionality include dextran (4–150 kDa), Lucifer Yellow (0.45 kDa), and cascade blue (5.9 kDa) [15,42,49,50] Moreover, Naumovska et al. found that the intestinal monolayer shows different permeability coefficients across identical leak-tight barriers for 150 kDa Dextran (4.03×10^{-6} cm/s) and 4.4 kDa Dextran (7.12×10^{-6} cm/s) [50].

Another promising avenue for microfluidic gut barrier permeability analysis involves electrochemical detection. Currently utilized for evaluating microvessel permeability and assessing the transendothelial migration dynamics of cancer cells, this method offers advantages over fluorescence-based detection [197–199]. That is, it allows manual sampling, optical instrumentation, and offline sample processing. In electrochemical assays, efficient electron transfer, bioinertness, nontoxicity, repeatability, and stability are vital criteria for selecting electroactive tracers. Wong et al. extensively evaluated three potential electroactive species (MB, RuHex, and FCN) for the on-chip electrochemical measurement of cell monolayer permeability, considering factors such as diffusive transport, cytotoxicity, and current stability [200]. RuHex emerged as the optimal tracer owing to its high electron transfer efficiency on the integrated electrode, robust electrochemical responses within the 0–200 μM range, and minimal impact on cell viability [200]. In the future, electrochemical detection could be instrumental in the integrity analysis of gut barriers, offering a critical metric for gut barrier-on-a-chip models.

6. Conclusions and outlook

Microfluidic gut barrier-on-a-chip models are emerging paradigms for *in vitro* human organ simulation, displaying considerable potential in recapitulating the intricate structural and functional complexity of human gut barriers. The central components of microfluidic gut barrier bioengineering include the configuration of the intestinal epithelium and vascular compartments, as well as the integration of extracellular microenvironmental factors (e.g., peristalsis, bioflow, and the gut-organ axis). The performance of these microchips has been significantly enhanced through the integration of multidisciplinary approaches, including biosensors, 3D imaging, and multi-omics techniques. These have enabled the *in situ* monitoring of system status, real-time measurement of oxygen and TEER levels, and the identification of junction proteins and key biomarkers. Evaluating the suitability of *in vitro* microfluidic gut barrier models is an important consideration for their subsequent application. This review provides a comprehensive guide for the design and functional validation of microfluidic-based gut barriers.

A number of research groups have developed the construction parameters and performance indicators of on-chip gut barrier biosystems. There are still limitations to overcome. It can be improved from two aspects: the model establishment and performance evaluation. In terms of model establishment, we can improve its performance from the perspective of increasing fidelity and authenticity. First, emerging stem cell engineering and patient-derived organoids can provide resources for obtaining abundant intestinal cell components, including Paneth cells, intestinal cells, and neuroendocrine cells [48,50,52,56]. Second, there are abundant flora (aerobic bacteria, anaerobic bacteria and facultative bacteria) in the human intestine, which regulate metabolism and immune response, and maintain intestinal homeostasis [7]. Admittedly, some studies have reproduced intestinal barrier dysfunction, stromal reshaping and probiotics translocation under inflammation induced by harmful bacteria [2]. However, they only highlighted the influence of a single factor. In my opinion, the collective behavior of complex microbial communities to intestinal homeostasis should be explored, and even the contributions of specific cytokines and mechanical movements should be synergically incorporated. Third, the matrix is necessary to reshape the *in vivo*-like microenvironment [201]. Biomaterials especially in hydrogels play an important role in guiding intestinal organogenesis. Fourth, multi-organ interactions should also be taken into account to more realistically simulate the effects of drugs *in vivo* on downstream organs after intestinal absorption and microbial metabolism. Based on the modular gut-liver, gut-kidney and gut-brain organ axis chips established earlier [57,59], in the future, it is an inevitable trend to integrate all organ systems of the human body on a mini-chip to systematically reproduce whole-body physiological responses *in vitro*. In a word, more abundant cell components (e.g., intestinal organoids, fibroblasts, smooth muscle cells, nervous cells and organ-organ axis) and more biomimetic microenvironment elements (e.g., peristalsis and matrix), and more realistic microbiome-derived from human samples (e.g., blood, stool, and tissue biopsies) should be incorporated into the gut barrier models. Finally, high variability in terms of cell compositions, structures and phenotypes limits the transition of intestinal engineering from basic research to clinical trials. The development of standard procedures for *in vitro* intestinal models will reduce the poor reproducibility of experimental results due to different protocols and enable the comparison of differences between different systems.

In terms of performance evaluation, some efforts need to be made in the following directions. Due to the high integration of the microfluidic chip, electrochemical sensors have been *in situ* integrated into the intestinal chip to observe transmembrane resistance values and oxygen levels online in real-time [107,202]. In my opinion, more advanced biosensors such as enzyme sensors, microbial sensors, and immune sensors should be integrated to detect gut-specific enzymes, microbes, markers and metabolites. In addition, the intersection of multiple disciplinary technologies can promote the comprehensive analysis of the on-chip gut biosystems. It is worth noting that artificial intelligence (AI) has great application potential in efficient multi-omics data analysis and microbiome big data mining through digital networks [203]. On the one hand, AI can also be used to evaluate the success of preset models through computer simulations and provide reliable genotypic and phenotypic data for specific diseases [204]. On the other hand, by detecting the composition and abundance of intestinal microbiota, it can realize the diagnosis and prediction of diseases, and help to improve the occurrence process of diseases [205]. Certainly, developing a fully automated read-and-analysis setup is needed. In the future, online AI-enabled monitoring modules, real-time physical, chemical and biological sensors can be integrated into the gut chip. Besides, *in vitro* on-chip test results need to be standardized and compared with *in vivo* data. Even though *in vivo* human data is difficult to obtain, animal data can complement the *in vitro* models. Hence, data from animal models should not be completely ignored. In the future, the improvement of technologies is expected to greatly advance and widen the application of *in vitro* microfluidic gut barrier models in academic research, the biomedical industrial sector, and clinical centers.

CRediT authorship contribution statement

Hui Wang: Writing – original draft, Investigation, Funding acquisition, Conceptualization. **Xiangyang Li:** Investigation, Data curation. **Pengcheng Shi:** Investigation, Data curation. **Xiaoyan You:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Guoping Zhao:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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.

Data availability

I have presented all the data in the manuscript.

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