Skin-on-a-Chip Microfluidic Devices: Production, Verification, and Uses in Cosmetic Toxicology

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Abstract

It is increasingly recognized that the use of 2D cell culture assays to model keratinocyte and fibroblast responses to cosmetic products is being rapidly modified and updated. While these probes provide a time-efficient, simple, and cost-effective model of the skin, they have been repeatedly shown not to fully incorporate the in vivo cutaneous environment. The European Union has already implemented legislation that prevents the sale of cosmetic products developed through animal testing. This highlights the urgent need for novel, reproducible, cost-effective, and mass-producible model systems that can offer a comparable resource for cosmetic testing. While this need was initially attempted to be met through the development of 3D models of the skin, these systems have been found to lack the more complex biochemical and biophysical properties present in vivo. Thus, considerable interest has been shown in the development and optimization of "organ-on-a-chip" technology to produce a reproducible and ethically isolated model system of the skin. The following chapter will explain how to produce a skin-on-a-chip microfluidic device, the various factors that must be considered in its design and production, the methodology required to verify its reproduction of the in vivo cutaneous environment, and its applications in cosmetic toxicology.

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4.1 Introduction

It is increasingly accepted that the use of 2D cell culture assays to model keratinocyte and fibroblast responses to cosmetic products needs to be revised and updated. While these assays provide a time-efficient, simple, and cost-effective model of the skin, they have been repeatedly shown to not fully encompass the full in vivo cutaneous environment (Duval et al. 2017). This issue of true representation has traditionally been resolved through the use of animal testing (Ngo and Maibach 2010). However, the growing movement in scientific and political fields to limit the use of animals in cosmetic product development due to ethical concerns may prevent the future use of animals as representative skin models. The European Union has already implemented legislation that prevents the sale of cosmetic products developed through animal testing (Adler et al. 2011; Hartung and Rovida 2009). This highlights the urgent need for novel, reproducible, cost-effective, and mass-producible model systems that can offer a comparable resource for cosmetic testing. As such, a significant effort has been made in recent decades to develop and improve in vitro systems to encompass all aspects of in vivo skin biology.

While this need was initially attempted to be met through the development of 3D models of the skin (Carlson et al. 2008; Antoni et al. 2015), which typically use commercially available biologically inert plastic scaffolds or ECM-derived hydrogels to produce a multicellular and multilayered model system of the skin, these have also been found to lack the more complex biochemical and biophysical properties present in vivo *(*Gangatirkar et al. 2007*;* El Ghalbzouri et al. 2009*)*. As such, considerable interest has been shown in the field for the development and optimization of "organ-on-a-chip" technology to produce a reproducible and ethically uncompromised model system of the skin.

Organ-on-a-chip technology originally emerged from material and computer research during the 1980s, which built upon advancements in micro-electric–mechanical systems (MEMS), a technology used in producing semiconductor chips and other electrical components that are only microns in size (Azizipour et al. 2020). Following this advancement, biomaterialists began to develop MEMS that incorporated biological material for potential use in multiple areas of life science research, including drug development and toxicology (Grayson et al. 2004). These biological MEMS (bioMEMS) are ultimately microfluidic devices, which are a type of device designed to allow for the precise control of extremely low volumes of liquid within microscopically small cell culture environments (Wu et al. 2020). This level of control allows for a new level of complexity to be introduced to tissue models previously lacking in all other systems. For example, these bioMEMS allow closer modeling of the in vivo tissue environment by mimicking the supply of nutrients and immune cells to tissues via a specifically designed and machined

mock–vascular network (Vargas et al. 2021). They also allow for the establishment of biological molecule gradients within tissues that give broader context to the model and allow more significant distinction in tissue-to-tissue interfaces (Bhatia and Ingber 2014). This advancement in fluid control, combined with the previously developed tissue-specific cell culture techniques, allowed the first "organ-on-a-chip" devices to be produced.

The first reports of the successful implementation of organ-on-a-chip technology for skin modeling, or "skin-on-a-chip," occurred in 2013. Since this founding work was published, there has been a significant advancement in this technology (Ataç et al. 2013; Wagner et al. 2013).

The following chapter will not attempt to explain this history of skin-on-a-chip development but will instead aim to explain how to produce a skin-on-a-chip microfluidic device. This will be achieved in three separate sections. The first (Sect. 4.2) will explain the various factors that must be considered in the fabrication and production of the microfluidic device itself. The second (Sect. 4.3) will explain the methodology that needs to be employed to verify that the device is able to fully capture the in vivo cutaneous environment, and the final section (Sect. 4.4) will explain how these skin-on-a-chip devices can actually be utilized in the field of cosmetic toxicology.

4.2 Production of Skin-on-a-Chip Devices

4.2.1 Sourcing and Production of Skin Models for Skin-on-a-Chip Devices

When developing a skin-on-a-chip microfluidic device, for the purpose of toxicity and cosmetic testing, there are many factors that need to be considered. However, the central and most vital factor to consider is how to source or produce the physiologically relevant skin model housed within the device itself. When considering the best skin model system for a given device, its intended use, access to patient tissue or cells, and the need for incorporating other vital components, such as those needed for its maintenance and monitoring, all must be considered (Zoio and Oliva 2022).

Typically, the skin models housed within skin-on-a-chip devices have two separate sources. The first is human skin biopsies, which are extracted from clinical patients and transferred directly into the housing units of the device (Risueño et al. 2021). Access to this source of material does pose a challenge, given the clinical training required to remove the sample and the patient/ethical permissions that need to be attained prior to removal. As such, humanized full-thickness skin equivalents have been increasingly seen as a viable source of the material. These skin equivalents are produced using a specific cell culture technique that can accurately replicate the three-dimensional structures of skin (Hill et al. 2015). These humanized skin equivalents can either be produced externally, then biopsied, and inserted into the microfluidic device, or they can be generated de novo within the skin-on-a-device itself. To date, multiple tissue culture protocols have been developed and

optimized to incorporate multiple cell types from various biological sources into humanized skin equivalent models.

Conventional full-thickness skin equivalent models are generally constructed using primary human cutaneous cells, such as keratinocytes and dermal fibroblasts, isolated from healthy human skin biopsies, as the use of these cells will capture an in vivo phenotype exceptionally well (Zoio and Oliva 2022; van den Broek et al. 2017). Previous studies have successfully demonstrated that this protocol can also be used to generate 3D psoriatic skin models using patient-derived cells (Rioux et al. 2021). Additional work has also shown that by incorporating activated T cells, isolated from whole human blood, into the skin model, they were able to create an immunocompetent model that can reflect the psoriatic inflammatory environment more precisely, thereby creating a suitable model that could be used for both fundamental and translational research studies (Rioux et al. 2021). Overall, using primary cells presents a unique set of advantages, such as capturing the in vivo phenotype to ensure cell–cell communication within the skin-on-a-chip device is consistent with the in vivo environment; however, there are still disadvantages associated with using primary cells. Some main disadvantages include the limited availability of donor skin and donor variation, which may affect experimental reproducibility. Also, given that the extraction, growth, and maintenance of primary cells require more specialized culture techniques than immortalized cell culture, laboratories with little experience in primary cell culture may find it harder to integrate primary cells into the design of their skin-on-a-chip devices.

An alternative to using primary cells in the construction of humanized skin equivalents is immortalized cell lines. Cell lines with validated purity and viability have significant advantages due to their high availability and reliability for cell population expansion (Zoio and Oliva 2022). However, cell lines are only approximations of primary cell function and can deviate from the original phenotype. This can be observed in the widely used keratinocyte cell line, HaCaT. While HaCaT cells can be used to form epidermal tissue, they have a low differentiation potential compared to primary keratinocytes, making generating a functional *stratum corneum* challenging. This highlights the potential drawbacks of incorporating immortalized cell lines into the skin model retained within the skin-on-a-chip device, which may be used to assess the specific toxicological effects of a given compound on skin barrier function (Brohem et al. 2011; Jung et al. 2016).

Another cell line that could be considered is human telomerase reverse transcriptase (hTERT)-immortalized keratinocyte and dermal fibroblast cell lines. Previous studies have demonstrated that hTERT-immortalized keratinocytes and dermal fibroblasts could form a high-quality full-thickness skin equivalent with a fully differentiated epidermis, comparable to that formed when using primary keratinocytes (Reijnders et al. 2015). As these hTERT-immortalized cell lines are both reliably expansive and able to generate a fully stratified epidermis, 3D skin models generated using these cells should be considered when designing skin-on-a-chip devices needed for higher throughput screening of new drugs and cosmetics. However, depending on the intended use of the skin-on-a-chip model, donor variation can be an essential feature to accurately resemble the population (Zoio and Oliva

2022). Cell lines lack patient specificity, which is particularly important for disease modeling. Furthermore, previous studies have suggested that some proteins overexpressed in cell lines are associated with toxicity-related pathways and, therefore, limit the use of these cell lines in 3D skin models for toxicity testing (Astashkina et al. 2012).

Another promising cell source for skin-on-a-chip is induced pluripotent stem cells (iPSCs). These cells are derived from adult somatic cells via reprogramming with octamer-binding transcription factor 3/4 (Oct 3/4), SRY (sex-determining region Y)-box 2 (Sox2), c-Myc, and Kruppel-like factor 4 (Klf4) expression factors (Rowe and Daley 2019). By expressing these factors, genes responsible for cell differentiation are suppressed, and the cells revert to a pluripotent state. Using iPSCs to construct 3D skin models could overcome the limitations of full-thickness skin models that are only comprised of keratinocytes, fibroblasts, and endothelial cells, as they have unlimited growth potential and the ability to differentiate into multiple cell types. Previous studies have demonstrated that iPSC-derived keratinocytes, dermal fibroblasts, and melanocytes generated a full-thickness skin equivalent that showed similar morphology and physiology to normal human skin (Gledhill et al. 2015). This model also reported efficient melanin production and transfer within epidermal–melanin units of the iPSC-derived skin equivalent, thereby demonstrating the potential to generate increased complexity in the model system, thus better mimicking normal skin function and physiology (Gledhill et al. 2015). Furthermore, cells that have been differentiated from iPSCs retain characteristics of the original donor, such as disease phenotypes and therapeutic response, thereby offering an alternative source of cells for modeling cutaneous diseases (Khurana et al. 2021). However, many factors still need to be considered before iPSC-derived skin-on-a-chip models can be viable, such as high cost, retention of epigenetic memory, and genomic instability (Zoio and Oliva 2022).

Overall, each cell type and skin model has its own set of advantages and disadvantages that need to be carefully considered when producing a physiologically relevant skin-on-a-chip model, especially when designing a skin-on-a-chip model for a specific downstream application such as toxicology and cosmetic testing.

4.2.2 Fabrication Methodologies of the Housing Units of Skin-on-a-Chip Devices

A central consideration when producing skin-on-a-chip devices for cosmetic toxicology is the design and fabrication of the housing units that hold all the components, producing the microfluidic system and device.

While these housing units can be produced through a number of different methods, all must contain several critical elements to ensure primary function. The first element is a central space capable of housing a functional skin model. As explained previously, this skin model will typically be either an extracted skin biopsy from either native skin or an externally produced humanized skin equivalent or a de novo generated skin equivalent produced within the device. The second

required element is a complete microfluidic system, which must either mimic or allow for the production of a vasculature that supplies the skin model with all required nutrition through the perfusion of cell culture medium. This system must also be accessible to replace the cell culture medium after nutrition depletion.

In addition to these required elements, the housing unit may also contain space for integrating a wide range of other potential components. This may include space for the installation of pumps to allow for powered perfusion of the cell culture medium, biosensors to capture an output of interest, or additional organ housing spaces, to allow for multi-organ testing within the same microfluidic device. This section will briefly describe the most common production methods used in building skin-on-a-chip microfluidic devices and examine some of the more complex and advanced methods.

The most common production method in fabricating microfluidic housing units is lithography, specifically a combination of two separate lithographic processes named "photolithography" and "soft lithography." In short, these processes begin with pouring a light-sensitive material onto a silicon-based support block. When this light-sensitive material, also named a "photoresist," is exposed to UV light, it will liquefy, allowing it to be removed. This property allows the photoresist material to be shaped into the desired design. Once this photoresist has been poured onto the support block and allowed to solidify, this material is overlapped with the housing unit design. This design is printed or etched onto a "photomask," which either blocks or allows light penetration into the photoresist material. Areas exposed to UV light by the photomask will be liquefied and removed, while any area blocked from UV penetration will remain solid. This allows the two-dimensional design on the photomask to become a three-dimensional solid object made by the photoresist. The solid remains of the photoresist material can either act as a mold, casting the desired shape when a desired material is poured into it, or it can be used as a stamp to imprint the design on other materials (Duffy et al. 1998; Lee et al. 2017). While this is a standard production method for the housing units of skin-on-a-chip devices, the number, complexity, design, and arrangement of these housing units themselves can produce unique devices with different uses and different levels of in vivo representation. It is important to note that the descriptions in this section are generalized and can be deviated from based on the required use.

The most common use of this lithography-based technique in skin-on-a-chip production is named membrane-based soft lithography. In this technique, photolithography and soft lithography are initially used to produce two separate housing units, an apical and a basal plate. While many materials can be used, which will be discussed later, they are typically cast using the elastomer material polydimethylsiloxane (PDMS) (Lee et al. 2017; Maschmeyer et al. 2015; Song et al. 2017a, b).

The apical housing plate is typically designed to have a hole in the center that spans its entire thickness. This central hole allows for the placement and support of either the externally sourced skin model or will act as the cell culture chamber itself for de novo skin equivalent generation. In addition to this structure, the upper plate will commonly have additional holes that allow access to the microfluidic system,

which will be housed in the basal plate. These can either be large holes at opposing ends of the plate, allowing access to cell culture medium reservoirs, which are also present in the basal plate, or smaller holes, which act as inlets and outlets for an external perfusion system. The cell culture medium reservoirs store the cell culture medium, which ultimately perfuses the microfluidic device and supports the skin model. This access in the upper plate has to be present to allow for the easy replacement of the medium after sufficient culture time and nutrient use.

The basal plate is typically more complex, as it must contain the entire microfluidic system within it. As previously mentioned, this system is typically comprised of two cell culture medium reservoirs, placed at opposite ends of the basal plate, which are connected to a central hole, which is aligned with the apical plate hole, and connected through either a single or a series of microfluidic channels. This allows cell culture medium to be perfused through the microfluidic system through either the use of pumps or gravity. In some designs, the medium reservoirs are only present in the apical plate, and the basal plate connects these to the microfluidic system (; Song et al. 2017a, b).

The entire housing unit can be assembled when both plates have been produced. Initially, the bottom plate is typically adhered to a glass slide through low-pressure plasma oxidization, which irreversibly seals these plates together, producing a fluid-tight seal. Following this, a porous membrane is fused to the top of the basal plate, using either glue or low-pressure plasma oxidation. This membrane must span the central cell culture hole present in both plates but be prevented from spanning the cell culture medium reservoirs. This porous membrane supports the external skin model or acts as a base for the construction of de novo skin equivalents. The membrane also allows for the transmission of nutrients to whichever functional skin model is used in the upper central cavity. It also prevents cell exposure to the direct flow of the medium solution, which can be damaging. Finally, the upper plate can be fixed to this structure to complete the arrangement.

This form of two-plate membrane-based soft lithography has been expanded on by adding a third PDMS plate into the structure (Wufuer et al. 2016; Jeon et al. 2020a). This additional middle plate, inserted between the apical and basal plates, typically has a central hole that spans its entire thickness. This ensures that when all the plates are fused, with each one separated by a porous membrane, there is a central cavity, which is not present when using a two-plate design. The presence of this central cavity serves a modeling function, as it allows for the epidermis of the skin model to be directly seeded onto the top of the upper membrane, with the dermis to be seeded in the central cavity. The bottom of the lower membrane can then be populated with endothelial cells, allowing for direct endothelial/dermal/epithelial cell contact, which better mimics the in vivo blood supply of the skin than using a single porous membrane to separate the dermis from the mock vasculature. While membrane-based soft lithography has been repeatedly used in the generation of skin-on-a-chip devices and can be argued to be the most popular form of production, there has been increased interest in the development of other production methods that can address some of its shortcomings, such as improving the reproducibility and speed of production of skin-on-a-chip devices. One of these emergent production methods is micromilling.

Micromilling is an ultra-precise production process that uses a cutting edge less than 1000 μm in size to machine complex 3D structures on a chip material (O'Toole et al. 2021; Câmara et al. 2012). The design of these complex 3D structures mirrors those produced by membrane-based soft lithography, such as a full microfluidic system and inlet/outlet ports. The cutting process is often guided through computer numerical control (CNC) and will often use poly methyl methacrylate (PMMA) as a base material rather than PDMS. Once multiple micromilled plates are produced, they can be assembled to form a full microfluidic device. This method ultimately produces a similar device to membrane-based soft lithography, with an apical plate used to house the skin construct and a basal plate with microfluidic channels to guide the medium solution, which is separated through the use of a porous membrane (Sriram et al. 2018). The best example of this production method is by Sriram et al. (Sriram et al. 2018), who utilized micromilling to produce this form of a device, with the addition of multiple other plates that are layered above the apical plate to allow for additional elements to be inserted into it, such as diffusion chambers, which allows for compound absorption testing.

Another method of production that does not employ any form of lithography is named the "layer-by-layer" production method, which has been previously used in many forms of organ-on-a-chip production. This process typically breaks down the aforementioned apical and basal plates into multiple smaller layers, which are then assembled to produce a fully functional microfluidic device. While this process can produce full devices, it can also produce the master molds, which allow for the repeated casting of PDMS plates for skin-on-a-chip production (Sasaki et al. 2019).

When directly producing microfluidic devices via layer-by-layer assembly, multiple vinyl UC or acrylic layers are cut from a single starting sheet. As this cutting process involves passing a single sheet through the narrow opening of the cutting tool, these layers must be much thinner than those plates produced via soft lithography. The multiple separate layers are then assembled to form the apical and basal plates of the skin-on-a-chip device. Some descriptions of this production method suggest that as many as seven separate sheets are required to produce a single plate, and assembly holes must be included in the design. These assembly holes allow for the correct alignment of the layers, which ensures the various microfluidic channels and housing units form correctly and provide a fluid-tight seal (Risueño et al. 2021; Valencia et al. 2021). These assembly holes prevent more complex structures from being included in the design of devices, as they occupy some of the limited available space. Still, cutting and assembly speed allows for a high-throughput approach to testing.

These production methods, layer-by-layer, micromilling, and membrane-based soft lithography have produced significant advancements in the modeling of skin and the production of skin-on-a-chip devices. However, these production methods have limited design potential compared to the housing units that can be produced through 3D printing technologies and have less in vivo representation compared to what can be achieved by incorporating 3D bioprinting. As such, multiple studies have investigated and developed viable production methodologies that employ 3D printing/bioprinting. This revolutionary technology can be utilized to either improve the

microfluidic device housing units produced by these other methods or directly fabricate a more representative model of in vivo skin for insertion into the device.

One of the simplest forms of the incorporation of 3D printing into skin-on-a-chip housing units is through the printing of accessory elements that either support more standard production methods or adapt an already established device to allow for de novo skin equivalent generation within it.

An example of 3D printing to produce support structures is described by Bajza et al. While the actual microfluidic device was produced using standard PDMS polymer techniques, the surrounding support structures of PMMA and PLC were printed using a variety of commercially available 3D printers. These printed support structures secure the entire microfluidic device together and allow for the placement of a heating element in close contact with the skin model (Bajza et al. 2020).

3D printing has also been shown to be able to alter the housing unit in an already established skin-on-a-chip device, allowing for a different skin model to be utilized. This relatively simple 3D-printed apparatus is made of three separate elements. The first is a "sample holder," a short cylindrical tube with a faired base produced through filament-based 3D printing. This holds a collagen-based gel within it, forming a viable dermal compartment to support different cell cultures. The second is a circular mesh structure produced through the electrospinning of polycaprolactone (PCL). After coating with an ECM protein, it is placed on top of the collagen gel's sample holder. This mesh further supports cell attachment, as keratinocytes are seeded directly onto its surface. The final element of the 3D-printed apparatus is a "ring holder," which holds the other two components together. This sample holder device, with functioning epidermal and dermal compartments, can be inserted directly into the established skin-on-a-chip system, allowing a full-thickness humanized skin equivalent model to be utilized rather than a human skin biopsy (Tárnoki-Zách et al. 2021).

3D printing in skin-on-a-chip devices has a broader application than these listed alterations of already established production methods. It can fully produce the entire housing unit itself, with more accurate and complex components.

One of the earliest incorporations of broader 3D printing into the design of skin-on-a-chip housing units was the work by Abaci et al., who utilized this method to integrate a more complex vasculature into a humanized skin equivalent model. While not a complete skin-on-a-chip device, this methodology could be readily incorporated with little difficulty.

Through computer-aided design (CAD), several separate components are designed, printed, and cured. The first part of this novel structure was a ring-shaped support, which attaches directly to the top of the transwell insert and provides connection points for the other components. The second set of components are the inlet and outlet pipes, which are connected directly to the ring support structure and reach the base of the transwell insert. Finally, separate molds were used to cast sacrificial alginate channels, which will be used to guide vasculature formation. These sacrificial channels are laid on the insert's base and connected to the inlet and outlet pipes. Once the 3D-printed structures and alginate channels are assembled in the transwell, a dermal collagen matrix was seeded over the sacrificial alginate

channels to form a functional dermal compartment and support base for the seeding of an epithelial layer. Once this matrix has solidified and cross-linked, the precast alginate channels are removed by passing a solution of sodium citrate through the 3D-printed inlet and outlet pipes. This dissolves the alginate, leaving continuous hollow channels in the dermal compartment. These channels can then be perfused with a solution containing a single endothelial cell suspension, which allows endothelial cells to adhere to the hollow channels' walls and form a de novo vasculature embedded in the dermal compartment, which is more representative of in vivo skin physiology.

Another methodology that employs 3D printing and de novo vasculature generation in the production of a skin-on-a-chip microfluidic device is the work conducted by Mori et al. (Mori et al. 2017). In this methodology, 3D printing technology initially produces a base plate that will go on to support the rest of skin-on-a-chip device. Following this, a hollow four-walled compartment was produced using the same extrusion-based printing technique. Each of these walls contained three hollow anchoring structures that spanned its length and projected in and out of the walls. These components are treated with perylene and subjected to O_2 plasma etching to increase ECM adhesion and general cell biocompatibility. To provide further support, nano-strings are fed between the anchoring structures in the walls to create an overlain grid of wire that provides a solid support on which a dermal compartment can be cast. A collagen solution containing fibroblasts is then added to the central cavity of the skin equivalent holder and allowed to solidify to form a dermal compartment. The anchoring structure and nanofibers continue to support the dermis as it contracts and detaches from the support structure walls. The nanowires are then slowly removed from the dermal compartment to create hollow channels in the dermis, which can be seeded with endothelial cells to form a network of de novo vasculature vessels that closely mimic those present in the in vivo dermal compartment. To form a full-thickness skin model, keratinocytes are seeded onto the dermal compartment within a silicone ring placed on its surface, which helps to promote keratinocyte adhesion and cornification directly on the dermis. The entire structure is then placed in a cell culture dish, with a series of silicone tubes connected to a pump to allow the perfusion of culture media (Mori et al. 2017).

This work by Abaci et al. and Mori et al. demonstrates that the use of 3D printing technology has advanced the production of skin-on-a-chip devices substantially, allowing for more complex incorporations of vascular networks into skin equivalents, the alteration of existing microfluidic devices to hold a wider variety of skin models, and the production of more specific support structures. These methodologies, however, only used standard plastic-based 3D printing. Further work has shown that 3D bioprinting, which utilizes bioink to produce a more comprehensive humanized skin equivalent model, has the potential to improve skin-on-a-chip devices further and replace animal-based model systems.

Studies by Kim et al. used 3D bioprinting to produce a total thickness skin equivalent and standard 3D printing to produce the surrounding housing unit (Kim et al. 2019). Again, while this skin equivalent production method is not a complete

skin-on-a-chip microfluidic device, it could be readily expanded upon or incorporated into existing devices.

Initially, 3D printing produces a PCL mesh base plate, which, once filled with gelatin, acts as the support plate for the entire housing unit and the skin model itself. Unlike other 3D-printed housing units, which often fully produce the housing unit first, with the skin model produced after, this methodology allows for support walls to be printed in tandem with the various skin compartments. The first compartment of the skin is the hypodermal compartment, which is printed directly onto the PCL/gelatin mesh base plate using an adipose-fibrinogen bioink containing preadipocytes. This is the first skin model that includes a hypodermal compartment of the examples in this section. Following this, a thrombin–gelatin hydrogel containing endothelial cells was printed directly onto the hypodermis in a continuous cylinder that connects to inlet and outlet channels printed during the production of the support structure. This vascular bioink is surrounded by a 3D-printed dermal compartment, produced by the extrusion of a fibrinogen-based bioink containing fibroblasts directly onto both the vascular and hypodermal compartments. With these different compartments printed in the correct architecture, a series of temperature changes are then conducted to cross-link and mature the printed bioink. Initially, the device is incubated at 30 °C, which induces partial cross-linking of the collagen proteins in the hypodermal and dermal bioink, increasing its structural integrity. The temperature is then further increased to 37 °C, which completes the cross-linking of ECM components in the hypodermal and dermal compartments while completely liquefying the vascular bioink. This liquidation allows the endothelial cells present in the vascular bioink to sink under the influence of gravity and attach to the solid walls of the dermal compartment. This means that as the device is rotated, endothelial cells will adhere to all the free surfaces of the dermal compartment, forming a complete vasculature. The entire device is then submerged in fibroblast supporting medium, and a peristaltic pump is used to circulate endothelial supporting medium through the vasculature, to further help the maturation of these tissue compartments. The final step in this production method is that keratinocytes are printed directly onto the dermis surface using an in-house printing method and allowed to form a functional epidermis. This completes a comprehensible skin equivalent model containing all three primary skin layers and a representative vasculature present in the dermis.

Overall, all the numerous techniques listed in this section produce viable skin-a-on-chip microfluidic devices that are well suited as apparatus for cosmetic toxicology.

4.2.3 Material Selection for Fabrication of Skin-on-a-Chip Housing Plates

As explained in the previous section, there are numerous methods for producing housing plates for skin-on-a-chip devices. Still, even with these differences, the materials used to manufacture the housing plates have a level of consistency.

Excluding those that use 3D printing and 3D bioprinting, which use extrusion of specific plastic and bioink material to produce the device design, the materials selected in the other listed methodologies are chosen due to their specific characteristics.

The most common material used in skin-on-a-chip fabrication is polydimethylsiloxane (PDMS), which is characterized as an elastomer (Ding et al. 2020). Elastomers are a class of polymers that are noted for their viscoelastic properties. Viscoelasticity is defined by viscosity, the solidity of the material, and elasticity, the ability of a material to stretch and return to its original shape and structure upon removal of the force (Gogoi et al. 2022; Touchet and Cosgriff-Hernandez 2016). The preference for PDMS of all the potential elastomers is well documented in organ-on-a-chip designs due to numerous factors (Nge et al. 2013). The first is its biocompatibility, being well tolerated by numerous body tissues and cell types (Miranda et al. 2021; Bélanger and Marois 2001; Guo and Liu 2017; Hassler et al. 2011). The second is its gaseous permeability, allowing oxygen and carbon dioxide to permeate throughout the device (Markov et al. 2014). The third is its ability to be used in rapid production processes, reducing manufacturing time compared to processes that use silicon and glass as base materials and being easily removable from molds (McDonald and Whitesides 2002; Friend and Yeo 2010). The final is its optical transparency, allowing for real-time analysis via microscopy, which is critical to the verification and implementation of organ-on-a-chip devices (Liu et al. 2020).

While the elasticity of elastomers makes them desirable materials for lithographic production methods, this quality makes them unsuitable for other methods, such as micromilling (Sriram et al. 2018). These types of production methods tend to employ plastics as a base material. Plastics are defined as synthetic or semisynthetic polymers that have thermoplastic and thermosetting properties, meaning they are easily shaped through the use of heat (Halden 2010). The specific plastic, poly (methyl methacrylate) (PMMA), has been demonstrated to be a suitable material in the micromilling production of skin-on-a-chip housing plates. This is chiefly due to the increased rigidity of PMMA compared to PDMS, which allows the micromilling cutting edge to shape the plastic without causing deformities that may occur in more elastic materials. PMMA specifically has this improved structural integrity while retaining the transparent and biocompatible properties of PDMS, with the additional benefit of reduced autofluorescence (Ding et al. 2020; Piruska et al. 2005). The use of PMMA as a fabrication material has been shown to have a reduced permeability to oxygen, which should be accounted for when designing the apical plate of the skin-on-a-chip device (Zoio and Oliva 2022; Zahorodny-Burke et al. 2011).

The production method, "layer-by-layer," has been shown to chiefly employ acrylic and vinyl UC as a base material for skin-on-a-chip devices. While these materials have been demonstrated to produce viable skin-on-a-chip devices able to support the extensive viability of the housed skin model, the complete and comparative biocompatibility of these materials is yet to be elucidated and requires further analysis (Risueño et al. 2021; Valencia et al. 2021).

While the materials listed are the most frequently used in non-3D-printed housing plate production techniques for skin-on-a-chip devices, other materials have been investigated for their potential use in other organ-on-a-chip device contexts. These include paper, a variety of hydrogels, and combinations of different materials (Ding et al. 2020; Seliktar 2012; Sapp et al. 2015). While not explicitly shown to produce working skin-on-a-chip devices, all these should be considered potential base materials.

4.2.4 Design of the Nutrient Support System Within the Skin-on-a-Chip Device

Nutrient support is an essential factor when designing a skin-on-a-chip system. The supply of nutrients, such as glucose, calcium, and hormonal growth factors to the skin model, retained within the device are important to improve the longevity of the housed skin model, the induction of differentiation and stratification of the epidermis, and its downstream applications (Bikle et al. 2012; Salameh et al. 2021).

The vasculature of in vivo human skin is located within the dermal layer and acts to deliver nutrients to cells while also removing unwanted metabolic waste products. The vasculature also functions as a conduit for immune system components and helps regulate temperature (Low et al. 2020). While important for normal cutaneous function, the cutaneous vasculature also plays a role in different pathological conditions such as inflammatory conditions, cancer metastasis, and wound healing (Kashani-Sabet et al. 2001; Huggenberger and Detmar 2011). Furthermore, previous studies have shown that the cutaneous vasculature can impact the transdermal diffusion of substances. Therefore, cutaneous vasculature and its distribution should be considered in the generation of 3D skin models, especially those used for topical or transdermal drug delivery studies (Cevc and Vierl 2007).

Conventional humanized 3D skin models do not incorporate a vasculature, limiting their ability to fully replicate the function of human skin properly. Furthermore, the lack of vascularization within a skin model reduces the perfusion of nutrients and oxygen within the model system, reducing cell viability (Magliaro et al. 2019). In particular, the diffusion limit of oxygen within cell-rich tissues is approximately 200 μm. This value can be used to determine the smallest cubic volume of cells that can function and survive without a vasculature (Magliaro et al. 2019; Ehsan and George 2013). Therefore, the culture of 3D skin models thicker than 200 μm is more likely to undergo hypoxia-induced apoptosis if they do not include a complete vascular system.

There have been many approaches to inducing vasculogenesis in vitro, such as endothelial cell seeding onto support matrixes, such as hydrogels or scaffolds, and cell encapsulation (Shafiee et al. 2021; Zhang et al. 2018; Heo et al. 2019). However, these approaches are complex and slow, and are thus unsuitable for high-throughput applications (Shafiee et al. 2021; Sorrell et al. 2007). Additionally, the vascular channels formed have a random formation, meaning that the vascular channels

within the 3D skin models cannot achieve complete perfusion, thereby limiting their applicability.

Advancements in biotechnology, which led to the development of microfluidic devices and 3D bioprinting, have allowed the development of perfusable vascular networks within full-thickness skin models (Salameh et al. 2021; Dellaquila et al. 2021). Various approaches have also been explored for perfusing culture medium. The two most frequent methods are either pump or gravity-driven approach (Kaarj and Yoon 2019).

The most common method for perfusing culture media is to use external pumps, such as a syringe or peristaltic pumps, as these systems can deliver an accurate, fine-tuned fluid flow (Mori et al. 2017; Salameh et al. 2021; Ramadan and Ting 2016). However, using these pumps can be time-consuming, and the need for external tubing and multiple connections can increase the risk of contamination (Zoio and Oliva 2022).

An alternative method is gravity-driven approach (Wufuer et al. 2016; Wang and Shuler 2018; Abaci et al. 2015). These approaches usually involve using custom-built rocking platforms to recirculate culture media through the microfluidic device. However, these gravity-driven approaches typically need more refined control as they do not perfuse fresh culture media and remove waste products.

The development of 3D bioprinting technology has allowed further advancements in mimicking the cutaneous vasculature within reconstructed skin models. Abaci et al. (Abaci et al. 2016) used 3D-bioprinted molds to micropattern sacrificial alginate channels that were used to cast a simulated vasculature inside a collagen matrix of the dermal compartment of a 3D skin model. Following stratification of the epidermis, the alginate was removed, leaving behind hollow tubes for perfusion. Additionally, Abaci et al. (Abaci et al. 2016) seeded either iPSC-derived endothelial cells or human umbilical vein endothelial cells (HUVECs) onto the inner surface of these channels. Adding these cells decreased the permeability and diffusivity compared to unseeded channels and demonstrated values similar to a real microvasculature. Similar 3D skin models were utilized by Mori et al. (Mori et al. 2017), with nylon threads used to create hollow channels before seeding with HUVECs. This perfusable skin model was used to study the percutaneous absorption of caffeine and isosorbide dinitrate solutions applied topically by measuring the amount of these molecules in the perfused medium flowing through the microvasculature and in the medium at the bottom of the culture device. Mori et al. (Mori et al. 2018) further improved the design of their perfusable skin model by introducing a motor to the system. This approach allowed them to apply mechanical force to the tissue, recreating skin stretching and thus demonstrating enhanced epidermal differentiation and stratification (Mori et al. 2018).

These advances in 3D bioprinting and microfluidics have allowed for better tissue formation and maturation ex vivo. Additionally, the incorporation of perfusable vascular channels demonstrates good vascular permeability properties, making skin-on-a-chip a promising platform for drug and cosmetic testing.

4.2.5 Biosensor Integration into the Skin-on-a-Chip Device

When analyzing 3D tissue models cultured on scaffolds or membranes, conventional microscopy techniques cannot be used as 3D cultures may be too thick and have high scattering effects, thereby limiting light penetration (Graf and Boppart 2010). Conventional 3D tissue morphology and function analyses mainly rely on endpoint assessment techniques such as immunohistochemistry of tissue sections. This usually requires the removal of the tissue from its original housing, chemical fixation, and labeling. Alternatively, assays using tracer compounds such as fluorescein isothiocyanate-labeled dextran can also be used. Furthermore, these compounds may affect tissue integrity and are not sensitive enough to detect subtle changes in tissue function (Arık et al. 2018). However, this limitation can be circumvented by integrating microsensors into organ-on-a-chip systems to measure physical or chemical parameters in situ.

Integrating sensors into organ-on-a-chip systems can help characterize engineered tissue models while also giving prompt insights into tissue interactions with different stimulants. Zhang et al. (2017) demonstrated that integrating an array of on-chip sensors, such as optical and pH, oxygen, and temperature monitors, allowed them to monitor and capture real-time changes in organoid behavior to obtain more information about what was happening within the organ-on-a-chip system. In the skin-on-a-chip platforms, integrating various sensors would be extremely beneficial as establishing a full-thickness skin model within the platform can be a long process ranging from around 2–6 weeks. Conventional endpoint assays provide no information about the period during skin formation and, therefore, may result in low experimental reproducibility. Ideally, skin-on-a-chip devices should incorporate physical sensors to monitor cell culture parameters, e.g., pH, oxygen, and temperature; electrochemical sensors to measure soluble protein biomarkers; and transepithelial electrical resistance (TEER) sensors to measure skin barrier integrity and function.

Currently, most skin-on-a-chip devices mainly use TEER sensors as it enables non-destructive real-time quantification of barrier integrity and function by quantifying alterations in the transcellular and paracellular permeability of epithelial and epidermal cell cultures (Zoio et al. 2021b; Petrova et al. 2014). TEER values will gradually increase during skin culture, correlating with epidermal differentiation and formation of the *stratum corneum,* thus allowing researchers to use these results as a quality control to ensure barrier integrity of skin models prior to their use in downstream assays (Gorzelanny et al. 2020; Zoio et al. 2022a). Additionally, TEER sensors can also be used for drug testing purposes, with TEER measurements used as a testing parameter for the Organization for Economic Cooperation and Development (OCED) test guidelines 430 (In vitro skin corrosion: Transcutaneous electrical resistance test method) (Zoio and Oliva 2022; Zoio et al. 2022a). Subsequently, various studies have investigated using TEER to assess the potential skin irritation of different compounds. Wei et al. (2020) demonstrated that TEER measurements could be used to evaluate the skin irritation potential of 46 compounds tested on 3D-bioprinted skin models. Groeber et al. (2015) used TEER as a

complimentary endpoint in cutaneous toxicity analysis to distinguish between the effects of solid irritants and non-irritants while concluding that TEER could be an instrumental measurement to identify sub-irritative effects such as burning and itching sensations in the skin.

TEER measurements can be performed on skin-on-a-chip devices by incorporating electrodes on either side of the cellular barrier; however, this can be difficult because of the micrometer-sized channels within the device (Henry et al. 2017). Electrodes can also be inserted into the chip's inlets and outlets. This method does not affect access to the cells; however, it has low reliability due to variations in the placement of the electrodes and geometry of the small channels (van der Helm et al. 2016). Alternative methods involve integrating the electrodes into the chip closer to the cell culture chamber, to decrease resistance from the cell culture medium and noise generated by the motion of the electrode (Arık et al. 2018). However, to ensure the successful integration of electrode systems into on-chip devices, researchers must consider the electrode size, geometry of the chip, and placement. Studies by Odijk et al. (2015) reported problems when integrating electrodes into their organ-on-a-chip device, resulting in overstimulation of TEER values. Other potential sources of error for TEER measurements include the presence of air bubbles within the microchannels and inadequate cell coverage. Previous studies have reported that even a small gap in tissue coverage (0.4%) can cause TEER values to drop by approximately 80% (Odijk et al. 2015). This problem is particularly relevant for full-thickness skin models generated using animal-derived collagen, as this matrix source is prone to shrinkage during culture over time.

The idea of a modular microfluidic device with interchangeable organ-on-a-chips and sensors has been investigated; however, a skin-specific system has yet to be achieved (Zhang et al. 2017). The ability to alter the circuits to suit the requirements of a skin-on-a-chip device will enhance the functionality of the skin models while allowing for automated analysis procedures and real-time monitoring of tissue health.

4.3 Verification of Skin Structure and Function Within the Skin-on-a-Chip Device

As explained earlier in this chapter, the design and production of skin-on-a-chip devices is a complicated process that requires the consideration of multiple factors, which must be chosen carefully to produce a microfluidic device that is best suited for its intended purpose. However, of all the elements considered in its design, the skin model housed within it is the most critical. As this skin model aims to reproduce in vivo skin, this skin model system must be verified to ensure that it retains the same structure, function, and physiology of in vivo human skin over the entire course of its time in culture within the microfluidic device. Without this verification, any toxicological information gathered about a given compound from its testing using this model would not be truly representative of the expected response in consumers.

During the course of the development of skin-on-a-chip devices, numerous verification methods have been established as standard assessments of the skin model. This section will aim to explain what those methods are and how best to apply them for verification of skin-on-a-chip performance.

4.3.1 Histological and Immunohistological Examination of Skin Model Structure

The use of classical histopathology stains is one of the main techniques that can be used to examine the physical structure of a housed skin model and determine whether it is representative of in vivo human skin. This has been achieved using three basic histopathology stains: hematoxylin and eosin (H&E), Masson's trichrome, and Sirius Red.

H&E staining can be applied to formalin-fixed paraffin-embedded (FFPE) or optimal cutting temperature (OCT) frozen sections of the skin model isolated at various stages of culture in the microfluidic device and exploits the different preferential binding of these stains to examine cell morphology (Song et al. 2017b; Abaci et al. 2016; Abaci et al. 2015). Hematoxylin has a high binding potential for nucleotide-based molecules, such as DNA, and strongly stains the cells' nucleus. In contrast, eosin has a solid binding affinity for proteins and, as such, binds nonspecifically to the cytoplasm. This staining technique allows all cells within the skin model to be visible, where noticeable phenotypic differences in cell morphology can be observed (Feldman and Wolfe 2014; Fischer et al. 2008).

Clinical histopathologists routinely use this stain to access tissue structure. While it does require a level of knowledge of typical skin structure to use, it gives the broadest indication of the structure and maintenance of the epidermal and dermal compartments of the skin.

While H&E staining broadly labels all skin compartments, other stains can be used to specifically examine the dermal compartment's structure (Lim et al. 2018). Two of these are Masson's trichrome and Sirius Red (Rieppo et al. 2019; Rittié 2017), which only stain the collagenous fibers and other connective tissue in the dermis. These fibers are secreted by dermal fibroblasts and are critical for forming an extracellular matrix required for normal skin function and resistance. The density and intensity of these stains correlate to the level of these fibers, which, when compared to the level in in vivo skin, can be used to ensure consistency between the two pieces of tissue.

While these histological stains are helpful in the verification of skin model structure, many studies use immunofluorescence/immunohistochemistry of FFPE or OCT skin model sections to label cell type-specific markers to achieve a closer examination of its structure. Numerous markers are available that identify cells at various stages of differentiation in various skin compartments. While examining all is not required, examining some in all the compartments is recommended.

One of the critical skin structures to investigate is the stratum corneum, the outermost layer of the epidermis. This stratum comprises fully differentiated

keratinocytes, or corneocytes, and provides the initial and strongest barrier to the external environment. Two markers are commonly used to judge these cell level of terminal differentiation, involucrin, and loricrin (Lee et al. 2017; Abaci et al. 2016). These two proteins, upon terminal differentiation, cross-link and help form the cornified envelope, which aids in providing the skin's barrier function. When staining skin models, there should be a continuous line of expression of both of these proteins in this stratum, which indicates a sufficient level of terminal differentiation (Eckert et al. 1993; Kalinin et al. 2001). If the expression is absent, the barrier function of the skin model should be questioned.

Before reaching the stratum corneum and undergoing terminal differentiation, keratinocytes progress through the multiple other squamous stratums in the epidermis, including the stratum spinosum and stratum granulosum. The cells in these layers can be identified as the keratinocytes begin to undergo differentiation, which causes visible phenotypic changes in their morphology. This change in keratinocytes' phenotype is accompanied by an alteration to their protein expression, allowing their demarcation from the cells in the more columnar basal stratum. One of the most commonly examined protein families, which marks this difference, is the keratin family (Maschmeyer et al. 2015; Abaci et al. 2016; Plaza et al. 2021). Keratins are essential structural fibrous proteins that aid in forming the skin barrier, and the expression of specific keratin proteins is specific to a given skin stratum. Keratin 1 and keratin 10 ensure normal differentiation of the epidermis, as they should only be detectable in differentiating keratinocytes (Totsuka et al. 2017). As such, no expression should be detected in the stratum basale. Keratin 19 may also be examined, as some have reported that this protein has weak staining throughout the epidermis and can be readily observed in normal skin, making it suitable for comparison between de novo and native skin (Kim et al. 2019).

To ensure that the epidermis is normally proliferating, keratin 16 can be examined, as this protein only appears in inflamed or hyperproliferating epidermises, such as those actively undergoing wound repair (McGowan and Coulombe 1998). An absence of this marker ensures that the epidermis is forming correctly and not increasing at a rate where it will be unable to maintain itself (Sriram et al. 2018).

Keratins are not the only proteins induced through keratinocyte differentiation (Jusoh et al. 2019). To ensure normal epidermal function, the adhesion and tight junctional proteins desmoglein-1 and claudin-1 can also be examined. The expression of these markers increases in line with differentiation; as such, a gradient of expression should be observed, increasing toward the stratum corneum (Tsukita et al. 2001; Hammers and Stanley 2013).

The combined expression of these markers indicates that the differentiation of keratinocytes is proceeding as expected and not unsustainably.

The differentiation of keratinocytes present in the upper stratum layers of the epidermis is critical to skin function. Still, the ability of the keratinocytes present in the basal layer to continuously proliferate to provide the replacement cells lost to shedding is equally as important. Without this activity, the epidermis will fully differentiate into corneocytes and ultimately be lost, reducing the longevity of the skin model. The structural proteins keratin 14 and keratin 15 have mostly restricted

expression to the stratum basale, thus acting as good markers for the preservation of this stratum (Abaci et al. 2016; Wei et al. 2016; Bose et al. 2013). Additionally, the expression of Ki-67 and p63 should also be examined (Abaci et al. 2016; Kim et al. 2019), as these proteins are involved in the initiation of proliferation, and as such, their expression is vital to maintain both the basal population and to replace the corneocytes in the stratum corneum (Pellegrini et al. 2001; Chang et al. 2010). Occasional identification of these proteins in cells within the stratum basale is a good indicator of a functional epidermis that can continuously replenish itself, mirroring in vivo behavior and suggesting the skin model has the potential for long-term cell culture.

The expression of these markers demonstrates the presence of differentiated, differentiating, and basal keratinocytes within the epidermis. However, this epidermis needs to be securely attached to the dermal compartment of the skin to capture in vivo biology fully. Observing that the epidermis and dermis are tightly attached can be achieved by staining the extracellular matrix (ECM) proteins that form the epidermal–dermal junction, which are a series of structural proteins that attach basal keratinocytes to the dermis. Markers of the epidermal–dermal junction include collagen VII, collagen XVII, integrin-β1, and laminin-5, which can be identified when using immunofluorescence or immunohistochemistry, as the expression of these proteins form a solid line of expression directly beneath the columnar basal keratinocytes at the base of the epidermis (Sriram et al. 2018; Chung and Uitto 2010; Gatalica et al. 1997; Nishiyama et al. 2000; Liu and Leask 2013).

The formation and structure of the dermal compartment also need to be examined to ensure sufficient secretion and cross-linking of the ECM proteins that infer the tensile strength and elasticity of human skin. Numerous ECM proteins ensure this integrity and rigidity, but the most common ECM fibrous proteins examined are collagen I, collagen IV, and fibronectin (Pankov and Yamada 2002; Matsuura-Hachiya et al. 2018; Hwang et al. 2021). When stained, these proteins should be visible throughout the dermis, and the staining levels can be compared to that seen in native skin to ensure a sufficient level of similarity between the two structures (Sriram et al. 2018; Lim et al. 2018).

As the previous sections explain, some skin-on-a-chip models have been constructed to contain a full vascular network produced using endothelial cells (Abaci et al. 2016; Mori et al. 2017; Kim et al. 2019). As these networks are commonly formed through the perfusion of hollow chambers with single endothelial cell suspensions, work must be undertaken to ensure they form a complete and tight endothelial cell sheet. This can be achieved by staining for ZO-1, which is an occludin protein that contributes to the formation of tight functions between endothelial cells, which should be readily observable in the membrane of these cells (Kim and Kim 2017).

This is not the only marker used; however, several "cluster of differentiation" (CD) proteins are also used to indicate the level of differentiation in endothelial cells (Abaci et al. 2016). Most skin models are commonly stained with CD31 and CD14, which helps identify cells as either endothelial progenitor cells or as less-proliferative mature endothelial cells, respectively (Krenning et al. 2009).

Functional markers can also determine the level of function within the cells. One of them is the expression of endothelial nitric oxidase synthase, an enzyme that maintains cardiovascular endothelial homeostasis (Shimokawa and Tsutsui 2010).

Finally, while the inclusion of a hypodermis is unusual within a skin model present in a skin-on-a-chip model, advancements in 3D printing have allowed this often neglected compartment to be utilized in skin modeling (Kim et al. 2019). A standard fluorescent stain that examines the formation of this compartment is boron dipyrromethene (BODIPY), which can determine the maturity of the adipocytes present in this compartment (Nicu et al. 2018). BODIPY is a probe that emits a fluorescent signal in the presence of lipids. As adipose cells accumulate lipids following maturation, the fluorescent intensity level indicates the level of adipocyte maturation (Sarantopoulos et al. 2018).

4.3.2 Determining Cell Viability of the Retained Skin Model

While using immunofluorescence to examine critical markers in the skin is vital, it can only demonstrate the maintenance of the skin's structure. Equally important is determining the viability of the cells in the model for the length of time required. Multiple direct and indirect measurements have been developed and utilized to assess the level of viability during the production of different skin-on-a-chip models.

Cell viability assays such as the use of calcein and ethidium homodimer-1 solution, more commonly known as the LIVE/DEAD viability assay, can directly measure the housed skin model viability. The housed model is harvested at several time points during culture, with the epidermal and dermal compartments separated before treatment with the LIVE/DEAD assay (Lee et al. 2017). Calcein is a derivative of fluorescein, which is converted to green fluorescent calcein after it passes the cell membrane of live cells. At the same time, ethidium homodimer-1 can only cross severely damaged cell membranes, where it binds to nucleic acids and increases in fluorescent intensity, identifying dead cells (Decherchi et al. 1997; Bratosin et al. 2005). Confocal microscopy can be used to capture fluorescent images, which can be quantified using standard image processing software. The proportion of alive cells can then be determined by calculating the live cell fraction (calcein positive) over the total cell fraction (calcein and ethidium homodimer-1 positive).

While this is a valuable method, using LIVE/DEAD viability assays is problematic as it is destructive and does not allow for continuous assessment of the skin model's viability. As such, other methods have been established for continual modeling, which often examines either metabolic byproducts or the secretion of damage-related markers.

One method that examines damage-related markers utilizes the measurement of lactate dehydrogenase (Maschmeyer et al. 2015). Lactate dehydrogenase is only released by cells that have suffered some damage. As such, the relative concentration in the circulating cell culture medium over time within the microfluidic device indicates the level of damage within the whole model system. To assess the levels of lactate dehydrogenase, cell culture samples can be extracted at any time during the

skin model culture and incubated with lactate. Lactate dehydrogenase converts lactate to pyruvate through the reduction of NAD+ to NADH. The addition of diaphorase then utilizes the available NADH formed by this reaction to convert endogenously added tetrazolium salt to red formazan (Kumar et al. 2019). The level of red formazan present in the culture medium, determined by standard optical density, acts as a reporter for the lactate dehydrogenase level, indicating the level of damage in the skin model.

Lactate dehydrogenase is not the only damage-related marker, and optical density is not the only measurement system used in determining cell viability. Standard experimental assays can also be utilized to examine the level of damage-related markers in the circulating medium, such as multiplex assays and ELISA that determine the concentration of a given inflammatory or damage-related marker, such as interleukin-1β, interleukin-6, interleukin-8, or TNF-α (Wufuer et al. 2016; Kim et al. 2020).

Another possible indicator of declining cell health is through the monitoring of the metabolic activity of the skin model. Regular sampling of the cell culture medium can be conducted, with the level of glucose and lactate analyzed through detection assays, such as GLU 142 and LAC 142, which allow the concentration of these two metabolites to be quantified with a photometer (Maschmeyer et al. 2015).

A similar method for determining metabolic activity is assessing extracellular acidification rate (EAR) (Alexander et al. 2018). This methodology exploits the charged nature of H+ ions, a natural byproduct of cellular metabolic processes. The skin model is submerged in a fresh medium in the presence of metal oxide sensors. The metabolites present in this fresh medium are actively utilized, producing an increase in H+ ions that alter the charge of the solution, which is detected and plotted as pH (mV) (Alexander et al. 2017). While a helpful assay, the requirement of submerging the whole skin model in a cell culture medium and the need for incorporating metal oxide sensors into the design of microfluidic devices, prevent its use in all microfluidic devices.

4.3.3 Accessing the Flow Rate and Perfusion of the Retained Skin Model by the Vascular/Microfluidic System

The validation methods explained so far act to verify that the skin's structure retained within the model reflects in vivo physiology and that the cells within the model remain viable during its use. However, given that the significant advantage of skin-on-a-chip devices is the mimicking or generation of a complex vascularization able to supply nutrition to the retained skin, this system also requires verification. This is to ensure that the fluid flow rate within the system closely aligns with that observed in the vasculature of native skin and that it can adequately perfuse the skin model.

The first of these verifications is achieved by analyzing microparticle image velocity. This can only be performed on machined microfluidic channels, and not endothelial-derived channels, as the channel must be transparent to allow for

observation (Maschmeyer et al. 2015). This methodology uses either polymeric microparticles or isolated red blood cells suspended in PBS. As these particles are circulated through the manufactured chip, several points of interest are defined within the microfluidic system. As the particle/PBS solution passes through these points of interest, a high-speed CMOS (complementary metal oxide semiconductor) camera connected to a microscope takes serial images at 4 μs exposures. This series of images can be used to track the distance moved by these polymer/cells, which in turn can be used in the calculation of the mean velocity of the solution using standard image analysis software (Stamhuis and Thielicke 2014; Schimek et al. 2013). With the mean velocity calculated, the flow rate of the solution can then be determined with the following equation:

$$Q = w \times h \times v_{av} = w \times h \times k \times v_{\max} \tag{4.1}$$

Q	Flow rate
w	Microfluidic channel width
h	Microfluidic channel height
k	Flow coefficient
v_{av}	Average velocity
v_{max}	Maximum velocity

Equation (4.1): Equation for calculating the flow rate within a microfluidic channel. Adapted from Maschmeyer et al. (2015), Schimek et al. (2013)

While the flow rate must match that of standard in vivo vasculature to prevent the buffeting of the cells present in the skin model, the ability of the chosen vasculature system to sufficiently perfuse a skin model must also be assessed.

This ability is most commonly determined using fluorescein isothiocyanate (FITC)-dextran, a fluorescent probe that can cross vascular cell barriers (Natarajan et al. 2017). The use of FITC-dextran, however, is skin-on-a-chip design dependent. For skin-on-a-chip devices that partially mimic endothelial/dermal transport by culturing endothelial and fibroblasts cells on opposing sides of a membrane, the central cavity in the microfluidic device is sampled at regular periods to determine the level of perfusion. The level of fluorescence in these samples, caused by the perfusion of FITC-dextran, plotted over time, indicates the level of perfusion and, thus, the performance of the microfluidic channels (Wufuer et al. 2016).

Skin models generated with de novo endothelial cell vasculatures are more complex to assess. Most commonly, FITC-dextran is added to the circulating medium solution and allowed to pass through the skin model for a given time. The permeability level is then assessed by tracking the fluorescent intensity in the dermal area surrounding the vascular structures. The changes in intensity are measured via fluorescent microscopy, with a time series of fluorescent images taken at a given dermal location. The level of fluorescence in these areas can be quantified with standard image processing software. The permeability of the vascular network can then be calculated using the following equation (Lee et al. 2017; Abaci et al. 2016; Kim et al. 2019):

$$Pd = \frac{1}{l_1 - l_b} \times \left(\frac{l_2 - l_1}{t} \right) \times \frac{d}{4} \tag{4.2}$$

P_d	Diffusion permeability coefficient
l_1	Initial average fluorescent intensity
l_2	Average fluorescent intensity after a given time (t)
l_b	Background fluorescent intensity
t	Time
t	Time
d	Microfluidic channel diameter

Equation (4.2): Equation for calculating vascular channel permeability using FITC-Dextran. Adapted from Kim et al. (2019).

4.3.4 Determining Barrier Performance of the Retained Skin Model

The verification methods explained so far provide a good indication of the health and structure of the housed skin model; however, none of those listed examine the skin's natural functions. One of the most critical in vivo functions of the skin is to prevent the penetration of foreign bodies and liquids into the body. The barrier performance needs to be assessed in the housed skin model.

The simplest form of assessment, which can be conducted on almost any skin-on-a-chip device, is a water-wicking test. A small volume of PBS can be applied to the skin model's surface, and the stratum corneum's ability to repel this solution into a single droplet can be observed by the eye, indicating a certain level of barrier function (Mori et al. 2017). As the easiest and simplest method, water-wicking does not provide quantifiable measurements, making the continuous monitoring of barrier function complex.

As such, the measurement of transepithelial resistance (TEER) is more frequently used as an assessment methodology (Alexander et al. 2018). TEER does not directly measure the permeability of the skin model. Instead, it is inferred from the level of resistance observed in a current passing through the skin model. This resistance is generated from the tight junctions that connect the cells in the stratum corneum, with a higher resistance equating to more tight junctions equating to a lower level of permeability (Benson et al. 2012). TEER is measured using two electrodes on opposing areas of the visible skin model. A defined DC voltage can then be applied between the two electrodes, with the outputted current produced measured. The ohmic resistance of the stratum corneum can then be calculated through Ohm's law.

$$V = I \times R \tag{4.3}$$

V	Voltage
I	Current
R	Resistance

Equation (4.3): Ohm's law

The level of resistance can be tracked over time in culture, indicating if the barrier function is maintaining its function or losing it over time.

This method has been improved upon due to the potentially detrimental effects of the direct application of current. One example is the epithelial voltohmmeter (EVOM), which uses alternating current at a fixed voltage rather than a direct current, to avoid the direct application of electricity to the cells (Srinivasan et al. 2015). As is the case in the measurement of the EAR, the potential need to incorporate electrodes into the design of the microfluidic device may be a limiting factor in its use.

A permeation methodology can be employed to avoid this potential limiting factor. The methodology utilizes oligonucleotides labeled with fluorescein amidites (FAM), which are applied to the apical side of the skin model at a given concentration, and sufficient time is given to allow their penetration into the skin model (Abaci et al. 2016). The circulating medium solution within the microfluidic device can then be sampled at regular time points and assessed for their level of fluorescence using a fluorescence spectrometer, which indicates the concentration of FAM-labeled oligonucleotides. The difference in concentration between that applied to the apical surface and that present in the circulating medium can be used to determine transepithelial and transdermal permeation, utilizing Fick's law:

$$J_{ss} = \frac{\left(\frac{C_s}{C_m}\right) D_s}{\delta_s} \times A_s \times (C_d - C_b) \tag{4.4}$$

J_{ss}	Steady-state mass transfer of compound
C_s	Skin concentration of compound
C_m	Medium concentration of compound
D_s	Diffusion coefficient of compound
δ_s	Skin model thickness
A_s	Skin model surface area
C_d	Compound concentration in vehicle
C_b	Compound concentration in bottom chamber

Equation (4.4): Equation to find the transepithelial/transdermal permeation of the FAM-labeled oligonucleotides. Adapted from Abaci et al. (2015).

This methodology is advantageous as the application, collection, and determination of the concentration of FAM nucleotides can be performed at any point during culture. It is not damaging and is suitable for all microfluidic devices.

Another potential method of barrier function assessment is confocal Raman spectroscopy. This method is destructive, as the skin model must be removed from the culture and placed directly on the instrument, making it unsuitable for continual assessment (Sriram et al. 2018). Confocal Raman spectroscopy is a novel method that exploits the physical phenomenon of the Raman effect, which observes that the vibrations that occur in the chemical bonds of molecular structures can cause light to either gain or lose energy predictably. This change in light energy can then be measured at specific wavelengths, allowing for the identification of specific types of bonds (Butler et al. 2016; Caspers et al. 2001). This phenomenon can be used to measure the presence of structural proteins, such as keratin, and base molecules, such as water. The water-integrated intensity, determined by the OH stretching vibrations in the range of 3350–3550 cm^{-1}, and the keratin-integrated content, determined by the CH stretching vibrations in the range of 2910–2960 cm^{-1}, can then be used to find the water content of the outer layer of skin using the following equation:

$$WC = 100\% \times \frac{\left(\frac{W}{P}\right)}{\left(\frac{W}{P} + R\right)} \tag{4.5}$$

WC	Water content
W	Water-integrated intensities
P	Protein-integrated intensities
R	Water-to-protein signal proportionality constant

Equation (4.5): Equation to find the water content of a skin model following confocal Raman spectroscopy. Adapted from Sriram et al. (2018).

The determined water content of the stratum corneum can be used to determine its thickness, and when confocal Raman spectroscopy is performed on native skin, a direct comparison between the model and in vivo skin can be made, inferring barrier function (Caspers et al. 2003; Mahrhauser et al. 2015).

4.3.5 Confirming Cell Position within the Retained Skin Model

The final verification that can be employed is an examination of the position of the cells within the housed skin model. As with other verification methods, this methodology depends on the skin model's form in the skin-on-a-chip device.

A cell tracker system is best for skin models that aim to mimic the cell–cell contacts present in the skin through the seeding of monolayer sheets on membranes within the skin-on-a-chip device (Wufuer et al. 2016). Prior to seeding, keratinocytes, fibroblasts, and endothelial cells are incubated with three different cell tracker solutions with three different colored fluorescent probes. The cells are then seeded onto the microfluidic device and allowed to reach confluence. The microfluidic device can then be imaged using a standard fluorescent microscope,

and by examining the z-axis, the various cell types and their 3D position can be easily visualized. This can be used to confirm the confluence of the cell layers and that the required cells are making contact.

For more complex skin models, such as those that utilize de novo skin equivalents or bioprinted models, a more complex microscopy method has to be used, two-photon excited fluorescence microscopy (Sriram et al. 2018). Due to the thickness of these retained skin models, standard microscopy techniques would be unable to visualize targets in the deeper compartments of the skin model without causing severe photodamage. Two-photon microscopy prevents this by visualizing targets using two photons aimed at converging on a single target area. Each photon has half the required energy to excite the required fluorescence, so converging on a single molecule supplies enough energy to visualize the target without causing photodamage (So et al. 2000; Denk et al. 1990). This form of microscopy can be used to visualize cell positions within any skin model without fluorescent labels, as nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FAD) have endogenous fluorescence. The fluorescence of these molecules can help examine the confluence of keratinocytes and fibroblasts in the epidermal and dermal compartments.

4.4 Advancements and Applications of Skin-on-a-Chip Technology

Cutaneous and subcutaneous disorders affect approximately one-third of the global population. Given the high prevalence of skin disorders, there has been an increase in new technological advancements for cutaneous drug development. Skin-targeted drug delivery includes topical (e.g., creams and repellents), dermal (e.g., corticosteroids and antibiotics), and transdermal approaches (Zoio and Oliva 2022). Traditional preclinical drug testing relies on in vitro 2D cell culture or animal models. While 2D cell culture systems are rapid and reproducible, they are unable to mimic complex interactions observed in vivo. Also, while animal models provide insight into systemic effects, they cannot replicate human skin anatomy and physiology. Furthermore, animal models have low throughput and interspecies variability, limiting their accuracy for human cutaneous drug testing. Additionally, from an ethical perspective, the replacement and reduction in the use of animal models fulfill the growing societal concern surrounding animal testing, with ethical guidelines dictating animal testing should be replaced, reduced, or refined (3R principle) (Zoio and Oliva 2022). Since 2009, the European Commission has been authorizing regulations on cosmetic testing, establishing a prohibition that prevents testing finished cosmetic products or ingredients on animals and commercializing any cosmetic product or ingredient tested on animals within the European Union (Taylor and Rego 2020). These restrictions on animal testing have thereby led to advancements in the development of more physiologically relevant skin models that can replace current inefficient methods. The need for more physiologically relevant and functional tissue models has led to the development of skin-on-a-chip

technology. Even though skin-on-a-chip technology is still in its infancy, these devices show promise to improve upon the current limitations of 3D-based cell culture platforms and increase the ability to determine the toxicity and efficacy of new drugs.

The most common application of skin-on-a-chip has been the maintenance of skin tissue under dynamic perfusion to increase longevity or to establish a co-culture of different tissues (Ataç et al. 2013; Abaci et al. 2015; Tavares et al. 2020). These studies provide valuable insight into the potential use of skin-on-a-chip for clinical applications such as multi-organ crosstalk and assessing drug sensitivity and toxicity. By culturing monolayers of cells on a chip with each layer separated by a porous membrane, researchers can co-culture and mimic the different skin compartments to observe and analyze interlayer communication (epidermal, dermal, and vascular) when testing new drugs or cosmetics (Wufuer et al. 2016; Jeon et al. 2020b). The measurement of dual parameters such as cell viability and tight junction has allowed researchers to use this skin-on-a-chip device to assess skin irritation and distinguish between irritants and non-irritants with 80% more sensitivity, specificity, and accuracy compared to in vivo data (Jeon et al. 2020b). Other studies have further adapted this design and used a porous membrane sandwiched between branched microchannels to an on-chip device to culture a HaCaT monolayer that can be used in permeation assays to test skin irritants (Sasaki et al. 2019). Studies by Ren et al. used a microfluidic skin-on-a-chip device to study transendothelial and transepithelial migration of T cells in a mimicked skin inflammatory microenvironment (Ren et al. 2021). This microfluidic device allowed Ren et al. to quantitatively study the effect of cutaneous inflammatory mediators on T-cell transmigration at a single cell level and identify potential anti-inflammatory drugs for treating skin diseases such as psoriasis (Ren et al. 2021). While these 2D skin-on-a-chip devices were able to culture cells directly within the microfluidic device and have shown the ability to stimulate cell responses to drug treatment, they do not fully mimic the complex 3D architecture of native human skin as the cells are cultured in 2D monolayers.

Various groups have developed skin-on-a-chip devices to overcome this limitation that transfer skin models to the device or the in situ formation of 3D skin equivalent models within the device. Lukács et al. developed a microfluidic diffusion chamber to monitor the transdermal delivery of topical drugs (Lukács et al. 2019). The device comprised three functional units: a top compartment where the drug of interest is applied, a middle compartment that houses an integrated skin sample, and a bottom compartment that houses the receptors. This device was capable of producing similar reproducible results when compared to traditional drug penetration assays such as the Franz diffusion cell system, as well as demonstrating other advantages such as small drug and skin consumption, low sample volume, and a dynamic arrangement with a continuous flow to mimic blood circulation through the dermal compartment (Lukács et al. 2019). Further studies by Bajza et al. used the same skin-on-a-chip device to study the role of P-glycoprotein in dermal drug delivery using two P-glycoprotein substrate model drugs: quinidine and

erythromycin to further demonstrate the suitability of the skin-on-a-chip device as a tool to investigate dermal drug delivery (Bajza et al. 2020).

While skin-on-a-chip can be used to assess new cosmetics and drug toxicity within the cutaneous microenvironment, these skin-on-a-chip models can be further modified to determine if any toxic effects are exhibited in other tissues and organs and observe any crosstalk between the skin and these other tissues and organs. Lee et al. developed a skin–nerve hybrid on-chip model by incorporating differentiated neural stem cells in a collagen matrix adjacent to and below an epidermal layer, thus enabling real-time quantification of skin sensitization by measuring alterations in neuronal activity following chemical treatment (Lee et al. 2022).

In addition, Lee et al. also developed a skin–liver hybrid on-chip model by incorporating hepatic cells derived from pluripotent stem cells in a matrix distant from the skin model (Lee et al. 2022). This model evaluated potential hepatotoxicity from topically applied chemicals to the cutaneous layer by quantifying glutathione and reactive oxygen species.

To further increase the complexity of skin-on-a-chip models to mimic the dermal microvasculature for systemic applications, researchers have used various techniques such as 3D bioprinting, templating, and sacrificial molding to generate full-thickness skin models with perfusable lumens (Abaci et al. 2016; Mori et al. 2017; Salameh et al. 2021). Abaci et al. used 3D printing to print sacrificial channels of cross-linked alginate embedded in a collagen I gel (Abaci et al. 2016). These microchannels were removed using sodium citrate following epidermal differentiation leaving behind hollow channels. Endothelial cells derived from HUVECs or iPSCs were then used to coat the inner surface of the channels, allowing the researchers to recapitulate endothelial barrier function. Other studies have used nylon wires to create perfusable vascular channels (Mori et al. 2017). However, this technique lacked a microvascular network, resulting in only one microchannel. Alternative approaches for generating perfusable vascularized skin models involve 3D bioprinting. Kim et al. used a bioink of gelatin, glycerol, and thrombin embedded with endothelial cells to print vascular channels (Kim et al. 2019). While proper tissue formation and good vascular permeability properties were reported, this model was still limited to one microchannel. More recently, studies have used 3D templating techniques to develop a vascularized full-thickness skin model (Salameh et al. 2021). This technique produces hollow channels similar to the work by Mori et al. (Mori et al. 2017); however, to induce vasculogenesis, the hollow channels were seeded with HUVECs, and perfusion was achieved using a peristaltic pump. This model generated a differentiated epidermis, a perfusable vascular network with angiogenic sprouts, and an adjacent microvascular network. Furthermore, the potential of this model for downstream topical and systemic applications was validated using various compounds such as caffeine, minoxidil, and benzo[a]pyrene pollutant.

Aside from skin-on-a-chip devices with perfusable lumens, some researchers have used microfluidic-based techniques to develop full-thickness skin models with a basal perfusion system for testing new drugs and cosmetics. These skin-on-a-chip devices consist of two layers of PDMS assembled on top of a glass base, with the bottom PDMS layer containing a fluidic chamber, while the top layer houses a

central chamber for skin model formation (Lee et al. 2017). After generating the reconstructed skin models, they were transferred to the on-chip device. They were cultured for an additional 6 days at an air–liquid interface before assessing skin barrier function using testosterone and caffeine as reference substances. Studies have also used these skin-on-a-chip models with perfusion platforms.

Applying mechanical forces and shear stress to the skin models to better mimic the in vivo cutaneous microenvironment (Strüver et al. 2017).

Recently, skin-on-a-chip models have been used for studying skin aging and testing the efficacy of new anti-aging cosmetics. Studies conducted by Lim et al. incorporated magnets into a dedicated cavity within a PDMS layer of the skin-on-a-chip device and applied an external electromagnetic field to uniaxially stretch the membrane and create a wrinkled skin-on-a-chip (WSOC) (Lim et al. 2018). This WSOC demonstrated reduced collagen production and decreased fibronectin and keratin 10 expression similar to that observed in aged skin, thus highlighting the potential of this tool to evaluate the efficacy of new anti-aging cosmetics and treatments without having to rely on ex vivo human skin biopsies.

Despite advancements in the development and application of skin-on-a-chip for cosmetic toxicology, most studies use animal-derived ECM proteins such as rat rail collagen to produce the dermal compartment. Consequently, this results in the formation of dermal compartments with inadequate biomechanical properties due to the contraction and degradation of the matrix by fibroblasts, thereby limiting the lifespan and reproducibility of the skin-on-a-chip models. To overcome this issue, chemical and physical modifications of the ECM matrix through the addition of synthetic polymers, natural polymers, or peptide motifs were considered. Due to the poor mechanical stability of collagen and fibrin, Sriram et al. combined fibrinogen with PEG polymers before pipetting into a device comprised of a multi-chamber microfluidic chip that contained two fluidic compartments separated by a permeable microporous membrane (Sriram et al. 2018). Using this technique, Sriram et al. produced a stratified epidermis with an enhanced basement membrane, demonstrated by increased deposition of collagens IV, VII, and XVII (Sriram et al. 2018). Studies by Zoio et al. used rapid prototyping techniques to develop a modular device integrated with electrodes for TEER measurements. This method combined the production of a fibroblast-derived matrix with an inert polystyrene porous scaffold integrated on-chip, thereby excluding the need for exogenous hydrogels and membranes (Zoio et al. 2021a, 2022b). The integration of electrodes allowed for TEER measurements to be obtained in situ during skin culture and also allowed the analysis of irritants on skin barrier function.

Overall, skin-on-a-chip technology shows promise to surpass current conventional drug testing assays and provide an alternative and more representative model than animal testing, especially given the ethical guidelines surrounding the use of animals for testing new cosmetics. Despite being in its infancy, skin-on-a-chip will continue to evolve, thus allowing its successful translation into the field of cosmetic toxicology and use as the new gold standard over conventional 2D assays and animals when testing new cosmetics and drugs.

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