jove

Building Up Skin Models for Numerous Applications - from Two-Dimensional (2D) Monoculture to Three-Dimensional (3D) Multiculture

Adrianna Maria Piasek[*,1], **Iryna Levkovych**[*,1], **Paulina Musolf**[1], **Hanna Chmielewska**[1], **Aneta Ścieżyńska**[2], **Anna Sobiepanek**[1]

[1] Chair of Drug and Cosmetics Biotechnology, Faculty of Chemistry, Warsaw University of Technology [2] Department of Histology and Embryology, Medical University of Warsaw

[*] These authors contributed equally

Corresponding Author

Anna Sobiepanek
anna.sobiepanek@pw.edu.pl

Citation

Piasek, A.M., Levkovych, I., Musolf, P., Chmielewska, H., Ścieżyńska, A., Sobiepanek, A. Building Up Skin Models for Numerous Applications - from Two-Dimensional (2D) Monoculture to Three-Dimensional (3D) Multiculture. *J. Vis. Exp.* (200), e65773, doi:10.3791/65773 (2023).

Date Published

October 20, 2023

DOI

10.3791/65773

URL

jove.com/video/65773

Abstract

Due to the complex structure and important functions of the skin, it is an interesting research model for the cosmetic, pharmaceutical, and medical industries. In the European Union, there has been a total ban on testing cosmetic products and their ingredients on animals. In the case of medicine and pharmaceuticals, this possibility is also constantly limited. In accordance with the 3Rs principle, it is becoming more and more common to test individual compounds as well as entire formulations on artificially created models. The cheapest and most widely used are the 2D models, which consist of a cell monolayer but do not reflect the real interactions between the cells in the tissue. Although the commercially available 3D models provide a better representation of the tissue, they are not used on a large scale. This is because they are expensive, the waiting time is quite long, and the available models are frequently limited to only those typically used.

In order to move the conducted research to a higher level, we have optimized the procedures of various 3D skin model preparations. The described procedures are cheap and simple to prepare as they can be applied in numerous laboratories and by researchers with different experiences in cell culture.

Introduction

The skin is a continuous structure with multicell interactions revealing the proper functioning and homeostasis of this complex organ. It is built from morphologically different layers: the inner layer - dermis, and the outer layer - the epidermis. On top of the epidermis, we additionally distinguish the stratum corneum (consisting of flattened dead cells - corneocytes), which provides the greatest protection against the external environment. Some of the most important passive and active functions of the skin are body protection against external factors, participation

in the immunological processes, secretion, resorption, thermoregulation, and sensing[1,2,3]. Because it is considered one of the largest organs in the body, it is impossible to avoid contact with various pathogens, allergens, chemicals, as well as ultraviolet (UV) radiation. Thus, it is structured with many types of cells with specific functions. The main types of cells present in the epidermis are keratinocytes (almost 90% of all cells, with structural and immunological functions in the deeper parts of the epidermis, but which later undergo the keratinization process to turn to corneocytes in the top layer of the epidermis), melanocytes (only 3%-7% of the epidermal cell population, which produce the UV protective pigment melanin) and the Langerhans cells (from the immune system). In the case of the dermis, the main cells are fibroblasts (producing growth factors and proteins), dendritic cells, and mast cells (both cell types of the immune system)[4,5,6]. Moreover, the skin is equipped with several extracellular proteins (such as collagen type I and IV, fibronectin, and laminin; **Figure 1**) and protein fibers (collagen and elastin), which ensure the specific structure of the skin but also encourage cell binding, cell adhesion, and other interactions[7].

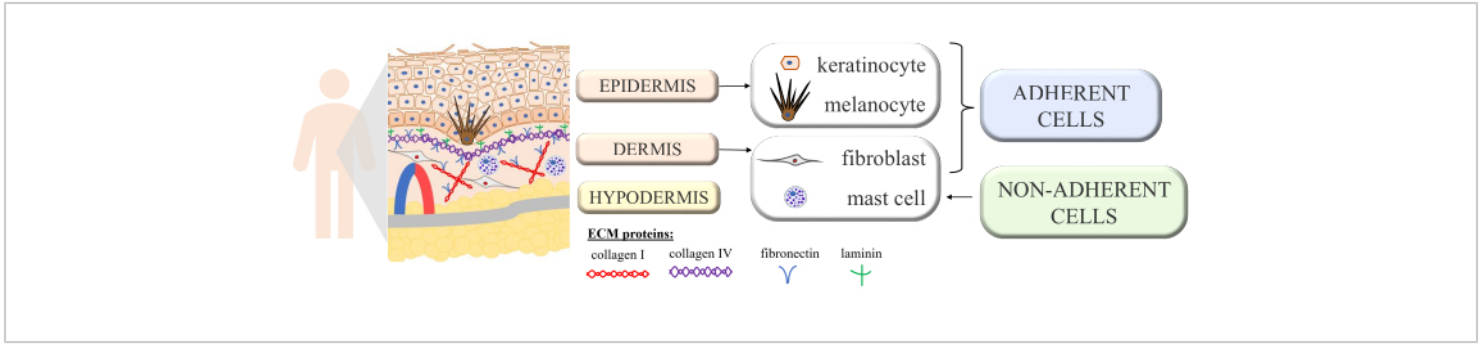

Figure 1: Schematic showing the skin structure. The skin structure marked four basic cell types occurring in its individual layers and distinguished proteins of the extracellular matrix. This figure was created with MS PowerPoint. Please click here to view a larger version of this figure.

The safety of cosmetics and pharmaceutical products is a very important issue, and protecting the health of consumers and patients is a priority[8]. Until recently, it was supposed to be guaranteed by numerous tests, including studies conducted on animals. Unfortunately, these often required the use of drastic methods, causing pain and suffering in animals used for research purposes (frequently mice, rats, and pigs). In 1959, the Principles of the Humane Experimental Technique (the 3Rs principle) were introduced: (1 - Replacement) replacing animals in research with *in vitro*, *in sillico,* or *ex vivo* models, (2 - Reduction) reducing the number of animals used for research, and (3 - Refinement) improving the well-being of the animals which are still needed for research and at the same time improving the developed alternative methods[9]. Furthermore, in the European Union (EU), cosmetic testing on animals is regulated by law. From September 11, 2004 the ban on animal-tested cosmetic products came into force. On March 11, 2009, the EU banned animal testing of cosmetic ingredients. The sale of cosmetic products made of newly animal-tested ingredients was not allowed; however, testing the products on animals for complex human health issues such as repeat dose toxicity, reproductive toxicity, and

toxicokinetics was still acceptable. Starting from March 11, 2013, in the EU, it is illegal to sell cosmetics where the finished product or its ingredients have been tested on animals[10]. Therefore, currently, in cosmetology, research is carried out at three levels: *in vitro* (cells), *ex vivo* (real tissues), and *in vivo* (volunteers)[11]. In the case of pharmaceuticals, the need for animal testing remains; however, it is significantly reduced and strictly controlled[12].

As alternative methods to animal testing and for the initial assessment of the effectiveness of a novel active ingredient, the *in vitro* skin cell cultures are used. Isolation of different types of skin cells and their cultivation in sterile laboratory conditions allows one to assess the safety and toxicity of active substances. Skin cell lines are also widely recognized models for research as the cells are sold by certified companies and the results can be comparable in different laboratories. These tests are usually performed on simple 2D models of the human skin cell monocultures. Some of the more advanced models are their co-cultures (such as keratinocytes with fibroblasts and keratinocytes with melanocytes), as well as the three-dimensional models, including scaffold-free cultures (spheres) and scaffold-based skin equivalents of the epidermis, dermis or even the full-thickness substitutes of the skin[13]. It is worth mentioning that apart from the last type (skin equivalents), the rest are not commercially available, and if needed, a scientist must prepare them on his/her own.

Even though plenty of these models have been maintained and are routinely sold nowadays (**Table 1**), additional models are constantly needed to validate most of the results. Thus, the newly engineered models ought to recreate better the real interactions taking place in the human body. When a mixture of cells of different types is used to form such models, the reproduction of the multicellular aspect of tissue *in vivo* can be achieved. As a result, an organotypic culture is developed (**Figure 2**).

	Name	Description
Normal skin	EpiSkin	Reconstructed Human Epidermis - Keratinocytes on a collagen membrane
	SkinEthic RHE	Reconstructed Human Epidermis - Keratinocytes on a polycarbonate membrane
	SkinEthic RHE-LC	Human Epidermal Model Langerhans Cells - Keratinocytes and Langerhans cells on a polycarbonate membrane
	SkinEthic RHPE	Reconstructed Human Pigmented Epidermis - Keratinocytes and Melanocytes on a polycarbonate membrane
	T-Skin	Reconstructed Human Full Thickness Skin Model - Keratinocytes on a layer of Fibroblasts, which were grown on a polycarbonate membrane
	Phenion FT Skin model	Keratinocytes and Fibroblasts in hydrogel

Skin with a disease	Melanoma FT Skin Model	Normal human-derived Keratinocytes and Fibroblasts with human Malignant Melanoma cell line A375
	Psoriasis Tissue Model	Normal human Keratinocytes and Fibroblasts

Table 1: The most popular commercial skin equivalents for various studies.

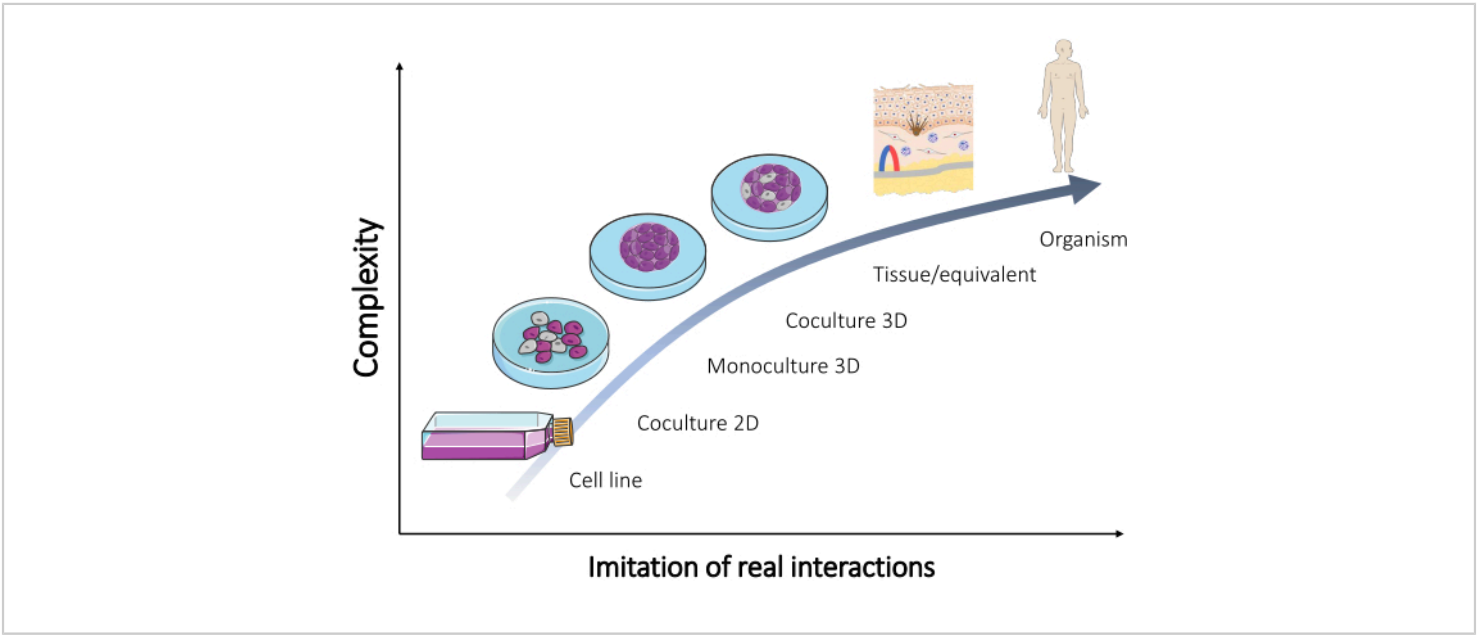

Figure 2: Complexity of different *in vitro* models. The relation between the complexity of different *in vitro* models to recreate an organism and the real interactions occurring directly in the human body. The figure has been modified from the set "Microbiology and cell culture" from Servier Medical Art by Servier (https://smart.servier.com/). Please click here to view a larger version of this figure.

One of the most important limitations of the commercial equivalents is the availability of only very general research models with a few types of cells (typically 1-2, seldom 3). Yet, there are many more cells present in the skin, and their interaction with each other can ensure better or worse tolerance of various ingredients[14]. The lack of some immune components can decrease its value in several kinds of research, including immunotherapy. This is a serious issue as melanoma is a life-threatening skin cancer due to the early onset of metastasis and frequent resistance to the applied treatment[15]. To improve the artificial skin model, researchers try to establish a co-culture of immune cells with cell lines and organoids[16] and this is considered a big improvement of the studied models. For example, mast cells take part in many physiological (wound healing, tissue remodeling) and pathological (inflammation, angiogenesis, and tumor progression) processes in the skin[17]. Thus, their occurrence in the model can significantly change the model's response to the studied compound. Finally, a lot of skin-related information is still missing, which can only be

discovered by performing basic research. This is why creating and refining different artificial skin models (**Table 2**) is such an important endeavor. This article presents several procedures to create advanced skin models, including spheres and skin equivalents.

***In vitro* skin model**	**Attempt to recreate interactions occurring in the tissue**	**Examples of the used cells**
2D or 3D cell culture	Epidermis	Keratinocytes
		Melanocytes
		Keratinocytes + Melanocytes
	Dermis	Fibroblasts
		Mast cells
		Fibroblasts + Mast cells
	Skin	Keratinocytes + Fibroblasts
		Keratinocytes + Mast cells
		Melanocytes + Fibroblasts
		Melanocytes + Mast cells
		Keratinocytes + Fibroblasts + Melanocytes
		Keratinocytes + Fibroblasts + Mast cells

Table 2: Examples of cell type mixture to recreate skin tissue in 2D and 3D culture.

Protocol

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Medical University of Warsaw (KB/7/2022). Informed consent was obtained from all subjects involved in the study.

NOTE: The described procedures of the advanced skin model preparation can be performed with the use of either commercially available primary skin cells and cell lines or with the primary cells isolated from patients. Commercial cells are supplied with the relevant documents, and their usage in research for most countries does not require any additional approval. However, for some countries, it is obligatory, thus, it must be checked with the Local Ethical Committee regulations. If the primary cells isolated from patients ought to be used in the research, first, the study must be approved by the Local Ethical Committee, and it needs to be conducted according to their strict guidelines. Furthermore, written informed consent must be collected from all skin tissue donors. The isolation of primary skin cells

was not the subject of this article, but exemplary isolation procedures can be found in Kosten et al. (keratinocytes)[18], Ścieżyńska et al. (melanocytes)[19], Kröger et al. (fibroblasts and mast cells)[20]. Most of the normal skin cells and cell lines have a safety level of BSL1 class; they do not cause any threats. However, the used laboratory equipment needs to meet the standards for animal and human cell culture under controlled conditions.

1. Skin cell culture

NOTE: Skin cell cultures must be carried out in flasks dedicated to adherent or suspension cells (depending on the cell type) at 37 °C and a carbon dioxide content of 5% in an incubator. Activities related to their cultivation and use for research require sterile conditions and must be performed in a laminar chamber after exposure to ultraviolet C (UVC) rays for 15-30 min. Obtaining cell suspensions, which are then used to create the two- and three-dimensional models, requires the implementation of procedures depending on the cell type (for adherent cells such as keratinocytes, fibroblasts, and melanocytes - step 1.1, for non-adherent cells of mast cells - step 1.2)(**Figure 3**). For different culture flask sizes, the volumes of all reagents (such as medium, phosphate-buffered saline, or trypsin solution) used in the described methods are listed in **Table 3**. Parameters dependent on the type of cells (e.g., concentration and composition of reagents, centrifugation method, etc.) are sorted in **Table 4**. Cell densities used for seeding are shown in **Table 5**. All these tables are included at the end of this section.

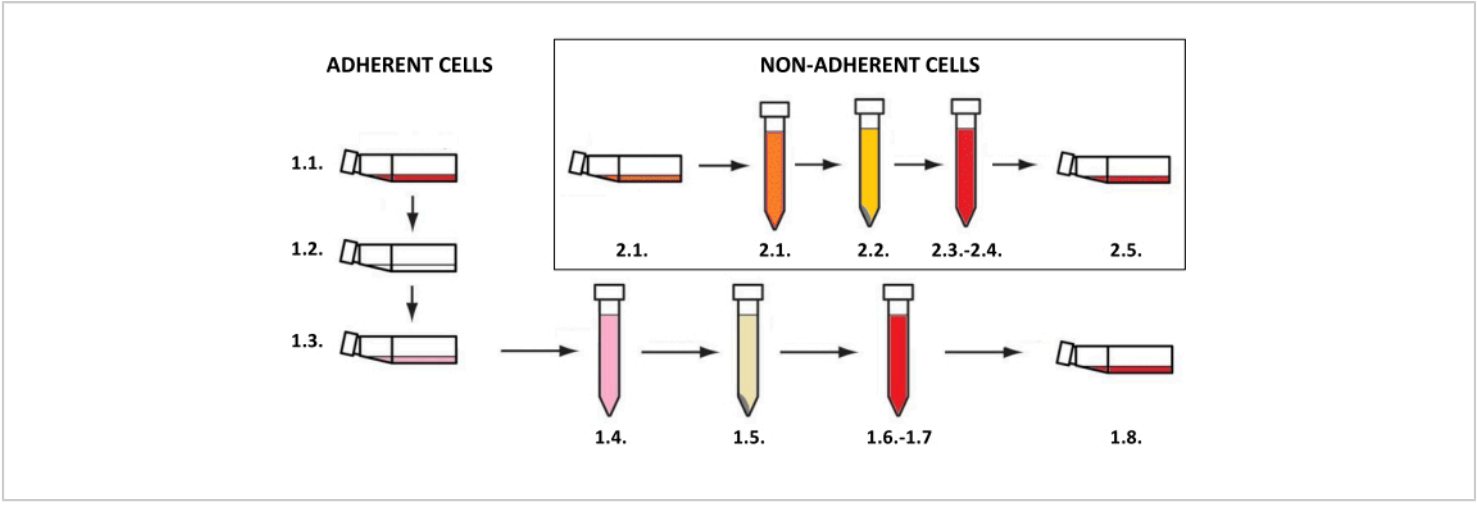

Figure 3: Adherent and non-adherent cell cultivation. The general step-by-step procedure of adherent and non-adherent cell cultivation (numbers correspond with the descriptions of steps 1.1 and 1.2). The figure was created with MS PowerPoint. Please click here to view a larger version of this figure.

1. Obtaining a suspension of adherent cells
    1. Remove the medium from the culture flask.
    2. Gently wash the cells with phosphate-buffered saline (PBS, **Table 3**).

3. Add trypsin solution in ethylene diamine tetra acetic acid (trypsin-EDTA solution, **Table 3**). Incubate the flask at 37 °C and control the detachment of cells from the surface on the optical microscope.
4. Suspend the detached cells in at least a double amount of full-growth medium or trypsin neutralizer to deactivate trypsin (2:1) (for volumes, refer to Table 3, and for reagents, refer to **Table 4**). Quantitatively transfer the content of the flask into the 15 mL tube.
5. Take a small volume (20 µL) of cell suspension into a 1.5 mL tube and count the cells with a manual or automatic hemocytometer.
6. Centrifuge the tube (parameters in **Table 4**), remove most of the supernatant, and resuspend the cell pellet in the small amount of the remaining liquid. Then, add enough fresh medium to obtain the pre-centrifugation volume (volume in **Table 3**) if the cell density is appropriate to seed or recalculate the required volume of the new medium.
   NOTE: Some cells, such as melanocytes, are very sensitive to centrifugation, thus avoid the necessity to centrifuge them again in a short time distance.
7. Prepare the cell suspension of the required density (cells/mL, cell density in **Table 5**) for the experiment (2D/3D mono- or multi-cultures of skin cells).
   NOTE: If cells are to be further cultured, return 5,000-8,000 cells/mL to a new flask and add fresh medium (volume in **Table 3**).

2. Obtaining a suspension of non-adherent cells
   1. Remove the medium with the cell suspension from the culture flask and quantitatively transfer it into the 15 mL tube.
   2. Take a small volume (20 µL) of the cell suspension into a new 1.5 mL tube. Count the cells with a manual or automatic hemocytometer.
   3. Centrifuge the tube (parameters in **Table 4**), remove most of the supernatant, and resuspend the cells in a small amount of the remaining liquid. Then, add fresh medium to obtain the pre-centrifugation volume (as indicated in **Table 3**) if the cell density is appropriate to seed or recalculate the required volume of the new medium.
   4. Prepare the cell suspension of the required density (cells per mL, as suggested in **Table 5**) for the experiment (2D/3D mono- or multi-cultures of skin cells).
      NOTE: If cells are to be further cultured, return 5,000-8,000 cells/mL to a new flask and add fresh medium.

	25 cm^2 culture flasks	75 cm^2 culture flasks
Culture medium [mL]	4–5	8–12
PBS [mL]	5	10
Trypsin–EDTA [mL]	0.5–1	1–2
Neutralization medium [mL]	1–2	2–4

Table 3: Volumes of reagents used during cultivation and preparation of cell suspensions.

Monoculture of skin cells	Trypsin	Trypsin deactivator	Centrifugation	Medium type for 2D monoculture
Keratinocytes	0.25%	with trypsin neutralizator	300 x g, 5 min, RT	Keratinocyte Growth Medium 2
Fibroblasts	0.25%	with medium	300 x g, 5 min, RT	DMEM, 10% FBS
Melanocytes	0.025%	with trypsin neutralizator	300 x g, 3 min, RT	Medium 254, PMA-Free Human Melanocyte Growth Supplement-2
Mast cells	not required	not required	300 x g, 3 min, RT	IMDM, 10% FBS, 1% non-essential amino acids, 226 µM α-monothioglycerol

Table 4: Trypsinization, centrifugation parameters, and type of media depend on the cell type.

Model type		Cell density [cell/mL]	
2D	Monolayer	Fibroblasts	2×10^5
		Mast cells	
		Keratinocytes	
		Melanocytes	
3D	Sphere (hanging drop method)	Fibroblasts	5×10^5
		Mast cells	
		Keratinocytes	
		Melanocytes	
		Mix of cells	
	Sphere (limiting cell adhesion method)	Fibroblasts	2×10^5
		Mast cells	
		Keratinocytes	
		Melanocytes	
		Mix of cells	
	Equivalent	Fibroblasts	1×10^5
		Mast cells	1×10^4
		Keratinocytes	8×10^5
		Melanocytes	5×10^4

Table 5: Cell seeding density for different types of skin models.

2. Preparation of skin cell spheres

NOTE: To create spheres, the use of the hanging drop method is described in step 2.1 (**Figure 4**), while the approach focused on limiting cell adhesion is shown in step 2.2 (**Figure 5**). Nevertheless, as spheres are very small and can be unstable, activities carried out under these methods require patience, delicacy, and slow actions.

1. The hanging-drop method
    1. Use an appropriate cell density to achieve the desired sphere size (recommended cell density 5 x 10^5 cells/mL, **Table 5**).

2. Pipette 20 µL of the cell suspension on the lid of a Petri dish or a multi-well plate (**Figure 4A**).
3. Cover the dish/plate with the bottom part and gently flip it (hanging drops will be automatically created on the lids, **Figure 4B**).
4. Fill the Petri dish/wells of the plate with a sterile water/PBS solution to avoid evaporation of the medium from droplets.
5. Incubate the droplets for 48-72 h at 37 °C.
   NOTE: The gravity pulls the cells down, and the lack of accessible surface prevents cell attachment to the vessel and promotes cell aggregation. However, some cell types may require longer incubation.
6. Before performing the next step, fill the wells of the new plate (or use the old plate with removed water/ PBS from the wells) with the full growth medium (100 µL).
   NOTE: Befor the next step, take 200 µL tips, remove 1/5 of each tip end, and sterilize before use.
7. Transfer the cell spheres to the wells of the multi-well plate by using sterile pipette tips with a cut end. Take 200 µL tips, remove 1/5 of each tip end, and sterilize before use (**Figure 4C**).
   NOTE: This step may be difficult, as the liquid flow during the dish/plate flipping can damage the spheres.
8. Incubate the transferred spheres for 1 day in the multi-well plate at 37 °C before performing any further experiments (for example, compound addition, cytotoxicity assays, sphere introduction to equivalents) (**Figure 4D**).

2. The method of limiting cell adhesion
   1. Before the cell seeding, cover the wells of the U-bottom plate with a surfactant solution (e.g., Pluronic F-127, polyethylene glycol, polyvinyl alcohol)[21]. Prepare and add 100 µL of 1% surfactant solution in PBS into each well. Incubate the plate with the solution for 24 h at 37 °C (**Figure 5A-C**). Store the plate longer if needed but maintain the fluid level by adding more PBS buffer.
   2. Prepare the cell suspension in the desired cell density in 50 µL per well (recommended cell density while seeding 2 x 10^5/mL, **Table 5**).
   3. Remove the surfactant solution from the wells before seeding the cells to avoid cell membrane disruption by lysis (**Figure 5D**).
   4. Add the cell solution to the plate and incubate for 24 h at 37 °C to reach cell aggregates (**Figure 5E**). After approximately 1-3 days, spheres will be formed (**Figure 5F**) and are ready to use for any further experiments.

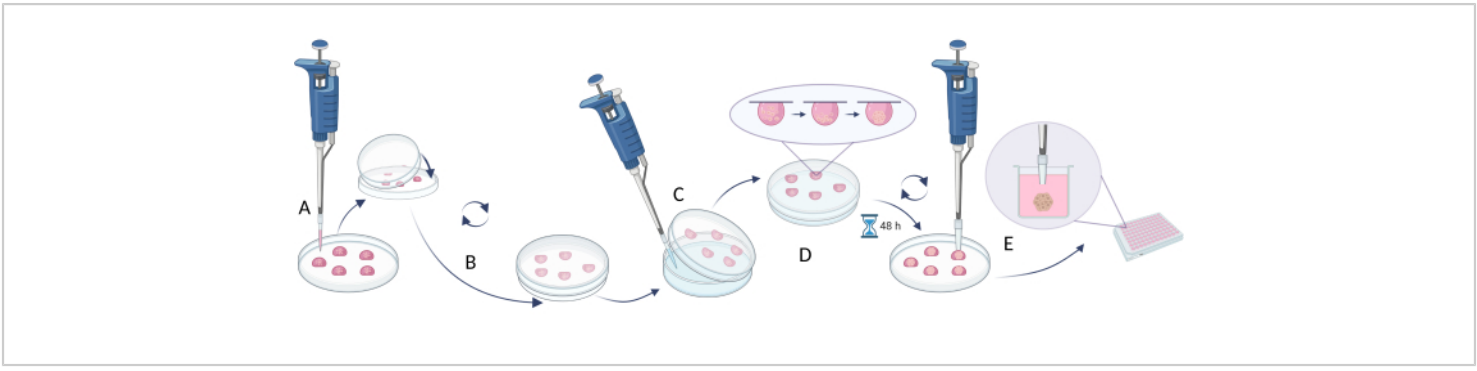

Figure 4: The hanging drop method. (**A**) pipetting cell suspension on the lid and covering the lid with the bottom part of the dish; (**B**) rotating the dish to create the hanging drops; (**C**) addition of water/PBS to the bottom part of the dish (limiting liquid evaporation); (**D**) incubation of the dishes with hanging drops to create cell spheres; (**E**) collection of droplets with formed spheres and stabilization of transferred spheres in multi-well plates. The figure was created with Biorender.com. Please click here to view a larger version of this figure.

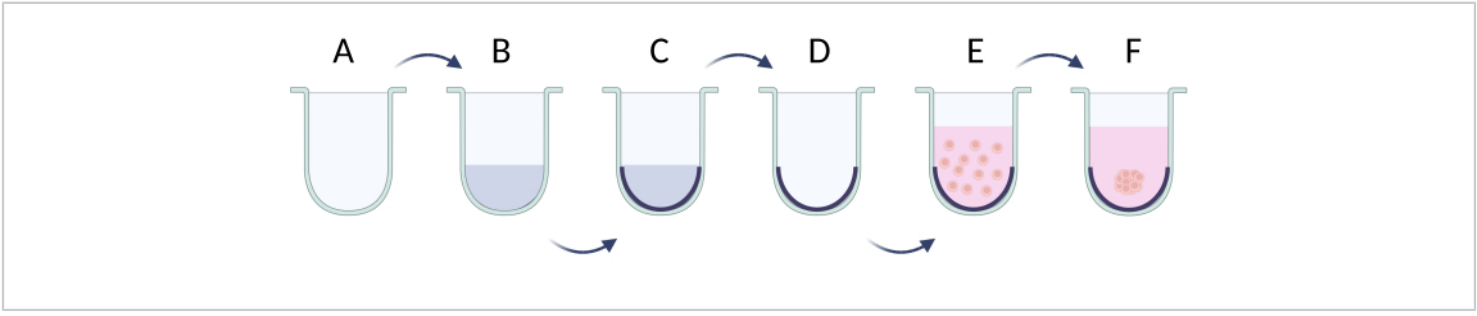

Figure 5: Step-by-step preparation of spheres by the limiting cell adhesion method. (**A**) U-bottom well; (**B**,**C**) limiting cell attachment by surfactant solution; (**D**) removing solution from wells; (**E**) seeding cells; (**F**) aggregation of cells and formation of a sphere. The figure was created with Biorender.com. Please click here to view a larger version of this figure.

3. Preparation of full-thickness skin equivalents

NOTE: The development of the full-thickness (epidermis and dermis) skin equivalents can be divided into three steps (**Figure 6**): the preparation of the artificial dermis layer with the occurrence of typical dermal cells (such as fibroblasts and mast cells, **Figure 6A**), seeding of cells included in the artificial epidermis (mostly keratinocytes and melanocytes, **Figure 6B**) and vertical growth of keratinocytes with a possible keratinization process (formation of the stratum corneum, **Figure 6C**). The preparation of full-thickness skin equivalents is described in step 3.1 (3.1.1-3.1.10). If a less advanced skin equivalent is needed (e.g., only the epidermis type), the selected cell type (such as keratinocytes) can be seeded directly onto the commercially available collagen or polycarbonate membranes and also cultivated

with the possibility of inducing the keratinization process (move directly to steps 3.1.9-3.1.10).

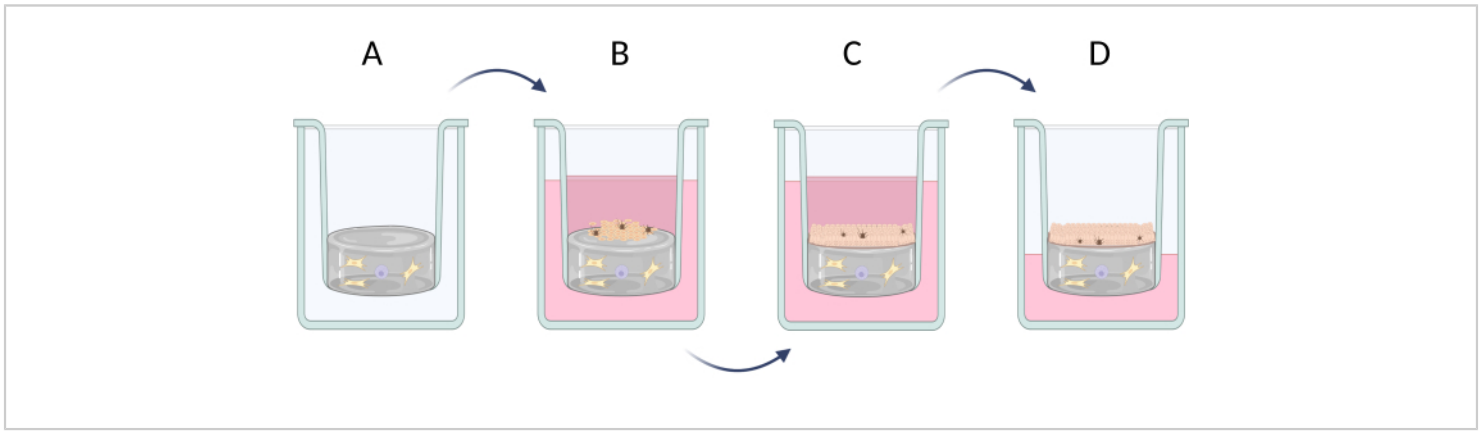

Figure 6: Step-by-step preparation of full-thickness skin equivalents in inserts. (**A**) preparation of the pseudo-dermis layer with dermal cells, (**B**) seeding of epidermal cells, (**C**) further culture of equivalent in medium, (**D**) air-liquid interface culture promotes the formation of stratified epithelium. The figure was created with Biorender.com. Please click here to view a larger version of this figure.

1. Preparation of full-thickness skin equivalents in a 24-well plate
    1. Place the tubes with water, PBS (10x), 1 M NaOH, and type I collagen solution on ice.
    2. Determine a proper number of dermal cells (e.g., fibroblasts and mast cells; in a ratio of 10:1) to be seeded in the hydrogel. Transfer a proper number of dermal cells to a 1.5 mL tube (500 µL of fibroblasts and 500 µL of mast cells, according to cell densities in **Table 5**) and centrifuge the cells (300 x *g*, 3 min, RT).
    3. Remove the supernatant and gently resuspend the cells in the mixture of 695 µL of water/100 µL of PBS (10x)/ 5 µL of NaOH.

        NOTE: If 1 mL of the total solution is not enough, use **Table 6** to recalculate the volumes of each reagent.
    4. Add 200 µL of collagen solution to the mixture and gently mix it by pipetting.

        NOTE: Be careful, as the consistency of the mixture will be dense.
    5. Add 200 µL of the prepared mixture to the insert in 24-well plate. For a model without the stratum corneum, add 500 µL to each well of the 24-well plate.
    6. Incubate the plate for 10 min at room temperature (RT) and then transfer it to the incubator for 30 min.

        NOTE: Check if the hydrogel has polymerized before any other action.

7. Before the cell seeding on the surface of the hydrogel, rinse it with a PBS buffer (500 µL/well).

8. Determine the proper number of epidermal cells (e.g., keratinocytes and melanocytes; in a ratio of 15:1) to be seeded on top of the hydrogel. Prepare the cell mixture in 500 µL of the DMEM medium supplemented with 10% FBS (add 250 µL of keratinocytes and 250 µL of melanocytes, cell densities are mentioned in **Table 4**) and add it gently to the wells.

   NOTE: In some cases, it is better to first seed melanocytes and allow them to spread well on the hydrogel, and after an additional 24 h, remove the medium and seed keratinocytes. In that case, add 250 µL of cell suspension and 250 µL of the medium.

9. Incubate the plates at 37 °C for 2-5 days, depending on the speed of cell growth, with medium exchange (with a decreasing concentration of FBS from 10% up to 1%) every 48 h and cell monitoring performed on the optical microscope.

10. If the keratinization process is to be induced after obtaining a monolayer of keratinocytes on top of the hydrogel, use the FBS-free medium additionally supplemented by calcium ions and L-ascorbic acid for an additional 2-7 weeks (in the concentration of 1.5 µM $CaCl_2$ and 50 µg/mL L-ascorbic acid).

    NOTE: The time of the incubation depends on the used keratinocytes and their differentiation speed.

Reagent	The equation for calculating the volume of a reagent	Example calculations (for a final volume = V_{total} of 1 mL)
type I collagen solution	$V_{collagen}(mL) = \frac{2(\frac{mg}{mL}) \cdot V_{total}(mL)}{C_{collagen}(\frac{mg}{mL})}$	($C_{collagen}$ = 10 mg/mL) 0.2 mL = 200 µL
PBS (10x)	$V_{PBS(10x)}(mL) = \frac{V_{total}(mL)}{10}$	0.1 mL = 100 µL
1 M NaOH	$V_{NaOH}(mL) = V_{collagen}(mL) \cdot 0.025$	0.005 mL = 5 µL
sterile H_2O	$V_{H_2O}(mL) = V_{total} - (V_{collagen} + V_{PBS(10x)} + V_{NaOH})$	0.695 mL = 695 µL

Table 6: Calculation of reagent volumes required for the 2 mg/mL collagen type I hydrogel preparation.

4. Identification of cell types in a 3D skin model by cell staining methods

NOTE: To confirm that the developed skin model consists of the expected cells it is good to perform cell staining. It is a crucial step before any target experiments can be performed on a given model[22]. In the case of 3D skin models, it is necessary to embed a given model in paraffin and to prepare

microscopic slides with the artificial tissue cut on a microtome (step 4.1) before cell staining (**Figure 7**).

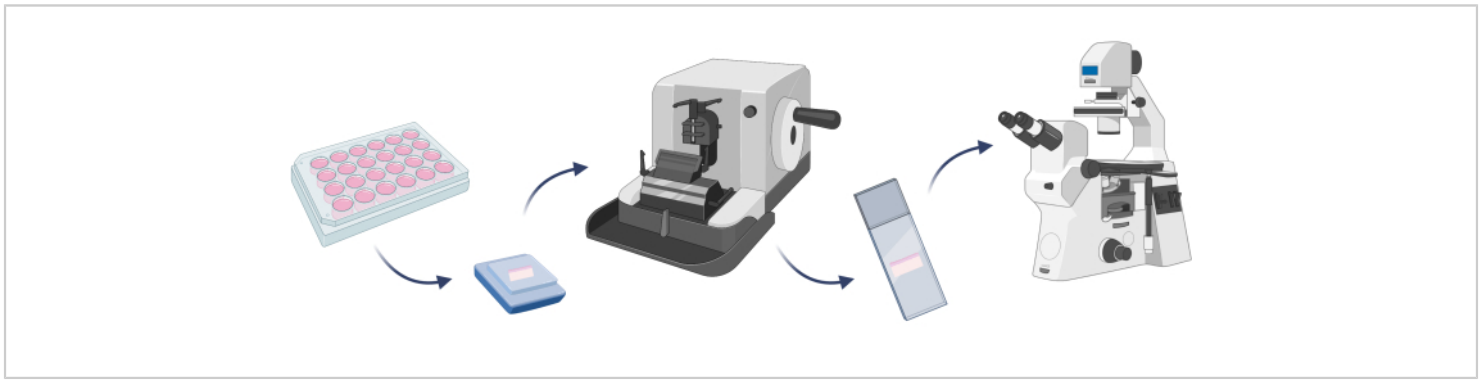

Figure 7: Basic steps of a 3D skin model embedding, cell staining, and microscopic observations. The figure was created with Biorender.com. Please click here to view a larger version of this figure.

1. Embedding of 3D skin models
    1. Wash the skin equivalent with PBS for 5 min at RT twice and fix it using a 3.7% paraformaldehyde solution in PBS (30 min, RT). Repeat the washing step with PBS.
    2. Before embedding, dehydrate the skin equivalent by incubating in increasing concentrations of ethanol solutions: 50% (15 min), 70% (15 min), 96% (2x, 30 min), and 99.8% (2x, 30 min).
    3. Put the fixed and dehydrated skin equivalent in the mold filled with paraffin.
    NOTE: Place the equivalent in an appropriate orientation.
    4. Cover the mold with the cassette and add more paraffin on top. Let it solidify for up to 30 min at RT.
    5. Freeze the paraffin-embedded skin equivalent for at least 1 h at -80 °C.
    6. Turn on the microtome, insert the paraffin-embedded skin equivalent, and cut 5 µm slices. Place the cut artificial tissue slices on the microscopic slides and dry them for at least 8 h at 37 °C.
    7. Immerse the slides in xylene (2x, 10 min), and next rehydrate the slides with the decreasing ethanol concentrations 99.8% (5 min), 96% (5 min), 70% (5 min), and 50% (5 min).
    8. Remove the slides from the ethanol solution and rinse them twice with water (5 min).
    NOTE: Traditional cell staining is performed either by applying specific dyes (Hematoxylin, Eosin[23]) or by using antibodies selectively targeting biomarkers (including collagen 1A2 for fibroblasts, cytokeratin 14 for keratinocytes, melan-A or tyrosinase for melanocytes[24]). A routine hematoxylin and eosin staining can be made by following the

protocols prepared by different companies (step 4.2). On the other hand, if immunofluorescent or immunohistochemical staining is required, the procedure is different and significantly longer (steps 4.3 and 4.4). To avoid unspecific reactions, use the primary antibodies produced in different species and next the dedicated secondary antibodies.

2. Hematoxylin and eosin staining of 3D skin models

    1. Stain the microscopic slide in hematoxylin solution for 3 min at RT.
    2. Wash the slides in an acidified alcohol solution for 1 min.

        NOTE: Prepare the acidified alcohol solution by mixing 2 mL of 35%-38% hydrochloric acid with 98 mL of 99.8% ethyl alcohol.
    3. Next, wash the slide in 0.1% sodium bicarbonate solution to obtain a visible, delicate blue-violet color.

        NOTE: To obtain 0.1% sodium bicarbonate solution, dissolve 100 mg of sodium bicarbonate in 100 mL of ultrapure water.
    4. Wash the slides with 95% ethanol for 1 min.
    5. Stain the microscopic slide in eosin solution for 1 min at RT.
    6. Wash the slides with 95% ethanol for 1 min and 99.8% ethanol for 2 min.
    7. Wash the slides with xylene for 2 min each twice.
    8. Mount in balsam and put a coverslip at the top of the slide. The samples are ready for microscopic observations.

3. Immunofluorescent staining of 3D skin models

    1. Rinse the slide with PBS (5 min).
    2. Prepare the blocking solution (3% bovine serum albumin [BSA] or non-fat milk in a PBS buffer with the addition of 0.1% Triton X-100 and 0.1% Tween 20) and incubate the slide in it for 1 h at RT.
    3. Wash the slides twice with a PBS buffer (5 min).
    4. Dilute the primary antibody in the PBS buffer (according to the producer's recommendations, **Table 7**) and incubate the slides for 1-2 h at RT or overnight at 4 °C.
    5. Wash the slides twice with a PBS buffer (5 min).
    6. Dilute the secondary antibody in the PBS buffer (according to the producer's recommendations, **Table 7**) and incubate the slides for 1 h at RT.
    7. Wash the slides twice with PBS (5 min).
    8. Prepare the dye solution for the nuclei staining (e.g., Hoechst 33342 or DAPI, **Table 7**) and incubate the slides for up to 15 min at RT.
    9. Wash the slides with PBS (5 min).
    10. Mount in balsam, cover the section with a coverslip, and visualize the effects of the cell staining using a fluorescent microscope.

4. Immunohistochemistry staining of 3D skin models

    1. Perform steps 4.3.1-4.3.5.
    2. Additionally, perform a washing step with a buffer appropriate for the enzyme with which the secondary antibody is conjugated.
    3. Dilute the secondary antibody in a buffer appropriate for the conjugated enzyme (according to the producer's recommendations) and incubate the slides for 1 h at RT.
    4. Wash the slides twice with PBS (5 min).

5. Prepare the solution of a proper substrate for the used enzyme and incubate the slides according to the producer's recommendations.
6. Wash the slides with a buffer (5 min) and mount them in balsam.
7. Visualize the effects of the cell staining using bright-field microscopy.

Cell type/ cell organoid detected	Staining agent	Dillution / Concentration
Mast cells	Avidin−Sulforhodamine 101	1 µg/mL
Fibroblasts	Col1A2 antibody produced in rabbit	1:50
	Goat anti-rabbit secondary antibody conjugated with FITC	1:250
Keratinocytes	Cytokeratin 14 antibody produced in mouse	1:50
	Goat anti-mouse secondary antibody conjugated with FITC	1:250
Melanocytes	Melan-A antibody produced in mouse	1:50
	Goat anti-mouse secondary antibody conjugated with FITC	1:250
Nuclei	Hoechst 33342	1 µg/mL
	DAPI	1 µg/mL

Table 7: Concentrations and dilutions of the reagents used for cell staining.

Representative Results

Before starting to create skin models in the laboratory a decision on the type of cells to be used (primary/cell line) and the selection of a proper medium for these cells must be made. Most cell banks recommend and can provide media for all types of cell culture. In the case of a multiculture model, it is necessary to select one medium that suits all of the cell types present in the culture. Some typical cell media used for both primary skin cell culture and most common skin cell lines were gathered in **Table 8**[18,19,20,25,26,27]. Typical media used for the primary cell cultures are rather expensive, and their composition is complex. On the other hand, cell lines are usually satisfied with media with a simple composition. Some cell types (mainly fibroblasts and mast cells) can produce and secrete factors stimulating the growth of other cells (such as keratinocytes and melanocytes)[28,29]. If their presence in a model is planned, additional supplementation of the medium is not necessary.

Cell type	Cell name	Medium	Reference
Keratinocytes	HaCaT cel line	DMEM, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin	According to the seller
	primary Normal Human Epidermal Keratinocytes (NHEK)	Keratinocyte Growth Medium 2 (basal medium + supplement mix)	According to the seller
	primary Human Epidermal Keratinocytes; Normal, Adult (HEKa)	Dermal Cell Basal Medium, 0.4% bovine pituitary extract, 0.5 ng/mL rh- transforming growth factor-alpha, 6 mM L-glutamine, 100 ng/mL hydrocortisone Hemisuccinate, 5 mg/mL rh-insulin, 1 mM epinephrine, 5 mg/mL Apo-Transferrin, 100 U/mL penicillin (if needed), 100 µg/mL streptomycin (if needed)	According to the seller
	primary keratinocytes	DMEM/F-12 (3:1), 1% Ultroser G, 1 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin, 1 ng/mL keratinocyte growth factor, 1% penicillin-streptomycin	18
Melanocytes	primary melanocytes	Medium 254, PMA-Free Human Melanocyte Growth Supplement-2, 1% antibiotic solution	19
	primary melanocytes	RPMI-1640, 10% FBS, 14.7 µg/mL phenol red solution, 1% L-glutamine, 1% penicillin/streptomycin	27
	HEMa-LP cell line	Medium 254, 5 µg/mL rh-insulin, 50 µg/mL ascorbic acid, 6 mM L-glutamine, 1 µM epinephrine, 1.5 mM calcium chloride, 100 U/mL penicillin (if needed), 100 µg/mL streptomycin (if needed)	According to the seller
	primary Normal Human Epidermal Melanocytes (NHEM)	Melanocyte Growth Medium (basal medium + supplement mix)	According to the seller
Fibroblasts	primary Human Tenon's Fibroblasts (HTFs)	EMEM, 5% FBS, 5 ng/mL rh-basic fibroblast growth factor, 5 µg/mL rh-insulin, 50 µg/mL ascorbic acid, 7 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B	28
	primary HTFs, and	DMEM, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B	20.28

	primary human dermal fibroblasts		
	HFF-1 cell line	DMEM, 15% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin	According to the seller
	BJ cell line	EMEM, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin	According to the seller
Mast cells	primary Human skin mast cells (hsMCs)	IMDM, 10% FBS, 1% non-essential amino acids, 226 µM α-monothioglycerol, 100 U/mL penicillin, 100 µg/mL streptomycin	20
	LAD2 cell line	StemPro-34, 2.5% StemPro-34 Nutrient Supplement, 2 mM L-glutamine, 100 ng/mL rh-stem cell factor, 100 U/mL penicillin, 100 µg/mL streptomycin	29
	HMC-1.1 and 1.2 cell lines	IMDM, 10% FBS, 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin	29

Table 8: An overview of the most used media for culturing primary skin cells and cell lines.

Legend: Dulbecco's Minimum Essential Medium (DMEM), Eagle's Minimum Essential Medium (EMEM), Fetal Bovine Serum (FBS), Nutrient Mixture Ham's F-12 (F12), Iscove's Modified Dulbecco's Medium (IMDM), recombinant human (rh), Roswell Park Memorial Institute (RPMI).

In this article, skin models were created with the primary cells of keratinocytes, melanocytes, fibroblasts, and mast cells. They are slightly more demanding than the cell lines, and the recommended media for their culture, which were used for single-cell culture, are: supplemented Keratinocyte Growth Medium 2 (for keratinocytes), supplemented Medium 254 (for melanocytes), supplemented DMEM medium (for fibroblasts) and supplemented IMDM medium (for mast cells). On these media, cells present their typical morphology assigned to their type (representative images are shown in **Figure 8**). In the case of multicultures of two or more cell types, it was important to choose a medium in which all cultured cell types can grow. After a few tests, a DMEM medium with 10% FBS and a 1% antibiotic mixture was selected for preparing the more advanced 3D models of spheres and equivalents.

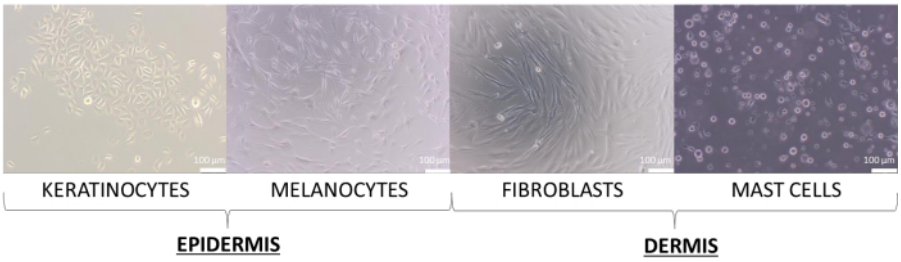

Figure 8: Skin cells present different morphology observed during the 2D monoculture. Scale bars: 100 µm. Please click here to view a larger version of this figure.

Spheres (commonly named spheroids) are one of the simplest 3D models developed by cell and tissue engineering researchers, although spheres created from skin cells are not that popular. Within this model, the creation of both mono- and multi-culture of spheres is possible. Multiple methods for spheres preparation (e.g., with a hanging drop, limiting cell adhesion, magnetic levitation, rotation, microfluidics, etc.) have been described in the literature[30]. Due to the easiness of preparation, low cost, readily available materials as well as equipment, the first two methods are recommended for the three-dimensional (3D) cell model beginners and protocols for their performance can be found above (step 2.1 and 2.2). According to literature[31,32], the most important parameters in these methods are the cell number, volume of the cell suspension, and time of incubation.

Spheres can be created with different numbers of cells, but the cell density for seeding should be optimized for every cell type separately as well as for cell co-cultures. The conducted optimization process for monocultures of keratinocytes and fibroblasts revealed that seeding 1 x 10^4 cells per well provided the best results for both cell types. Spheres presented in **Figure 9** were prepared using the method of limiting cell adhesion in the U-bottom plates (step 2.2). It is advised to prepare at least 4 wells with technical repetitions (optimal is 6 wells). Spheres consisting of 1 x 10^4 cells were easier to manipulate as they were visible in the wells. As a consequence, it was even possible to remove the old medium from wells during assays without draining the spheres. After the described operations, the spheres' shapes were mostly unchanged and repeatable. The stability of bigger spheres was low during the process. It is also worth mentioning that different cell types can form spheres of different colors (e.g., keratinocytes form darker spheres, while fibroblast spheres are significantly lighter).

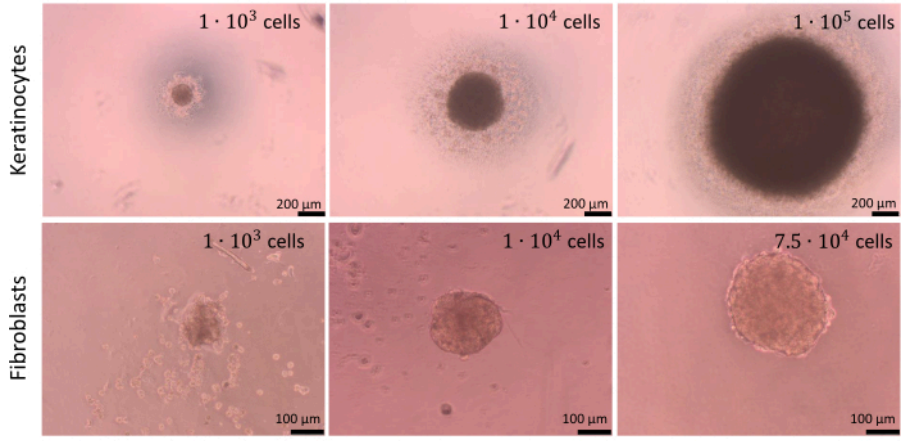

Figure 9: Creation of spheres. Spheres created by different types and numbers of skin cells with the use of the limiting cell adhesion method. Scale bars (top panel): 200 µm. Scale bars (bottom panel): 100 µm. Please click here to view a larger version of this figure.

As mentioned in the hanging drop method (step 2.1), at a certain point spheres require transferring from the lid to the well. This process can be potentially damaging for the spheres. Thus, for this step, high accuracy of work is essential. The created spheres are prone to losing proper shape if they are not carefully handled (**Figure 10**). The first image (**Figure 10A**) presents a good sphere equally rounded on all sides. In the second and third images (**Figure 10B**,**C**) a gentle loss of shape by the sphere was observed, but the cell aggregate remains rounded. The last three images (**Figure 10D-F**) show different phases of sphere damage. Gaining experience is needed to obtain repeatability of the created sphere shapes and structures. With the first attempts to develop the spheres, it is recommended to use the method of limiting cell adhesion for inexperienced researchers (step 2.2) as it provides more comparable results with the limited influence of the researcher's activity.

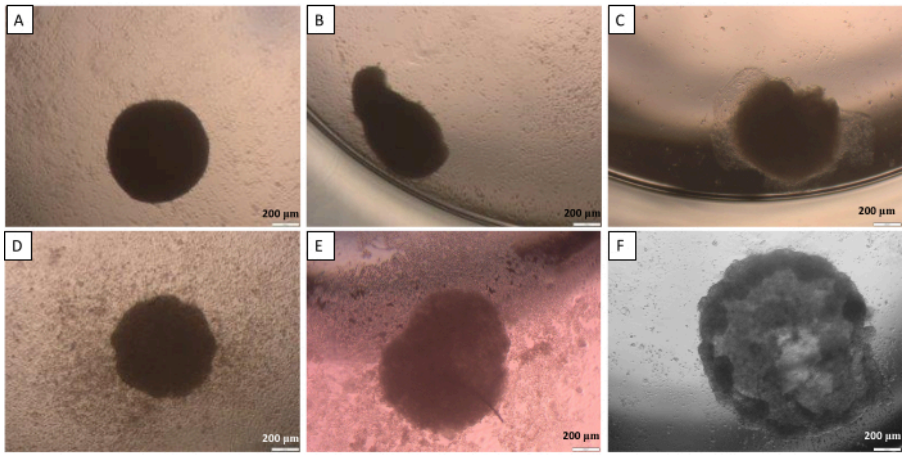

Figure 10: Possible difficulties in sphere transfer from the lid to the well - the hanging drop method. (**A**) a good sphere, (**B**,**C**) slightly damaged spheres, (**D-F**) highly damaged spheres. Images were taken 24 h after the transfer. Scale bars: 200 µm. Please click here to view a larger version of this figure.

Equivalents are far more advanced 3D models of artificial skin than spheres. During the construction process of a skin model, various aspects should be considered, including the number of layers in the model (only epidermis, only dermis, dermis with epidermis and stratum corneum), used cell types, applied materials, preferable size of the equivalent, type of research in which it will be further used, etc.[14]. The skin equivalents can be arranged in special inserts placed in the standard multi-well plates of a desired size (96-, 48-, 24-wells, etc.). Even though inserts are easier to transfer from one well to another and during the medium exchange, the equivalent cannot be damaged; they are quite expensive. If the model does not require the presence of the stratum corneum, a cheaper solution is to prepare the equivalent in a well of the multi-well plate.

The artificial dermis layer is typically constructed as a scaffold-based model by using natural (gelatin, collagen, fibrin, hyaluronic acid, chitosan-alginate, etc.) or synthetic (polyethylene glycol diacrylate and polylactic acid) hydrogels[33]. To be similar to the real skin dermis, this layer must contain mostly water with some extracellular matrix (ECM) components (including collagen or fibronectin), which mediate cell binding, cell-cell interactions, and other cell actions[34]. In this research, type I collagen was selected as it is easy to prepare in the form of hydrogel, and its flexible structure ensures the easiness of further potential research activities (e.g., transfer of the equivalent from one dish to another). The solution of type I collagen obtained from rat tail is normally prepared by powder dissolution in 20 mM acetic acid. To achieve the collagen polymerization step, it is necessary to provide appropriate pH conditions ranging from 6.5-7.5. This can be assured by the addition of a strict amount of sodium hydroxide. For easiness, some companies have introduced specific calculations, which may help determine the exact volumes needed to prepare such hydrogels (**Table**

6). Although in literature, different concentrations of collagen in the hydrogels can be encountered (for example, 0.5-2 mg/mL[35]; 5-30 mg/mL[36]; low and high collagen content[37]), in the described model, the 2 mg/mL solution was used as the hydrogel had still a flexible structure, but was compact enough to be moved out from the well if necessary.

To prepare a quite realistic full-thickness skin, equivalent cells should be seeded in the proportion possibly as close to this present in our body. In the case of the epidermis, depending on the body site, the relationship between a melanocyte and a pool of associated keratinocytes is a ratio of about 1:36, which is defined as the Epidermal Melanin Unit (EMU)[38]. Thus, the applied proportion in the artificial epidermis was 1 melanocyte to 15 keratinocytes (**Table 5**). To create an artificial dermis layer, the collagen type 1 hydrogel was used in which fibroblasts and mast cells were incorporated in the proportion of 1 mast cell to 10 fibroblasts. Each layer of the constructed equivalent is possible to be monitored in real-time on the inverted optical microscope by changing the depth of the observed sample (exemplary images are shown in **Figure 11**).

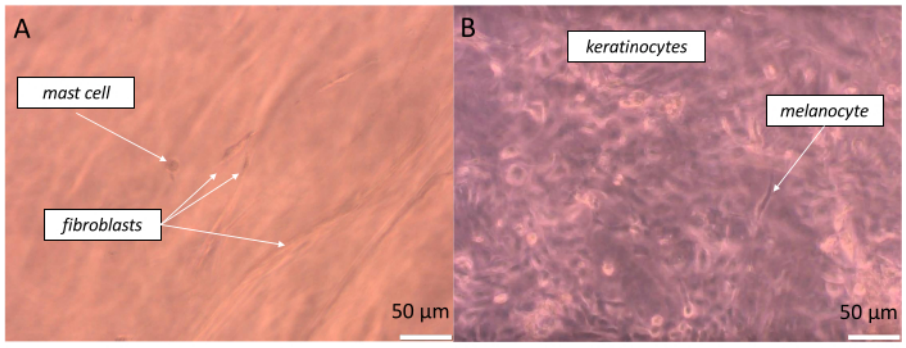

Figure 11: Real-time observation of different cells in particular layers of the created full-thickness skin equivalent. (**A**) pseudo-dermis and (**B**) pseudo-epidermis) as visualized by the bright-field microscopy. Scale bars: 50 µm. Please click here to view a larger version of this figure.

More accurate observations with the confirmation of obtaining the intended structure of the model can be made by performing the staining of the equivalent. The fixed equivalent needs to be first embedded in paraffin and next cut on a microtome. The slides with thin artificial tissue can be later stained with different dyes, including hematoxylin and eosin (basic staining performed in medical laboratories). Thanks to that action, it is possible to distinguish the artificial dermis and epidermis in the equivalents as well as to identify individual skin cells (**Figure 12**). In **Figure 12**, not only the specific cell types are shown, but it is also possible to see the keratinocytes in different phases of the cell division process (telophase and metaphase). In the case of mast cells, specific granules are well-recognizable inside the cell. These images initially confirm that the created skin equivalent is alive (the cells are growing in it) and they are able to function normally

in the developed model. However, with the 3D epidermis and full-thickness models of the skin, it is especially important to check the quality and functionality of the obtained construct. To check the permeability of the stratum corneum, the Transepithelial Electrical Resistance (TEER) measurements or Lucifer-Yellow staining should be applied[39,40]. Moreover, in a properly composed artificial skin, specific markers should be present including differentiation markers (e.g., Filaggrin, Involucrin, Loricrin, Keratin 10, Keratin 5, lipid classes comprising ceramides), dermal-epidermal junction markers (e.g., Type IV collagen, Laminin V, Alpha6Beta4-integrin, BP antigen)[41], tight-junction markers in the epidermal layers (e.g., claudin-1, occludin, zonula occludens protein (ZO)-1)[42] as well as basal layer proliferation markers (Ki67)[41].

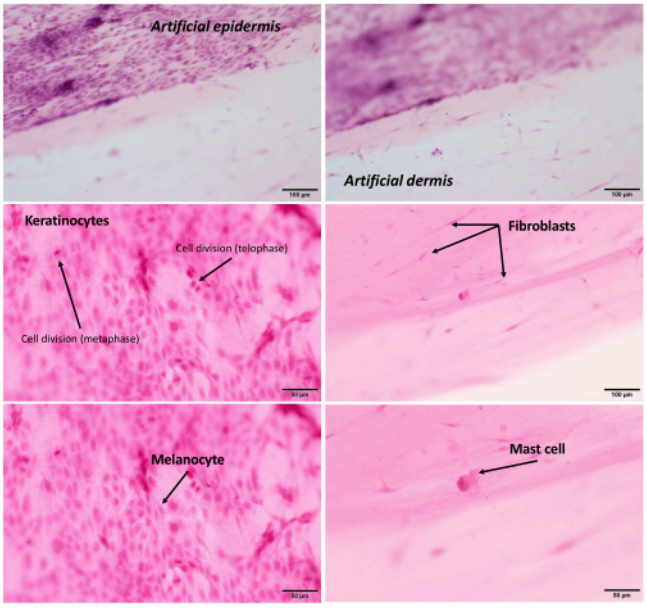

Figure 12: Skin cell morphology and functioning. Overview of skin cell morphology and functioning (observation of cell divisions) in the full-thickness skin equivalent stained with hematoxylin and eosin. Scale bars: 100 µm (top panel), 50 µm (middle panel, left), 100 µm (middle panel, right), 50 µm (bottom panel). Please click here to view a larger version of this figure.

The most frequently used way to confirm the presence of a biomarker is by carrying out specific staining, such as immunohistochemical or immunofluorescent. Different antibodies and fluorescent dyes can be applied for microscopic visualization of particular cells in the models. The result of exemplary staining of cells in culture can be seen in **Figure 13**. To observe keratinocytes, an antibody against cytokeratin 14 was used. In the case of melanocytes, it was melan-A specific antibody. Collagen 1A2 antibody was used to stain fibroblasts and the fluorescent Sulforhodamine 101 conjugated with avidin detected heparin present in mast cells.

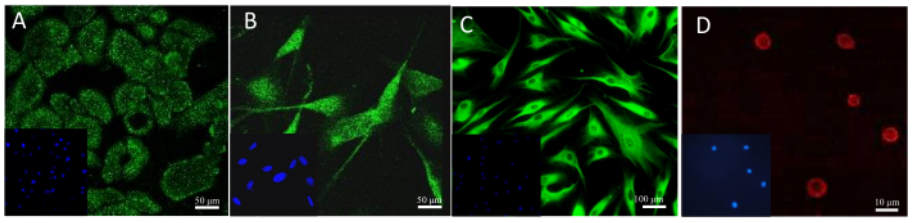

Figure 13: Results of fluorescent cell staining. (**A**) Cytokeratin 14 in keratinocytes. Scale bar: 50 µm. (**B**) Melan-A in melanocytes. Scale bar: 50 µm. (**C**) Collagen 1A2 in fibroblasts. Scale bar: 100 µm. (**D**) Heparin in mast cells. Scale bar: 10 µm. Please click here to view a larger version of this figure.

Discussion

This article presents the methodology that can be applied to prepare one's own advanced artificial skin models. It is a good solution whenever the planned research needs strictly defined research models that may turn out to be unavailable on the market or very expensive. As mentioned earlier, several commercial skin equivalents are available on the market (e.g., EpiSkin, EpiDerm FT). However, their cost (€100-€400 per piece) and delivery time (a few days-weeks) may encourage the researcher to attempt to prepare such a model on their own. The proposed procedures are easy to perform even for inexperienced scientists, and at the same time, allow to obtain very advanced skin models. It is worth emphasizing that the decision on the cellular composition of a given model is fully dependent on the researcher. Apart from the created model, it can be further developed and improved, which opens up completely new research perspectives. In the case of commercial models, it is necessary to buy a different equivalent.

Although the 3D cell cultures may be advanced with multiple cell types, easy to handle and accessible, they are still just artificial models which cannot fully recreate the complexity and functionality of the tissue (e.g., immunological functions, vascularization). That is why, in most studies, several models are required to confirm the obtained results. Some advantages and disadvantages of these models were gathered in **Table 9**, as well as their limitations. On the other hand, commercial models guarantee high qualitative standards with reproducibility of experiments and comparability of data between the laboratories. To implement the use of a new compound for research, it will certainly be necessary to purchase the appropriate commercial equivalent. But at the preparatory stage, such a self-made 3D model of the skin (multicell type sphere or equivalent) can help to reduce the number of experiments needed to be carried out on a commercial equivalent. The goal of producing and using the described models is not to bypass the need to apply certified research models but to facilitate research and reduce related expenses.

Compared pair of models	Advantages	Disadvantages
cell culture vs. animals	Minimalized animal suffering	Limited information on the influence of a tested factor on the whole body
	High experiment standardization - better reproducibility of the results	A single model is not enough to reflect the processes occurring in the body
	No side effects for the whole organism	-
	Better control over the conditions of the experiment	-
	Possibility of automatization (e.g., bioprinting)	-
	Lower costs	-
	The small size of the sample needed	-
	Limited amount of waste generated	-
3D vs. 2D cultures	Better reflect the full organism	Time-consuming culture
	Possibility to create a functional tissue	Higher costs
	Possibility to create a model tailored to the needs of the carried research	Spontaneous formation of a 3D structure is almost not possible
	-	Lack of standardized tests to quantify the effects of various compounds
	-	Limited access to different 3D cultures available on the market
cell line vs. primary cells	Certificated and approved models	Only a limited number of cell lines are available
	High experiment standardization - better reproducibility of the results	Limited possibility to obtain several types of cells from the same donor
	Longer life span	May possess changed properties from the native cells
	Rather quick proliferation rate	Frequently disturbed functionality of cells

	Less sensitive to several activities (e.g., freezing, centrifugation)	-

Table 9: Comparison of the usage of different models in research - advantages vs. disadvantages

Several articles describe how to prepare 3D skin models (apart from review articles summarizing commercially available models[14,43,44], they are usually focused on a single methodology to obtain spheres[45] or equivalents[46]).

In this article, two methodologies were described for the sphere formation with skin cells. The hanging drop method is widely used, but its repeatability and stability may be insufficient in some cases. Most steps require specific actions, such as high-speed work due to the evaporation of water from droplets during transfer. Gentle movements are also recommended, as lack of such a skill may result in cell aggregate damage[31,32]. Thus, an easier method for sphere preparation is focused on limiting cell adhesion. The absence of a good surface for cell attachment promotes higher interactions between cells. As a consequence, cell aggregates are generated. Its repeatability is much higher as there is no necessity for sphere transferring. With these methods, the optimal number of skin cells to create a sphere was established at 1 x 10^4 cells/sphere.

Next, procedures describing the preparation of skin equivalents were shown. Their appearance and functionality in research may strongly depend on the elements from which they are constructed, including cells (**Table 2**), scaffolds and media. The 3D scaffolds used for the preparation of artificial skin can be divided into synthetic hydrogels and those formed from natural sources. Depending on the used material and its properties to compose the hydrogel, the necessity to additionally supplement the medium may occur. Synthetic hydrogels require incorporating bioactive molecules (proteins, enzymes, and growth factors) into the synthetic hydrogel network to mediate specific cell functions[47]. The main approaches presented in the literature for achieving controlled delivery of growth factors to hydrogels include direct loading, electrostatic interaction, covalent binding, and the use of carriers[48]. Hydrogels formed from natural sources such as ECM proteins and polymers can generate fluid pathways throughout the 3D scaffold, accelerating the distribution of nutrients; thus, there is no need for additional supplementation of the medium. Investigations have shown that small molecules (like cytokines and growth factors) and macromolecules (including glycosaminoglycans and proteoglycans) can be transported through the ECM by diffusion[47]. However, the molecular diffusion of oxygen, nutrients, and other bioactive molecules may be hindered by the properties of the ECM hydrogel itself. Lower diffusion was correlated with the higher thickness of the hydrogel but also with a very high concentration of collagen[37]. In this study, to create the skin equivalent, a low collagen concentration equal to 2 mg/mL was used, which suggests that the molecular diffusion through the hydrogel should be good and rapid. Thus, no additional supplementation to the medium at this stage nor to the hydrogel itself was provided. To mimic the dermis, mast cells and fibroblasts (1:10) were embedded into the collagen hydrogel. Next, melanocytes and keratinocytes (1:15) were seeded onto the hydrogel and the whole equivalent was cultured in the medium. It is worth mentioning that the basic medium is

composed of several amino acids, inorganic acids, and vitamins, and it is additionally supplemented with serum (consisting of multiple: growth and attachment factors for cells, lipids, hormones, nutrients, and energy sources, carriers, binding and transfer proteins, etc.). To achieve the proper structure of the epidermis, different supplements to the medium should be added at a certain time. The most important stimulator to initiate epidermal differentiation is calcium, as it activates intracellular signaling. Ascorbic acid stimulates a similar signaling pathway as the one mediated by calcium, but its effect is also accompanied by enhanced ascorbate transport and prevention of hydrophilic antioxidant depletion[41]. Furthermore, the differentiation of cells was improved when other components were added to the medium (such as caffeine, hydrocortisone, triiodothyronine, adenine, and cholera toxin)[41,44]. It is important that the prepared models should always be checked for the presence of a given cell type in the appropriate layer. The presence of all four types of skin cells was confirmed in the structure of the created equivalent by H&E staining.

The most common problem encountered is the delicacy and intuition in the handling of the obtained models. Some difficulties may be connected with the cell sphere formation as well as with the hydrogel preparation. During the cell culture, several other problems can also occur; these include microbial infections, low proliferation rate of cells, aging of primary cells used in the models, maximum cultivation time of 2D and 3D models reconstructed from primary cells vs. cell lines, etc. In **Table 10**, some practical advice were gathered on what to do when one of the following problems is encountered.

Common problems in cell culture	Suggestions
Microbial infection	If a microbial infection occurs in one of the flasks/dishes with cells, it is better to remove the infected culture as fast as possible (not to contaminate the remaining flasks/ dishes with cells). Refreeze a new vial with cells. If the infection returns, it is good to try to widen the spectra of the applied antibiotics and increase their concentration.
Low proliferation rate of cells	Some cells have a long doubling time. To stimulate their proliferation, several cell-specific growth factors can be added to the basic medium. Also increasing the concentration of FBS or L-glutamine in the basal medium may help to stimulate the growth of the cells.
Aging of primary cells used in the models	After a few passages, the primary cells enter senescence and stop dividing. To overcome this problem in the models, it is recommended to use the cells from as early passage as possible to build the model.
Maximum cultivation time of 2D and 3D models reconstructed	The time of cultivation of a model depends strongly on the type of used cells. With primary cells, the time of cultivation will be shorter due to their short life span.

from primary cells vs. cell lines	
Difficulties in the cell sphere formation	Some cells may require a longer time for sphere formation. If after a few more days the spheres have not been formed, collect the cells from the sample and check their viability with, for example, trypan blue staining.
Problems with sphere stability	If the spheres are not stable and get destroyed while handling, try to create spheres from a lower number of cells. Make sure to always gently transfer the dishes in which the spheres are growing.
Difficulties with the hydrogel preparation	Check if the proportion of the ingredients (water, PBS [10x], NaOH, collagen type 1) was correct. The stock solution of collagen is usually very dense, thus make sure to slowly pipet it. Air bubbles disturb the morphology of the hydrogel, thus reverse pipetting of the gel may help with this issue.

Table 10: Cell culture troubleshooting

The established models after fabrication can be used in multiple fields, beginning with (1) cytotoxicity and genotoxicity experiments of novel compounds with biological activity for use in drugs and cosmetics[49], (2) experiments with various factor stimulation[50], (3) basic research increasing our knowledge about skin cells, their biological functions, interactions with other cells and the environment[51,52], (4) research on selected disease entities where a specific type of cell can be introduced into the created model (cancer cells, cells with a mutation in a given gene, *etc.*[14,53]) and many more. It is needless to say that the application of these models stays in agreement with the 3Rs principle for more ethical use of animals in product testing and scientific research and does not violate the prohibition law of cosmetic product testing on animals.

Disclosures

The authors declare no conflicts of interest.

Acknowledgments

The authors are grateful for the financial support given by the Warsaw University of Technology from the program 'Excellence Initiative - Research University' in the form of two grants: POB BIB BIOTECHMED-2 start (no. 1820/2/ZO1/POB4/2021) and the Rector's grant for Student Research Groups (SKIN-ART, no. 1820/116/Z16/2021). Moreover, the Authors would like to acknowledge the support received from Prof. Joanna Cieśla and the Chair of Drug and Cosmetics Biotechnology as well as the Biotechnology Science Club 'Herbion' at the Faculty of Chemistry, Warsaw University of Technology. Special thanks go to Dr. Michał Stepulak for providing the compound Pluronic F-127.

References

1. Farage, M. A., Miller, K. W., Elsner, P., Maibach, H. I. Characteristics of the aging skin. *Advances in Wound Care.* **2** (1), 5-10 (2013).
2. Zhu, H., Alikhan, A., Maibach, H. I. *Biology of Stratum Corneum: Tape Stripping and Protein Quantification.* In *Textbook of Aging Skin.* Farage, M. A., Miller, K. W.,

Maibach, H. I., Eds. Springer Berlin Heidelberg: Berlin, Heidelberg (2015).

3. Boer, M., Duchnik, E., Maleszka, R., Marchlewicz, M. Structural and biophysical characteristics of human skin in maintaining proper epidermal barrier function. *Postepy Dermatogogii I Alergologii.* **33** (1), *1.*-5 (2016).
4. De Falco, M., Pisano, M. M., De Luca, A. *Embryology and Anatomy of the Skin.* In *Skin Cancer: A Practical Approach*; Baldi, A., Pasquali, P., Spugnini, E. P., Eds. *Current Clinical Pathology.* Springer, New York (2014).
5. Dehdashtian, A., Stringer, T. P., Warren, A. J., Mu, E. W., Amirlak, B., Shahabi, L. *Anatomy and Physiology of the Skin.* In *Melanoma: A Modern Multidisciplinary Approach.* Riker, A. I., Ed.; Springer International Publishing: Cham. 15-26 (2018).
6. Graham, H. K., Eckersley, A., Ozols, M., Mellody, K. T., Sherratt, M. J. *Human Skin: Composition, Structure and Visualisation Methods.* In *Skin Biophysics*; Limbert, G., Ed. *Studies in Mechanobiology*, *Tissue Engineering, and Biomaterials.* Springer International Publishing: Cham. **22**, 1-18 (2019).
7. Piasek, A. M., Musolf, P., Sobiepanek, A. Aptamer-based advances in skin cancer research. *Current Medicinal Chemistry.* **30** (8), 953-973 (2023).
8. Bergfeld, W. F., Belsito, D. V., Marks, J. G., Andersen, F. A. Safety of ingredients used in cosmetics. *Journal of the American Academy of Dermatology.* **52** (1), 125-132 (2005).
9. Hubrecht, R. C., Carter, E. The 3Rs and humane experimental technique: Implementing change. *Animals.* **9** (10), 754 (2019).
10. Sreedhar, D., Manjula, N., Ajay, P., Shilpa, P., Ligade, V. Ban of cosmetic testing on animals: A brief overview. *International Journal of Current Research and Review.* **12** (14), 113-116 (2020).
11. Silva, R. J., Tamburic, S. A state-of-the-art review on the alternatives to animal testing for the safety assessment of cosmetics. *Cosmetics.* **9** (5), 90 (2022).
12. Xu, Y., Shrestha, N., Préat, V., Beloqui, A. An overview of *in vitro*, ex vivo and *in vivo* models for studying the transport of drugs across intestinal barriers. *Advanced Drug Delivery Reviews.* **175**, 113795 (2021).
13. Boelsma, E., Ponec, M. *Basics (Guidelines) on Cell Culture Testing for Topical/Dermatological Drugs/ Products and Cosmetics With Regard to Efficacy and Safety of the Preparations.* In *Dermatopharmacology of Topical Preparations.* Gabard, B., Surber, C., Elsner, P., Treffel, P., Eds. Springer Berlin Heidelberg, Berlin, Heidelberg. 37-57 (2000).
14. Suhail, S., Sardashti, N., Jaiswal, D., Rudraiah, S., Misra, M., Kumbar, S. G. Engineered skin tissue equivalents for product evaluation and therapeutic applications. *Biotechnology Journal.* **14** (7), 1900022 (2019).
15. Sobiepanek, A. et al. Novel diagnostic and prognostic factors for the advanced melanoma based on the glycosylation-related changes studied by biophysical profiling methods. *Biosensors and Bioelectronics.* **203**, 114046 (2022).
16. Yang, H., Sun, L., Liu, M., Mao, Y. Patient-derived organoids: A promising model for personalized cancer treatment. *Gastroenterology Report.* **6** (4), 243-245 (2018).

17. Baran, J. et al. Mast cells as a target-A comprehensive review of recent therapeutic approaches. *Cells.* **12** (8), 1187 (2023).

18. Kosten, I. J., Buskermolen, J. K., Spiekstra, S. W., de Gruijl, T. D., Gibbs, S. Gingiva equivalents secrete negligible amounts of key chemokines involved in langerhans cell migration compared to skin equivalents. *Journal of Immunology Research.* **2015**, 627125 (2015).

19. cieżyńska, A. et al. A novel and effective method for human primary skin melanocytes and metastatic melanoma cell isolation. *Cancers.* **13** (24), 6244 (2021).

20. Kröger, M. et al. *In vivo* non-invasive staining-free visualization of dermal mast cells in healthy, allergy and mastocytosis humans using two-photon fluorescence lifetime imaging. *Scientific Reports.* 10 (1), 14930 (2020).

21. Liu, D., Chen, S., Win Naing, M. A review of manufacturing capabilities of cell spheroid generation technologies and future development. *Biotechnology and Bioengineering.* **118** (2), 542-554 (2021).

22. Rossi, A., Appelt-Menzel, A., Kurdyn, S., Walles, H., Groeber, F. Generation of a three-dimensional full thickness skin equivalent and automated wounding. *Journal of Visualized Experiments: JoVE.* **96**, 52576 (2015).

23. Kim, K., Kim, J., Kim, H., Sung, G. Y. Effect of α-lipoic acid on the development of human skin equivalents using a pumpless skin-on-a-chip model. *International Journal of Molecular Sciences.* **22** (4), 2160 (2021).

24. Curto, E. V., Lambert, G. W., Davis, R. L., Wilborn, T. W., Dooley, T. P. Biomarkers of human skin cells identified using DermArray DNA arrays and new bioinformatics methods. *Biochemical and Biophysical Research Communications.* **291** (4), 1052-1064 (2002).

25. Godwin, L. S. et al. Isolation, culture, and transfection of melanocytes. *Current Protocols in Cell Biology.* **63**, 1.8.1-1.8.20 (2014).

26. Przekora, A., Zarnowski, T., Ginalska, G. A simple and effective protocol for fast isolation of human tenon's fibroblasts from a single trabeculectomy biopsy - a comparison of cell behaviour in different culture media. *Cellular & Molecular Biology Letters.* **22**, 5 (2017).

27. Rådinger, M., Jensen, B. M., Kuehn, H. S., Kirshenbaum, A., Gilfillan, A. M. *Generation, Isolation, and Maintenance of Human Mast Cells and Mast Cell Lines Derived from Peripheral Blood or Cord Blood.* In *Current Protocols in Immunology.* Coligan, J. E., Bierer, B. E., Margulies, D. H., Shevach, E. M., Strober, W., Eds. John Wiley & Sons, Inc., Hoboken, NJ (2010).

28. Artuc, M., Muscha Steckelings, U., Henz, B. M. Mast cell-fibroblast interactions: Human mast cells as source and inducers of fibroblast and epithelial growth factors. *Journal of Investigative Dermatology.* **118** (3), 391-395 (2002).

29. Panos, R. J., Rubin, J. S., Csaky, K. G., Aaronson, S. A., Mason, R. J. Keratinocyte growth factor and hepatocyte growth factor/scatter factor are heparin-binding growth factors for alveolar type ii cells in fibroblast-conditioned medium. *Journal of Clinical Investigation.* **92** (2), 969-977 (1993).

30. Lv, D., Hu, Z., Lu, L., Lu, H., Xu, X. Threedimensional cell culture: A powerful tool in tumor research and drug discovery (Review). *Oncology Letters.* **14** (6), 6999-7010 (2017).

31. Amaral, R. L. F., Miranda, M., Marcato, P. D., Swiech, K. Comparative analysis of 3D bladder tumor spheroids obtained by forced floating and hanging drop methods for drug screening. *Frontiers in Physiology.* **8**, 605 (2017).
32. Gao, B., Jing, C., Ng, K., Pingguan-Murphy, B., Yang, Q. Fabrication of three-dimensional islet models by the geometry-controlled hanging-drop method. *Acta Mechanica Sinica.* **35** (2), 329-337 (2019).
33. Zhang, C. et al. 3D culture technologies of cancer stem cells: Promising ex vivo tumor models. *Journal of Tissue Engineering.* **11**, 204173142093340 (2020).
34. Sobiepanek, A., Paone, A., Cutruzzolà, F., Kobiela, T. Biophysical characterization of melanoma cell phenotype markers during metastatic progression. *European Biophysics Journal: EBJ.* **50** (3-4), 523-542 (2021).
35. Jin, G. -Z., Kim, H. -W. Effects of Type I collagen concentration in hydrogel on the growth and phenotypic expression of rat chondrocytes. *Tissue Engineering and Regenerative Medicine.* **14** (4), 383-391 (2017).
36. Osidak, E. O. et al. Concentrated collagen hydrogels: A new approach for developing artificial tissues. *Materialia.* **20**, 101217 (2021).
37. Antoine, E. E., Vlachos, P. P., Rylander, M. N. Review of collagen I hydrogels for bioengineered tissue microenvironments: Characterization of mechanics, structure, and transport. *Tissue Engineering Part B: Reviews.* **20** (6), 683-696 (2014).
38. Jimbow, K., Salopek, T. G., Dixon, W. T., Searles, G. E., Yamada, K. The epidermal melanin unit in the pathophysiology of malignant melanoma. *The American Journal of Dermatopathology.* **13**(2), 179-188 (1991).
39. Van Den Bogaard, E. et al. Perspective and consensus opinion: Good practices for using organotypic skin and epidermal equivalents in experimental dermatology research. *Journal of Investigative Dermatology.* **141** (1), 203-205 (2021).
40. Bednarek, R. *In vitro* methods for measuring the permeability of cell monolayers. *Methods and Protocols.* **5** (1), 17 (2022).
41. Idrees, A. et al. Fundamental *in vitro* 3D human skin equivalent tool development for assessing biological safety and biocompatibility - towards alternative for animal experiments. *4 Open.* **4**, 1 (2021).
42. Park, H. -Y., Kweon, D. -K., Kim, J. -K. Upregulation of tight junction-related proteins by hyaluronic acid in human HaCaT keratinocytes. *Bioactive Carbohydrates and Dietary Fibre.* **30**, 100374 (2023).
43. Choudhury, S., Das, A. Advances in generation of three-dimensional skin equivalents: Pre-clinical studies to clinical therapies. *Cytotherapy.* **23** (1), 1-9 (2021).
44. Zhang, Z., Michniak-Kohn, B. B. Tissue engineered human skin equivalents. *Pharmaceutics.* **4** (1), 26-41 (2012).
45. Woappi, Y., Altomare, D., Creek, K. E., Pirisi, L. Self-assembling 3D spheroid cultures of human neonatal keratinocytes have enhanced regenerative properties. *Stem Cell Research.* **49**, 102048 (2020).
46. Ouwehand, K., Spiekstra, S. W., Waaijman, T., Scheper, R. J., de Gruijl, T. D., Gibbs, S. Technical advance: Langerhans cells derived from a human cell line in a full-thickness skin equivalent undergo allergen-induced maturation and migration. *Journal of Leukocyte Biology.* **90** (5), 1027-1033 (2011).

47. Akther, F., Little, P., Li, Z., Nguyen, N. -T., Ta, H. T. Hydrogels as artificial matrices for cell seeding in microfluidic devices. *RSC Advances.* **10** (71), 43682-43703 (2020).

48. Silva, A. K. A., Richard, C., Bessodes, M., Scherman, D., Merten, O. -W. Growth factor delivery approaches in hydrogels. *Biomacromolecules.* **10** (1), 9-18 (2009).

49. Lee, H. -R. et al. Effect of Aronia extract on collagen synthesis in human skin cell and dermal equivalent. *Oxidative Medicine and Cellular Longevity.* **2022**, 4392256 (2022).

50. Mulder, P. P. G., Raktoe, R. S., Vlig, M., Elgersma, A., Middelkoop, E., Boekema, B. K. H. L. Full skin equivalent models for simulation of burn wound healing, exploring skin regeneration and cytokine response. *Journal of Functional Biomaterials.* **14** (1), 29 (2023).

51. Goncalves, K. et al. Investigation into the effect of skin tone modulators and exogenous stress on skin pigmentation utilizing a novel bioengineered skin equivalent. *Bioengineering & Translational Medicine.* **8** (2), e10415 (2023).

52. Michel, M., L'Heureux, N., Pouliot, R., Xu, W., Auger, F. A., Germain, L. Characterization of a New Tissue-Engineered Human Skin Equivalent with Hair. *In Vitro Cellular & Developmental Biology. Animal.* **35** (6), 318-326 (1999).

53. Müller, I., Kulms, D. A 3D organotypic melanoma spheroid skin model. *Journal of Visualized Experiments: JoVE.* **135**, 57500 (2018).