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TITLE:

Generation of a Simplified Three-Dimensional Skin-on-a-chip Model in a Micromachined Microfluidic Platform

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SUMMARY:

Here, we present a protocol to generate a three-dimensional simplified and undifferentiated skin model using a micromachined microfluidic platform. A parallel flow approach allows the *in situ* deposition of a dermal compartment for the seeding of epithelial cells on top, all controlled by syringe pumps.

ABSTRACT:

This work presents a new, cost-effective, and reliable microfluidic platform with the potential to generate complex multilayered tissues. As a proof of concept, a simplified and undifferentiated human skin containing a dermal (stromal) and an epidermal (epithelial) compartment has been modelled. To accomplish this, a versatile and robust, vinyl-based device divided into two chambers has been developed, overcoming some of the drawbacks present in microfluidic devices based on polydimethylsiloxane (PDMS) for biomedical applications, such as the use of expensive and specialized equipment or the absorption of small, hydrophobic molecules and proteins. Moreover, a new method based on parallel flow was developed, enabling the *in situ* deposition of both the dermal and epidermal compartments. The skin construct consists of a fibrin matrix containing human primary fibroblasts and a monolayer of immortalized keratinocytes seeded on top, which is subsequently maintained under dynamic culture conditions. This new microfluidic platform opens the possibility to model human skin diseases and extrapolate the method to generate other complex tissues.

INTRODUCTION:

Recently, advances have been made toward the development and production of *in vitro* human skin models for the analysis of the toxicity of cosmetic and pharmaceutical products[1]. Researchers in pharmaceutical and skin care industries have been using animals, mice being the most common, to test their products[2–5]. However, testing products on animals is not always predictive of the response in humans, which frequently leads to drug failure or adverse effects in humans and consequently to economic losses[5,6]. The UK was the first country that prohibited the use of animals for cosmetic testing in 1998. Later, in 2013, the EU banned the testing and approbation of cosmetics in animals (EU Cosmetics Regulation No. 1223/2009)[7].

This prohibition is also being considered by other countries such as in 'The Humane Cosmetics Act' in the USA[8]. In addition to ethical concerns, the anatomical differences between animal and human skin make animal testing time-consuming, expensive, and often ineffective. Furthermore, the global *in vitro* toxicology testing market size is expected to reach USD 26.98 billion by 2025[9]. For these reasons, there is a need to develop new methods and alternatives for those *in vitro* studies, such as bioengineered human skin models, that enable testing for safety and toxic effects of cosmetics and drugs without the use of animals.

There are two different kinds of commercially available, *in vitro,* human skin models. The first type consists of stratified epidermal equivalents containing multiple layers of differentiating keratinocytes that are seeded on different materials. Some of them have been approved by the Organization for Economic Co-operation and Development (OECD) and validated by the (European Centre for the Validation of Alternative Methods (ECVAM) for skin corrosion and irritation testing, such as EpiDerm or SkinEthic[10–12]. The second type are full-skin equivalents with a layer of differentiating human keratinocytes seeded on a three-dimensional (3D) scaffold that contains fibroblasts, such as T-Skin and EpiDerm-FT. However, these models are cultured under static conditions, which makes them unable to accurately represent human physiological conditions.

Recent interest has focused on generating *in vitro* 3D skin models in cell culture-insert (CCI) formats with dynamic perfusion[13–19]. However, these systems cannot be considered *stricto sensu* as microfluidic skin-on-chips as per their classical definition in the field. Ingber's definition for organs-on-a-chip states that the organ must be placed inside the microfluidic channels, which is a condition that only a few devices fulfil[20,21]. Skin-on-chips have so far modelled mostly simple epithelia as single-cell layers and/or dermal cell layers separated by a porous membrane[22,23]. Although there have been some advances modeling skin in microfluidic systems[16,24], there is currently no literature showing an organ-on-a-chip system that fits Ingber's definition, capable of producing a multilayered skin *in situ* and including both epithelial and stromal components.

In this work, a new, cost-effective, robust, vinyl-based microfluidic platform for skin-on-a-chip applications is presented. This platform was produced by micro-machining, which provides more simplicity in the fabrication process, as well as increased flexibility and versatility in the layout of the device, overcoming some of the limitations of PDMS[25]. A way to introduce a simplified skin construct through a parallel flow controlled with syringe pumps was also designed. Parallel flow

allows two fluids with very different viscosities (a buffer and fibrin pre-gel in this case) to be perfused through a channel without mixing with each other. As a proof of concept, a dermo-epidermal construct containing fibroblasts embedded in a fibrin matrix mimicking the dermis was introduced in the device, on top of which a monolayer of keratinocytes was loaded to emulate the undifferentiated epidermis. The dermal compartment height can be modulated by modifying the flow rates. The main novelty of this work, compared to previously described models[22,26–29], is the development of a 3D construct inside a microchamber by means of microfluidics. Although this article presents a simplified undifferentiated skin, the long-term goal is to generate and characterize a fully differentiated skin construct to demonstrate its viability and functionality for drug and cosmetic testing purposes.

PROTOCOL:

1. Chip design and micromachining parameters

1.1. Design the microfluidic chip layers with FreeCAD open-source design software; refer to **Table 1** for the dimensions of the channels. Include four 2.54 mm diameter holes in the design to use a custom-made aligner for a correct layer superposition.

[Place **Table 1** here]

1.2. Cut 95 µm-thick, adhesive, transparent vinyl sheets into 30 cm x 30 cm squares to fit in the plotter properly.

1.3. Use Brother CanvasWorkspace software to create a 30 cm x 30 cm workspace, and fill it with the designed patterns for the different layers of the chip (**Figure 1A**). Store it in a .svg file.

1.4. Cut the 30 cm x 30 cm vinyl sheets with the edge plotter (**Figure 1B–D**).

1.4.1. Stick the vinyl sheet to a low tack adhesive mat, and eliminate all the air bubbles if necessary.

1.4.2. Upload the .svg file to the plotter, and set the cutting parameters: cutting blade: level 3; cutting pressure: level 0; cutting speed: level 1. Place the adhesive mat with the vinyl into the plotter, and start the cutting process.

1.5. Cut the top channel pattern on 12 µm-thick double-sided tape vinyl by following the previous steps.

[Place **Figure 1** here]

2. PDMS layer fabrication

2.1. Mix the PDMS and curing agent in a ratio of 10:1 (v/v), and place the mixture under

vacuum for 15 min to remove air bubbles. Pour 55 mL of the mixture into a 55 cm^2 square culture dish to obtain a 2 mm-thick layer. Remove bubbles with a needle.

2.2. Cure the mixture (step 2.1) in an oven for 1 h at 80 °C. Unmold the PDMS and cut it into rectangles with the chip dimensions. Make holes for the tubing with an 18 G syringe needle.

3. Chip assembly

NOTE: For better understanding, see **Figure 2**.

3.1. Assemble the whole device using an aligner to adjust channels, inlets, and outlets properly. Pile up four vinyl layers (with the corresponding bottom micropattern) for assembling the lower channel, keeping the cover tape of the bottom layer to avoid sticking to the aligner.

3.2. Cut and place the polycarbonate (PC) porous membrane on top of the lower channel to separate it from the upper one. Be careful not to cover the inlets of the lower channel.

3.3. Add ten vinyl layers with the upper chamber design. Stick a double-sided tape vinyl layer with the top-channel pattern on top. Remove the chip from the aligner and stick it on the glass slide.

3.4. Place a 2-mm-thick PDMS sheet on top of the double-sided tape vinyl layer to provide appropriate anchoring for the tubing and to avoid leakage. Leave a weight on top of the chip overnight to ensure the chip is completely watertight. Sterilize the chip by flushing 70% v/v ethanol for 5 min, and then wash with distilled H_2O.

[Place **Figure 2** here]

4. Pump connections

NOTE: The graphical representation of pumps connections is shown in **Figure 3**.

4.1. Connect Pump 1 to the Upper Chamber Inlet 1 (UCi1).

4.2. Connect Pump 2 to the Upper Chamber Inlet 2 (UCi2).

4.3. Connect Pump 3 to the Lower Chamber Inlet (LCi).

4.4. Connect Upper Chamber Outlet (UCo) and Lower Chamber Outlet (LCo) to a waste tube.

4.5. Connect the syringes to each inlet using polytetrafluoroethylene (PTFE) tubes and 18 G stainless steel connectors.

[Place **Figure 3** here]

5. Cell culture

NOTE: The HaCaT cell line has a commercial origin. Human primary fibroblasts come from healthy donors and were obtained from the collection of biological samples of human origin registered in "Registro Nacional de Biobancos para Investigación Biomédica del Instituto de Salud Carlos III".

5.1. Work in a cell culture hood, previously sterilized under ultraviolet light and wiped with ethanol.

5.2. Thaw H2B-GFP-HaCaT cells (human immortalized skin keratinocytes, hKCs) and GFP-human primary fibroblasts (hFBs) at 37 °C, add 2 mL of culture medium, and centrifuge for 7 min at 20 °C at 250 × *g*.

NOTE: H2B-GFP-HaCaT cells are human immortalized keratinocytes modified to express a hybrid histone H2B-green fluorescent protein (GFP), providing their nuclei with green fluorescence. GFP-hFBs are human primary fibroblasts transformed with the vector pLZRS-IRES-EGFP to express cytoplasmic green fluorescence. These cells were modified following previously published protocols[30,31]

5.3. Culture both hKCs and hFBs in 1x DMEM supplemented with 10% fetal bovine serum and 1% of antibiotic/antimycotic solution. Pre-warm the culture medium at 37 °C before use.

5.4. Detach the cells by washing them with 1x phosphate-buffered saline (PBS), adding 2 mL of trypsin/ethylenediamine tetraacetic acid (EDTA) and incubating them for 10 min at 37 °C.

5.5. Inactivate trypsin adding 4 mL of culture medium. Resuspend the cells, and transfer them to a 15 mL tube. Remove 10 µL to count cells on a Neubauer chamber and determine the appropriate concentration.

5.6. Centrifuge the 15 mL tube for 7 min at 20 °C at 250 × *g*. Remove the supernatant, and resuspend the pellets at the desired concentration: hFBs at 50,000 cells/mL and hKCs at 5·× 10^6 cells/mL.

6. Fibrinogen pre-gel preparation

6.1. Activate thrombin by adding 1mL of $CaCl_2$ (1% w/v in NaCl) to the vial.

6.2. Add the following components to obtain 1 mL of a fibrin hydrogel at a final concentration of fibrin of 3.5 mg/mL: 59 µL of activated thrombin (10 NIH units/mL), 59 µL of tranexamic acid (**Table of Materials**, 100 mg/mL), 764 µL of culture medium containing 50,000 hFBs/mL, 118 µL of fibrinogen (20 mg/mL in NaCl (0.9% w/v)).

NOTE: Fibrinogen must be added at the last moment.

7. Parallel flow protocol

7.1. Pump 1x PBS with pump 3 through the LCi at 50 µL/min during the whole process.

7.2. Pump sacrificial fluid (1x PBS) with pump 2 through the UCi2 at 100 µL/min.

7.3. Load the syringe with the pre-gel, rapidly place it into pump 1, and run it at 200 µL/min (**Figure 4A**).

7.4. Stop pumps 1 and 2 once the pre-gel exits the UCo.

7.5. Leave the chip without removing the tubing at 37 °C for at least 10 min to allow gelation.

7.6. Pump the culture medium at 50 µL/h with pump 3 through UCi2 overnight.

7.7. Block UCi1 with a cap.

8. hKCs monolayer seeding

8.1. Check that hFBs are spread 24 h after the generation of the dermal compartment.

8.2. Introduce the hKCs with pump 2 through UCi2 at 5 ×·10^6 cells/mL at 40 µL/min for 1 min (**Figure 4B**).

8.3. Leave the chip overnight at 37 °C in a humidity-saturated incubator for cell attachment.

8.4. Pump fresh culture medium with pump 3 only through LCi at 50 µL/min (**Figure 4C**).

[Place **Figure 4** here]

9. Cell viability assay

NOTE: Live/Dead kit stains cells with green or red fluorescence depending on their live or dead state. For proper viability differentiation, non-fluorescent hKCs and hFBs must be used in this step. All the steps in the procedure are carried out through UCi2 with pump 2.

9.1. Wash the top channel with 1x PBS for 5 min at 50 µL/min to remove culture medium.

9.2. Pump air to remove the 1x PBS at 50 µL/min.

9.3. Prepare Calcein AM/Ethidium homodimer-1 Kit (Live/Dead) solution by following the manufacturer's instructions.

9.4. Pump Live/Dead solution at 50 µL/min for 2 min.

9.5. Incubate 30 min at 37 °C in the dark.

9.6. Wash top channel by pumping 1x PBS at 50 µL/min for 2 min to remove any remaining reagent.

9.7. Observe the sample under the confocal microscope. Use an excitation wavelength of 495/590 nm and an emission wavelength of 519/617 nm for live and dead cells, respectively.

REPRESENTATIVE RESULTS:

The designed chip is composed of two fluidic chambers separated by a 5 µm pore size PC membrane that allows the growth of the cell by allowing the passage of growth-promoting molecules from the lower chamber. The upper chamber holds the tissue construct, in this case, a monolayer of hKCs on a fibrin hydrogel containing hFBs.

The height of the channels is determined by the number of adhesive sheets added to each channel. The lower chamber is composed of 4 layers (380 µm) and the upper one of 10 one-sided tape layers and a double-sided one (962 µm). The dimensions of the chip are 4 cm x 2 cm, which enhances its manipulation. The adhesive vinyl sheets provide water-tightness and transparency for visual inspection of the device. The PDMS layer was useful for the proper anchoring of llthe tubing to avoid any leakage from the holes where the tubes were fixed.

According to published literature, the injectability of cell-containing hydrogels into microfluidic chambers using syringe pumps has not been reported to date. For this reason, the injectability of the fibrin pre-gel had to be assessed. We observed that under a flow of 50 µL/min, the syringe was blocked. However, flow rates higher than 200 µL/min could damage the cells. Rheological studies were performed to test the shear thinning behavior of the fibrin pre-gel, obtaining viscosities from 10 to 50 cP within the selected flow rate range (50–200 µL/min). These results helped to establish the working conditions of this system.

In this work, a parallel flow method has been developed based on the generation of two superimposed laminar flows the lower one being the pre-gel, while the upper one was sacrificial fluid (PBS). Numerically solving Navier-Stokes equations and imposing the appropriate boundary conditions, we found that there were multiple possible solutions to obtain the desired height. Considering the shear rate limits established earlier, to achieve a hydrogel of approximately 500 µm height, the flow rates were 104 and 222 µL/min for the sacrificial PBS and the pre-gel, respectively. In practice, microflow rates of 100 and 200 µL/min were used for simplicity. When re-introduced in the model, these values were found to result in a gel height of 576 µm, very similar to the expected values (**Figure 5B**). To experimentally check the functioning of the proposed method, the height of the hydrogel along the upper chamber was measured. An average height of 550 µm was observed (**Figure 5A**), quite similar to the prediction of our mathematical model.

[Place **Figure 5** here]

When establishing the protocol at the beginning, PBS was not flushed through the lower channel, leading to discrepancies between the theoretical height of the gel and the one measured. This difference in height was ~40% compared with the estimated one (**Figure 6**). Once the protocol was optimized and PBS pumped through the lower chamber, this loss in height was resolved (**Figure 5**).

[Place **Figure 6** here]

Figure 7A shows a fluorescent top view image of the upper chamber containing a fibrin hydrogel with embedded GFP-hFBs, demonstrating that 24 h after loading, cells are uniformly distributed along the chamber and well spread. **Figure 7B** shows confluent GFP-hKCs seeded on top of the hydrogel.

[Place **Figure 7** here]

It is important to keep the system closed overnight until the hKCs sediment and attach to the hydrogel surface. When the tubing is removed before cell attachment, air bubbles enter the channel and displace cells, leading to a nonuniform confluent monolayer, as shown in **Figure 8**.

[Place **Figure 8** here]

The cell viability test performed on hFBs embedded in the hydrogel was carried out 24 h after loading using the parallel flow method, showing a cell viability of ~95%. The same test performed on hKCs cells 24 h after seeding them on the fibrin hydrogel showed similar results. The next step was to generate a dermo-epidermal construct and study its structure using confocal microscopy after 24 h (**Figure 9**).

[Place **Figure 9** here]

It is crucial to find an equilibrium between the shear thinning behavior of the fibrinogen gel and its gelation time: if it takes too long to establish the parallel flow, it coagulates and blocks the system; if the gelation process is too slow, hFBs in the hydrogel will sediment as shown in **Figure 10**. The behavior of this transient state can be regulated by varying the thrombin concentration.

[Place **Figure 10** here]

During the device planification and posterior experimental practices, some complications arose that had to be solved to obtain an optimally functioning device and well-structured tissue. These problems are shown in **Table 2**, along with the solutions for troubleshooting.

[Place **Table 2** here]

FIGURE AND TABLE LEGENDS:

Figure 1: **Chip design and micromachining process.** (**A**) Software layout showing the working space filled with both the top and bottom patterns designed for the chip. (**B**) Edge plotter during cutting process; cutting blade, whole vinyl sheet, and adhesive mat are shown. (**C**) Patterned vinyl being detached from the cut sheet. (**D**) Sample of an adhesive vinyl layer patterned with the top channel design.

Figure 2: **Microfluidic chip assembly.** (**A**) General scheme of the assembly of the device. Lower and upper chambers are composed of four and eleven superimposed vinyl sheets, respectively. (**B**) Top and lateral views of the microfluidic chip. Top and bottom channels are represented in pink and blue, respectively. (**C**) Image of the chip assembly using a custom-made aligner. (**D**) Chip image after complete assembly.

Figure 3: **Pump connections and inlets/outlets location.** (**A**) Diagram showing the connection of the three different pumps to their respective inlets. Outlets connect to a waste container. (**B**) Chip image with labeled inlets and outlets. Abbreviations: LCi = lower chamber inlet; LCo = lower chamber outlet; UCi2 = upper chamber inlet 2; UCo = upper chamber outlet.

Figure 4: **Microfluidic protocol for the generation of the dermo-epidermal construct.** (**A**) Transverse cross-section showing the parallel flow process to generate the dermal compartment. (**B**) Keratinocyte monolayer seeding 24 h after dermal compartment generation. (**C**) Cell culture maintenance inside the microfluidic device. (**D**) Cross-sectional recreation of the skin inside the chip.

Figure 5: **Parallel flow mathematical solutions to choose the appropriate pre-gel and sacrificial fluid flows to obtain the desired dermal height.** (**A**) Front view of the confocal image of hKCs seeded on top of the fibrin gel to assess its height. (**B**) Mathematical solution for different heights depending on the pre-gel and PBS flow rates. Abbreviations: hKCs = human immortalized skin keratinocytes; PBS = phosphate-buffered saline.

Figure 6: Confocal view of hKCs seeded on top of the hydrogel to measure its height when PBS was not pumped through the lower chamber. Abbreviations: hKCs = human immortalized skin keratinocytes; PBS = phosphate-buffered saline.

Figure 7: Fluorescence images of the upper channel showing different cells seeded in the device. (**A**) Top view of the upper chamber 24 h after parallel flow protocol showing the hFBs embedded in the fibrin gel. (**B**) Confluent GFP-hKCs seeded on top of the hydrogel 24 h after hydrogel generation. Dashed red line indicates channel walls. Scale bars: 400 µm. Abbreviations: hFBs = human primary fibroblasts; GFP-hKCs = green fluorescent protein-expressing human immortalized skin keratinocytes.

Figure 8: Top view of the hydrogel surface when removing tubing immediately after hKCs

seeding. Dashed red line indicates channel walls. Scale bar: 400 µm. Abbreviations: hKCs = human immortalized skin keratinocytes.

Figure 9: **Reconstructed 3D confocal image of the undifferentiated skin model in the microfluidic chip**. Yellow dashed line indicates the surface of the hydrogel separating hKCs (top) from the hFBs embedded in the gel (bottom). Abbreviations: hFBs = human primary fibroblasts; hKCs = human immortalized skin keratinocytes.

Figure 10: Confocal image of a fibrin hydrogel showing sedimented hFBs (in red) due to a slow fibrin gelling time. A hKCs monolayer (in blue) is shown on top of the gel. Abbreviations: hFBs = human primary fibroblasts; hKCs = human immortalized skin keratinocytes.

Table 1: Dimensions of the upper and lower channels of the device.

Table 2: Issues found during the development of the current work and solutions applied.

DISCUSSION:

The motivation to develop this method was the desire to model skin diseases and study the effects of new and innovative therapies in a high-throughput platform. To date, this laboratory produces these dermo-epidermal equivalents by casting—either manually or with the help of the 3D bioprinting technology—the fibrin gel with fibroblasts into a cell culture insert plate and seeding the keratinocytes on top of it. Once the keratinocytes reach confluence, the 3D culture is exposed to the air-liquid interface, which induces keratinocyte differentiation, generating a stratified epidermis and consequently, a fully developed interfollicular human skin[32–34]. However, these 3D cultures, albeit very relevant clinically, are expensive, time-consuming, and do not mimic physiological conditions.

Tissue-on-a-chip technology provides a platform to emulate physiological conditions in cell culture, thus enabling a better understanding of the toxicity, efficacy, and delivery of drug candidates[13–19,35]. Most of the tissues modeled on microfluidic “classical” chips are composed of single layers of cells, mostly epithelial cells[22,26,42,27,28,36–41]. The modelling of more complex, multi-layered tissues has been hampered by the difficulty of generating homogeneous and superimposed tissue layers.

The main novelty of this work, as compared to previous devices[22,43], is the development of a method to generate an undifferentiated and simplified skin construct by means of microfluidics. Moreover, another important innovation with respect to other published technologies[44] is the design of a relatively simple, cost-effective, easy-to-use disposable chip system made of biocompatible vinyl sheets allowing *ad hoc* fabrication. This system avoids the use of silicon wafers and complicated plasma bonding procedures needed with PDMS[25]. Chips based on this material require the generation of wafers specific for each different chip design, which raises the price of the prototyping process. All of this makes the current “classical” technology expensive, complex, and not very flexible.

To overcome these limitations, using skin as a tissue model, we present a very flexible, cheap, and robust microfluidic platform based on vinyl layers, produced by micromachining. We also present a new methodology based on the parallel flow of laminar fluids that allows the *in situ* generation of a bilayer skin construct with a lower dermal compartment containing hFBs and an upper epidermal compartment composed of a monolayer of hKCs. The chip consists of two chambers separated by a porous PC membrane. The upper channel contains the skin construct and leaves free space to allow hKC differentiation and stratification and/or perfusion of culture medium, air, or even drugs in the future. The lower chamber is continuously perfused with culture medium to promote cell growth. The use of a porous membrane may lead to the loss of a part of the pre-gel from the upper chamber to the lower one when subjected to high pressure due to pumping force. Introducing a PBS flow in the lower chamber compensates this pressure difference and avoids this leakage.

The distribution of hKCs on the hydrogel surface is an important aspect when generating a skin model. When they are not homogeneously spread, the generation of a uniform monolayer and therefore, the epidermal differentiation could be hampered. In the same way, hFBs must be equally distributed within the hydrogel to resemble their natural location in which they are found in real skin. To avoid cell sedimentation or tubing blockage, hydrogel composition (especially thrombin concentration) must be carefully studied to control gelation times and shear thinning behavior.

Despite these encouraging findings, future work is needed to demonstrate the long-term functioning of the system necessary to promote the correct proliferation and differentiation of the hKC monolayer to form a well differentiated human skin including a stratum corneum. Besides skin, this system would allow the generation of other complex, multilayered tissue constructs.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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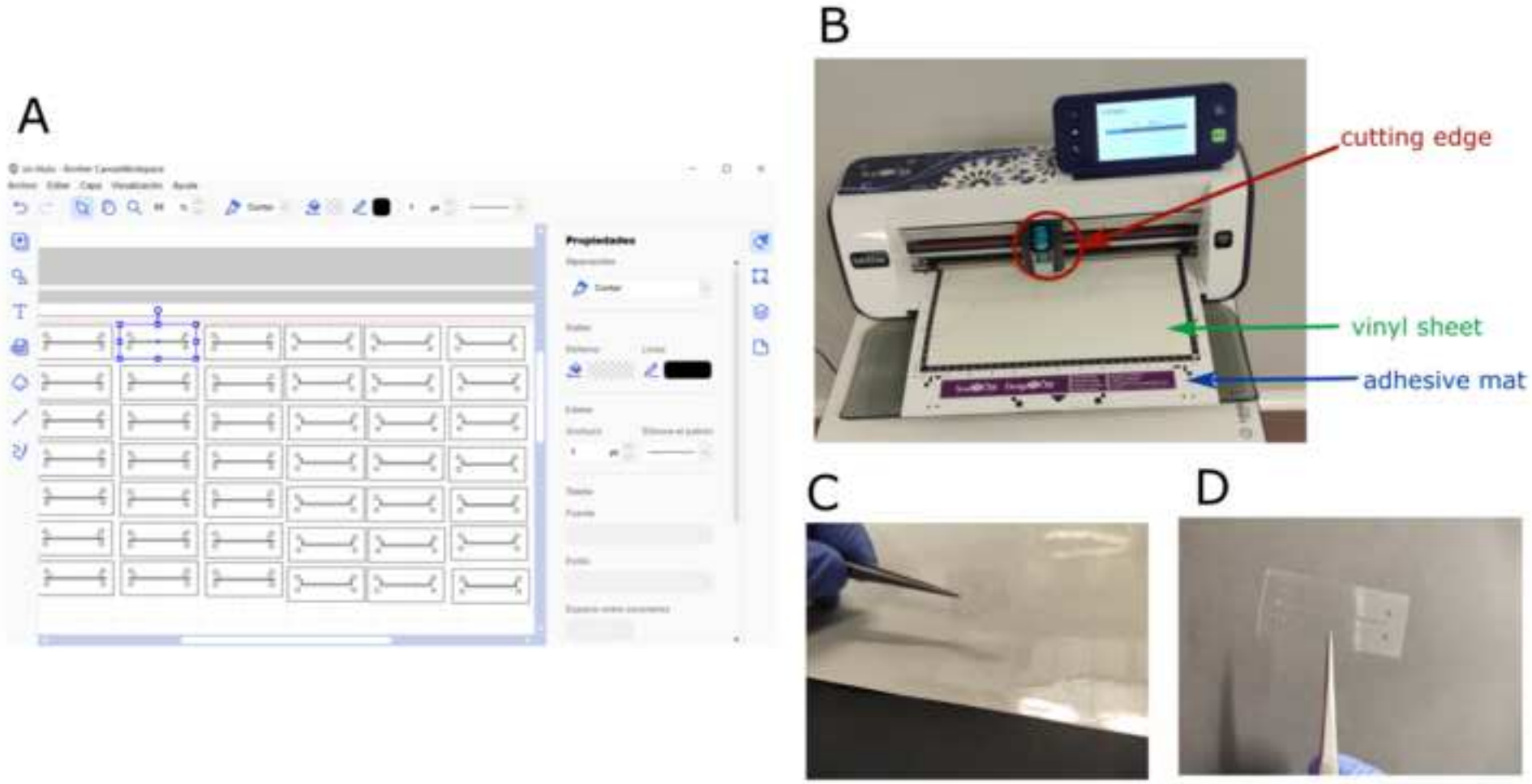
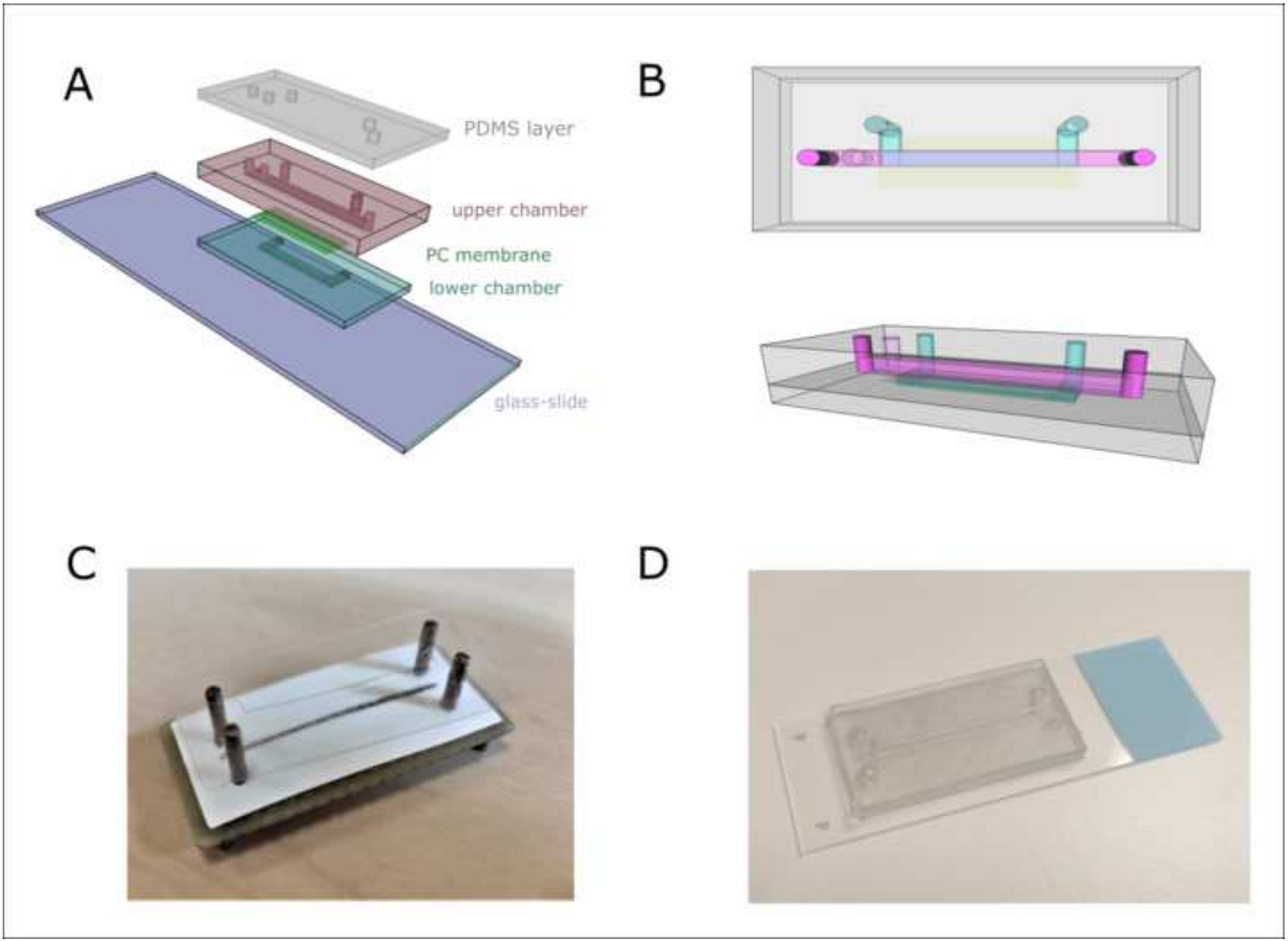
Figure

Click here to access/download;Figure;Figure1_plotter.png



Figure



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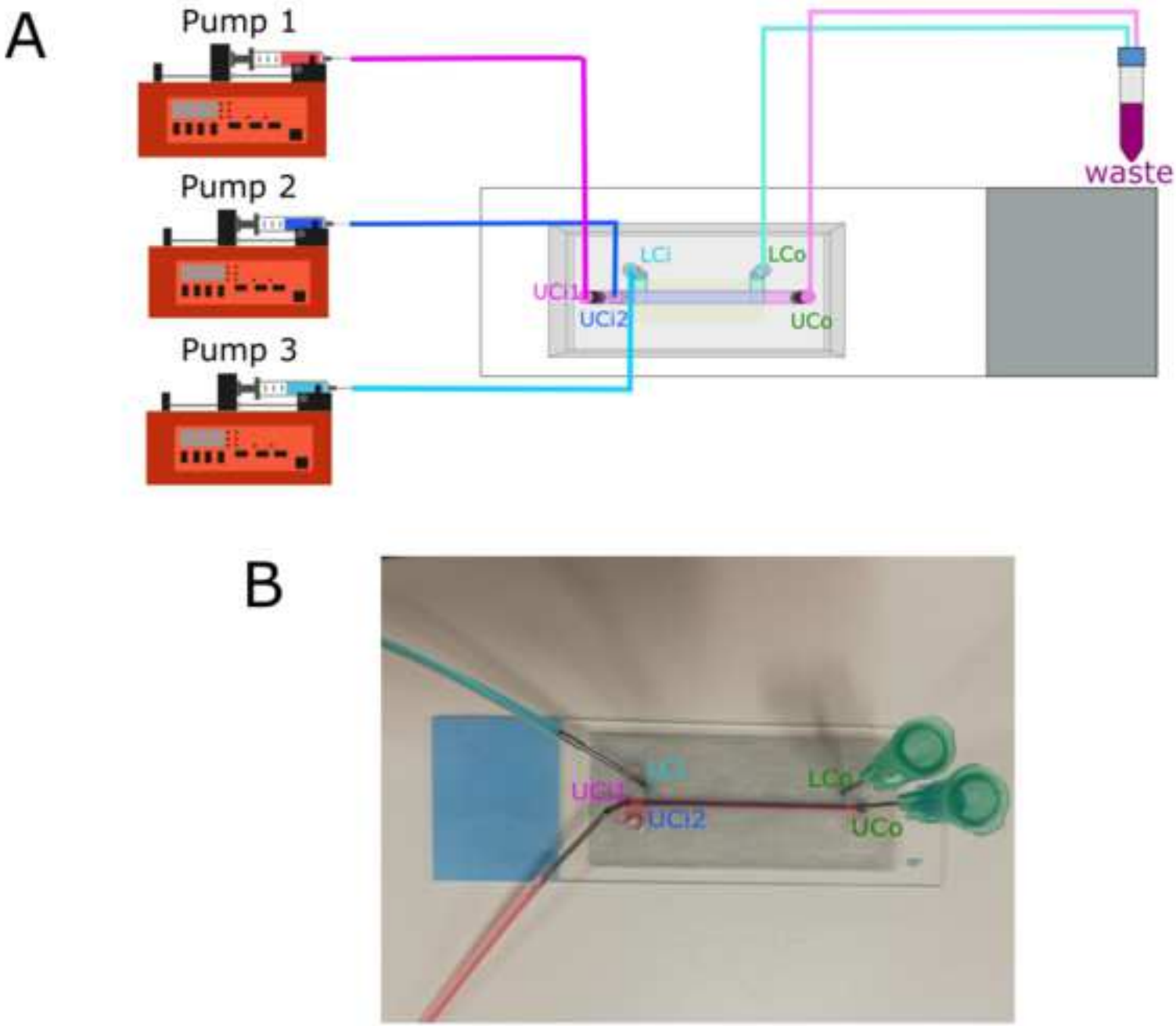

Figure

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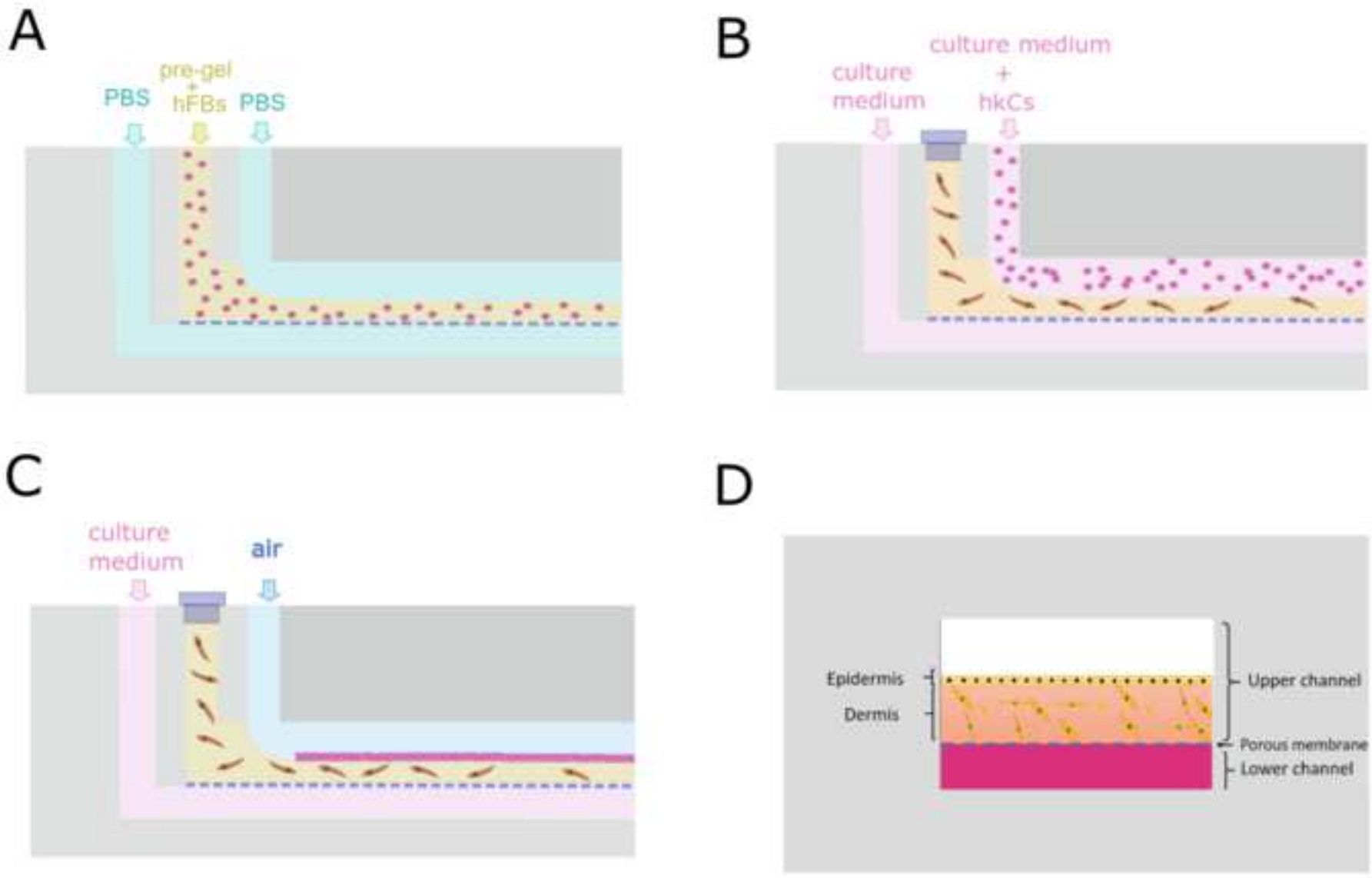

Figure

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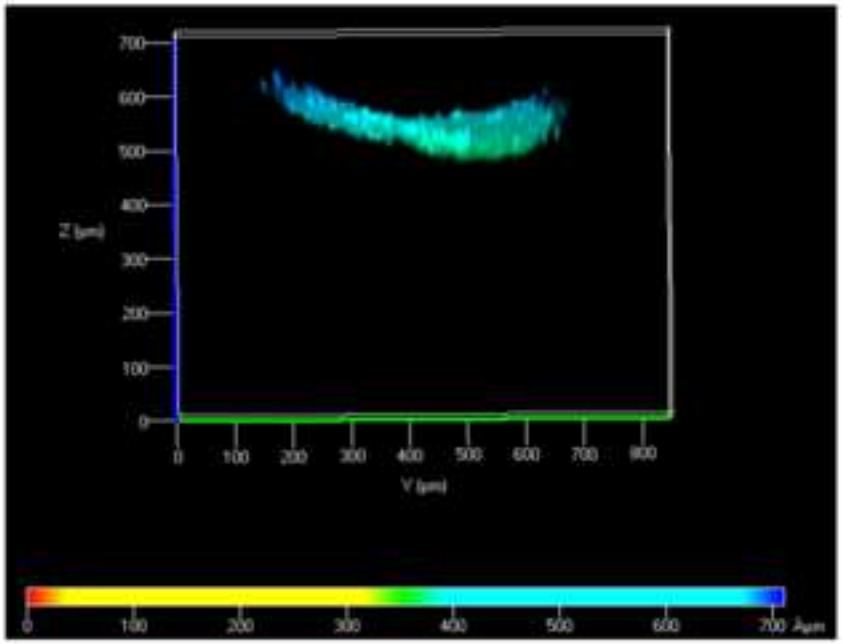
A



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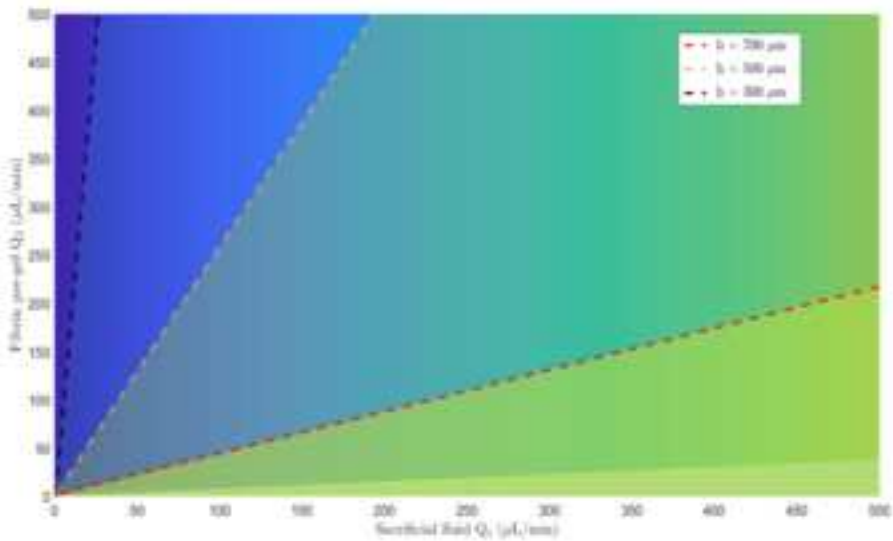

Figure

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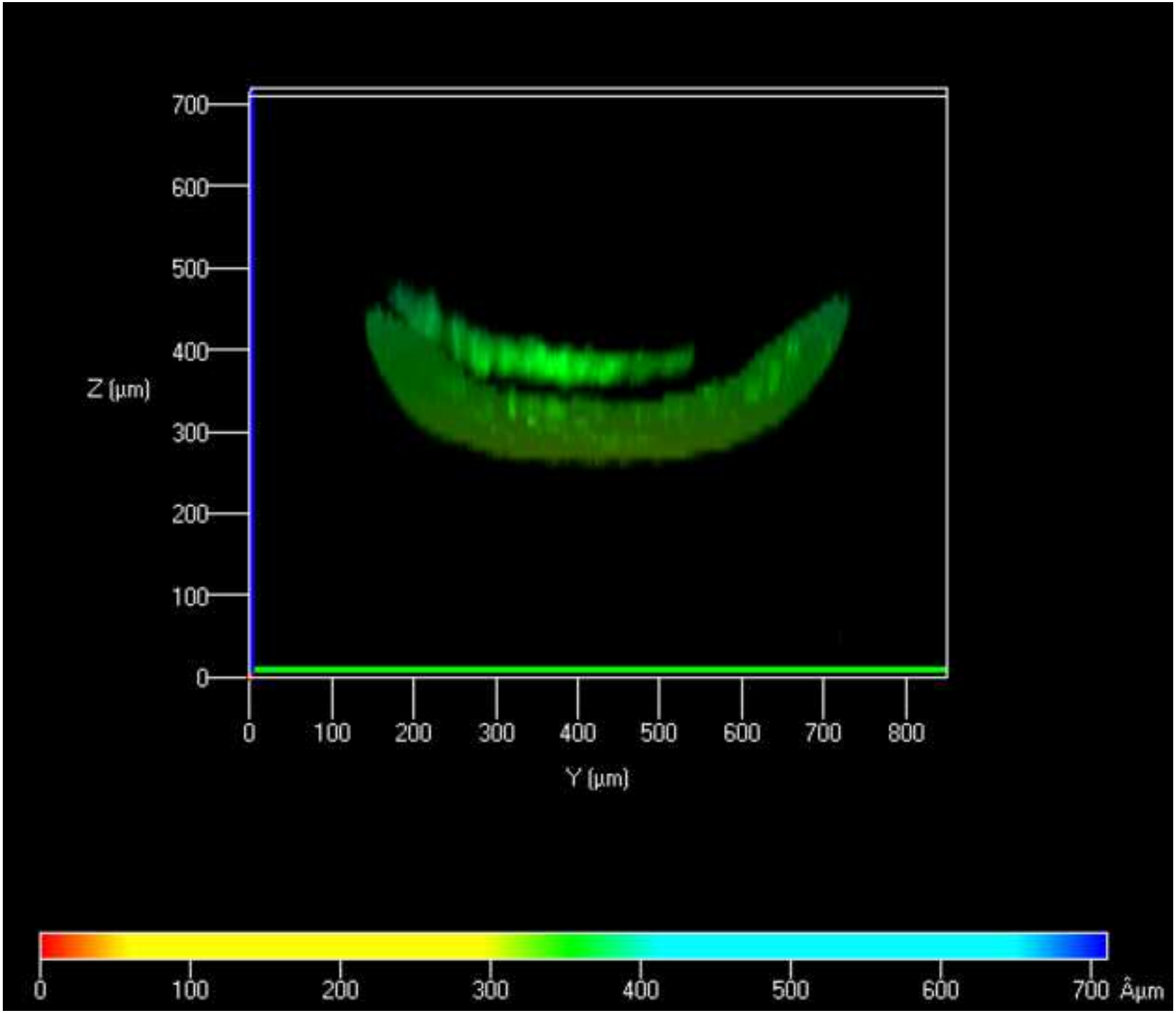
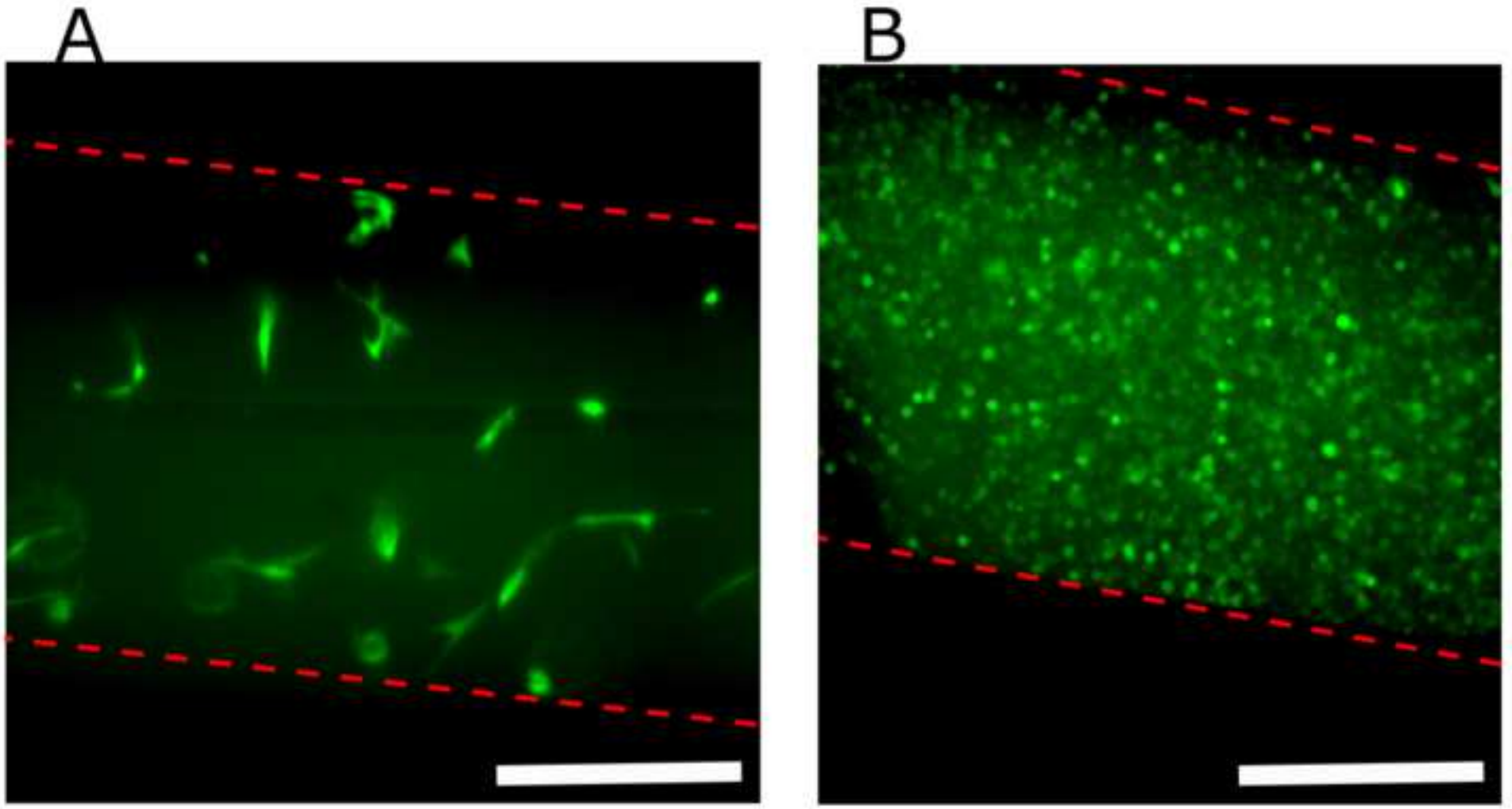


Figure



Figure

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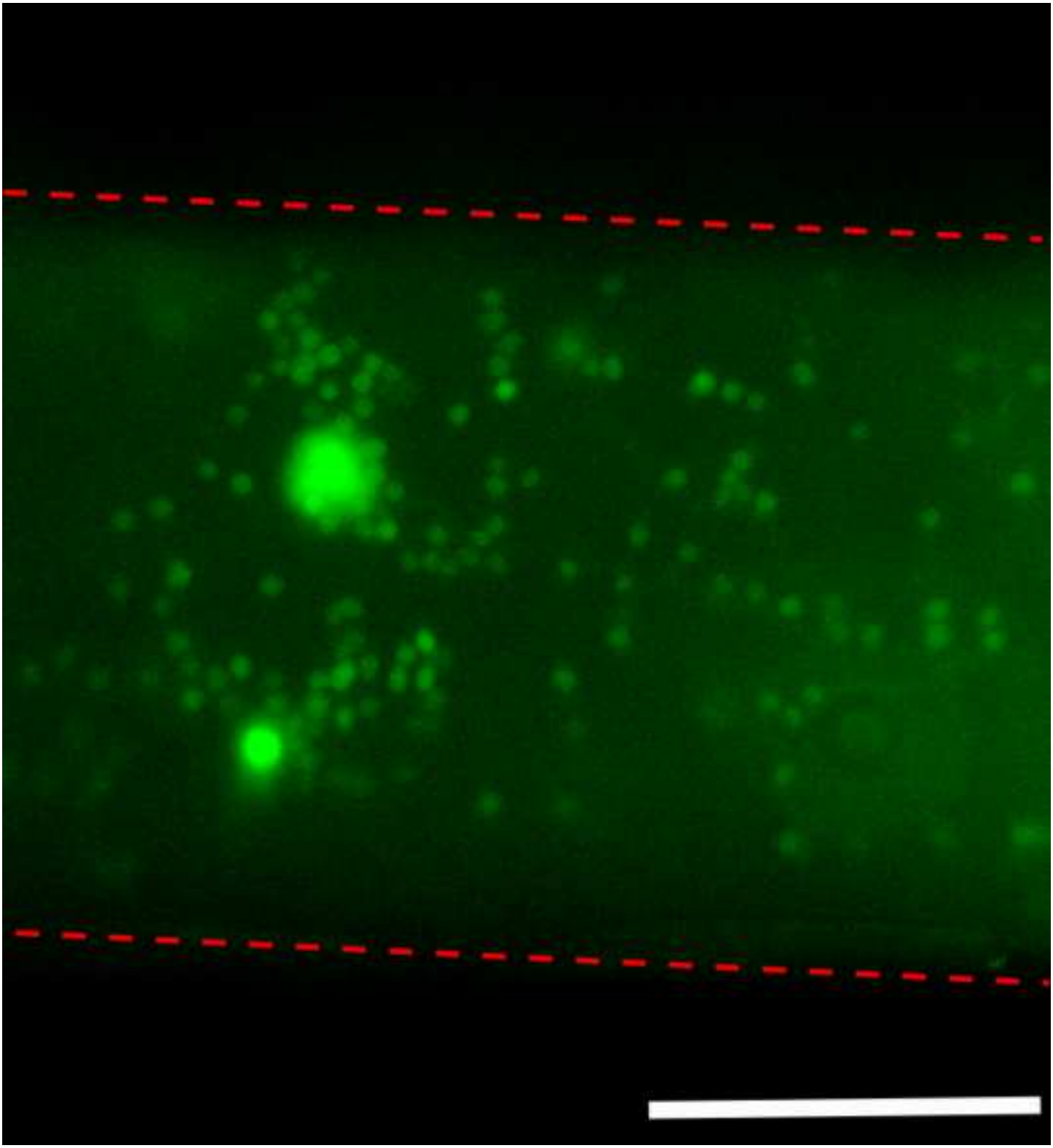
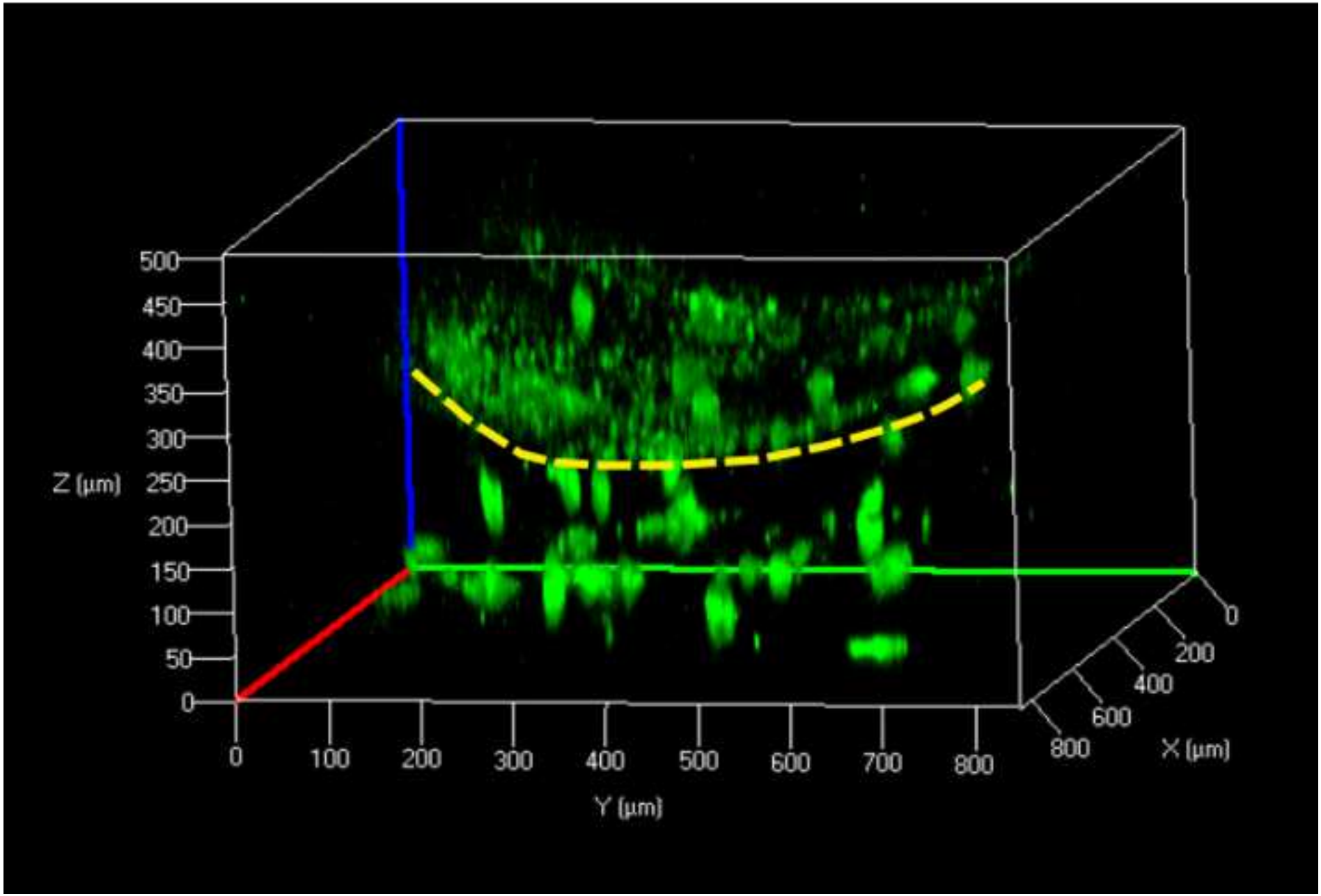


Figure

Click here to access/download;Figure;Figure9_confocal-dermis-epidermis.png



Figure

Click here to access/download;Figure;Figure10_fibrosdeposited.png

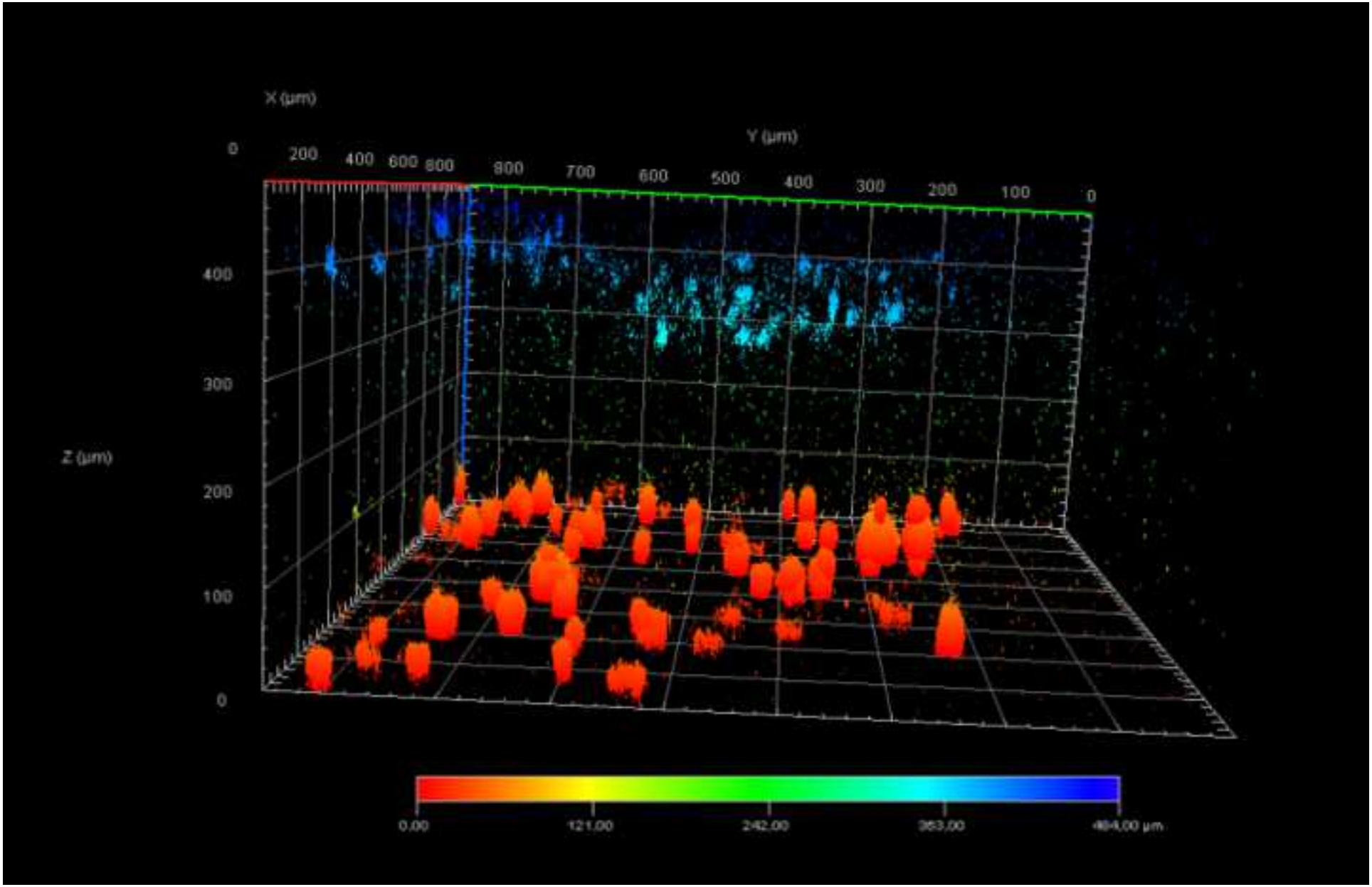


Table 1

	Length (µm)	Width (µm)
Lower chamber	28,400	800
Upper chamber	31,000	800

Table 2

Problems	Solutions
Introducing a 3D hydrogel in the upper chamber, leaving free space above to seed keratinocytes on top after dermal generation	Apply a parallel flow of two fluids (PBS and pre-gel) with different viscosities to create a dermal compartment with a controlled height.
Channel misalignment during vinyl sheet stacking	Use of a custom-made aligner to pile up all the sheets in the correct place
Achieve a confluent keratinocyte monolayer on top of the fibrin gel to simulate the epidermis	Allow cell attachment to the gel prior to removing the tubes from the chip, preventing air bubbles from entering the channel and displacing the cells.
Hydrogel height loss along the channel due to pre-gel leaking to the lower channel during parallel flow protocol through the porous membrane.	Pump PBS through the lower channel during parallel flow establishment.
Fibroblast sedimentation inside the gel	Thrombin concentration was slightly increased to accelerate fibrin gelation

Table of Materials

	Company	Catalog Number	Comments/Description
Amchafibrin	Rottafarm		Tranexamic acid
Antibiotic/antimycotic	Thermo Scientific HyClone		
Calcium chloride	Sigma Aldrich		
Culture plates	Fisher		
DMEM	Invitrogen Life Technologies		
Double-sided tape vynil	ATP Adhesive Systems		GM 107CC, 12 µm thick
Edge plotter	Brother		Scanncut CM900
FBS	Thermo Scientific HyClone		
Fibrinogen	Sigma Aldrich		Extracted from human plasma
Glass slide	Thermo Scientific		
GFP-Human dermal fibroblasts	-		Primary. Gift from Dr. Marta García
H2B-GFP-HaCaT cell line	ATCC		Immortalized keratinocytes. Gift from Dr. Marta García
Live/dead kit	Invitrogen		
PBS	Sigma Aldrich		
Polycarbonate membrane	Merk TM		5 µm pore size
Polydimethylsiloxane	Dow Corning		Sylgard 184
Sodium chloride	Sigma Aldrich		
Syringes	Terumo		5 mL
Thrombin	Sigma Aldrich		10 NIH/vial
Transparent adhesive vinyl	Mactac		JT 8500 CG-RT, 95 µm thick
Trypsin/EDTA	Sigma Aldrich		
Tubing	IDEX		Teflon, 1/16” OD, 0.020” ID

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March 22, 2021

Manuscript ID: JoVE62353

Dear Prof. Malaguarnera,

We are pleased to resubmit a revised version of our manuscript entitled “Generation of a simplified 3D skin-on-a-chip model in a micromachined microfluidic platform”. We are grateful to the reviewers for their time and constructive comments. We have considered their comments and have modified the manuscript accordingly.

On the following pages of this letter, you will find a detailed point-by-point response to the reviewer comments. In the revised manuscript, the changes introduced to address these comments are highlighted in red, blue and green.

We trust you will find this version of the manuscript to be a major improvement on our original submission, and that you will therefore be able to consider it suitable for publication in JoVE.

Yours sincerely,

Diego Velasco Bayón

Universidad
Carlos III de Madrid
Department of Bioengineering and Aerospace Engineering

Editorial comments:

The authors thank the editor for the comments on our manuscript, which have made us produce a revised version which, we believe, improves its quality. The modifications to the original manuscript are highlighted in **RED**.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

> **Answer:** Following the editorial's advice, we have sought help to improve grammar and have made a significant effort to improve the presentation of the information. However, the results or discussion have not been altered in the manuscript.

2. Please consider revising the title to "Generation of 3D organ-on-a-chip in a micromachined microfluidic platform". We cannot have punctuations in the title.

> **Answer:** Following the editorial and reviewer 1 comments, we propose to change the title to "Generation of a simplified 3D skin-on-a-chip model in a micromachined microfluidic platform"

3. Please revise the following lines to avoid previously published work: 57-58, 59-61, 61-62, 64-66, 68-71, 72-74.

> **Answer:** We apologize for that mistake. These sentences have been modified in order to avoid plagiarism with previously published works.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

> **Answer:** We have rewritten an important part of the introduction and results in order to avoid personal pronouns.

5. Please define all abbreviations before use (PDMS, etc.).

> **Answer:** All abbreviations have been correctly defined before the first use in the main text.

6. Line 104: Please elaborate on the micro machining steps. A citation would suffice.

> **Answer:** More details have been added to section *"1. Chip design and micromachining parameters "* elaborating on the micromachining process. Details

on the software used for creating the plotter files and the procedure to introduce the vinyl into the edge plotter are now provided. Additional pictures of the design, cutting process and assembly have been added to the manuscript to enhance reader comprehension (Figure 1).

7. Line 116/146/253/257: For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks.

Answer: Time units abbreviations have been properly written in the revised manuscript.

8. Line 120: Please mention if there is any specific dimension for the holes made.

Answer: There are not specific dimensions for the holes made in the PDMS layer. They are made using a syringe needle with the same diameter (18G) as the connectors latter used with the tubes. This method enhances the anchoring of the tubes to the chip. We have highlighted this in the text.

9. Line 165: Please mention how were the keratinocytes modified? Please elaborate on the steps. Please cite a reference, if necessary.

Answer: Keratinocytes were not modified in our laboratory, they were kindly donated by Dr. Marta García. She was the person who performed the modification following protocol described by Kanda et al. (Ref1).
This point has been clarified explaining the origin and modification of the cells with a proper reference.

References:

Ref1: W. G. Kanda T, Sullivan KF, “Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells,” Curr Biol., vol. 8, no. 7, pp. 377–85, 1998.

10. Line 168: Please mention how were the fibroblasts modified? Please elaborate on the transformation steps. Please cite a reference, if necessary.

Answer: Primary fibroblasts were not modified in our laboratory, they were kindly donated by Dr. Marta García. She was the person who performed the modification following protocol described by Holguín et al. (Ref1)
This point has been clarified explaining the origin and modification of the cells with a proper reference.

References:

Ref1: A. Holguín et al., “Assessment of Optimal Virus-Mediated Growth Factor Gene Delivery for Human Cutaneous Wound Healing Enhancement,” J. Invest. Dermatol., vol. 128, no. 6, pp. 1565–1575, 2008.

11. Line 202: Please ensure that the units specified for the rate of flow is correct.

Answer: Flow rate units have been fixed.

12. Line 206: For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2.

Answer: Liter units have been changed from l to L.

13. Line 225-229: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Answer: Non-imperative sentences have been changed and a note about fibrinogen gel preparation has been added in the point 6.2. of the protocol. A new point (5.1.) about cell culture hood has been included in the cell culture section.

14. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Answer: The key steps of the protocol have been highlighted in yellow for the video.

15. Please remove the Figure Legends from the Protocol and the Representative results section. After the Representative Results please add a separate Figure and Legends section. The information provided in the Figure Legends after the Representative Results is sufficient. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

Answer: We have added a separate section to include figure legends and kept the location of the figures in the main text.

16. Please do not use any abbreviations for journal titles and book titles. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Answer: We have changed the bibliography style so they match JoVE specifications.

17. Figure 5: Please revise that the units mentioned in the X and Y-axis using standard abbreviations (Replace "µl" with "µL").

Answer: Units in X and Y-axis in figure 5 have been modified.

18. Figure 7: Please include scale bars for both the images of the panel.

Answer: Scale bars have been added to both images in Figure 7.

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Reviewer #1:

The authors thank the referee for the comments on our manuscript, which have made us produce a revised version which, we believe, improves its quality. The modifications to the original manuscript are highlighted in **BLUE**.

Manuscript Summary:

1.In general, the paper presents a brief protocol. The reader may find it hard to get how the device looks like or works and how the in vitro model within it is constructed, functioning and beneficial.

> **Answer:**The referee is right and we have worked on the protocol to provide a more detailed step-by-step procedure. The order of the figures have been changed for a better understanding of the microfluidic chip. In addition, a new figure was included to illustrate the fabrication method (Figure 1). We have highlighted the advantages of this microfluidic device in the Discussion section.

2.Title: I would recommend changing the title and not using the word "new". For example, A "micromachined microfluidic device for generation of 3D organ-on-a-chip: Application to skin in vitro model"

> **Answer:** Following the recommendation, the title has been proposed to be "Generation of a simplified 3D skin-on-a-chip model in a micromachined microfluidic platform".

3. Abstract: the abstract, the authors do not explain how the developed microfluidic device overcomes the drawback of PDMS-based devices.

> **Answer:** We thank the reviewer to point this out and we have modified the abstract so the advantages of this device compared to traditional PDMS-based chips are highlighted. One of the main advantages here is the possibility of changing the design for free by changing dimensions and geometries in the CAD design. On the contrary, photolithography would imply the production of a new silicon wafer each time the design changes. Cut and assembly adhesive vinyl layers do not require specific skills and expensive facilities or equipment, such as clean room, spin-coater, plasma-bonding.

Introduction:

Line 57: "leads to economic problems" is a very wide expression. The authors may use more relevant words such as drug/product failure, adverse effect in human, etc.

Answer: According to the reviewer comment, the expression has been changed to clarify side effects apart from those economical. Drug failure and adverse effects have been added as additional problems to different drug responses in animals.

Line 68: Are commercially available may sound better. Authors may name few examples of commercially available skin equivalents such as EpiDerm, Episkin, Stratatest, etc.

Answer: Regarding the reviewer suggestion the expression has been changed and examples of both commercially available epidermal and dermo-epidermal equivalents have been added.

Lines (74-76): "However, these models are cultured under static conditions which makes them unable to accurately represent human physiological conditions." But these models can be maintained in microfluidic dynamic environment. The authors may provide some evidence that the full thickness that they are reporting is better than these commercially available skin equivalents.

Answer: The work presented in this article is at an early development stage and it is more focused on the development of a platform that allows the generation of a skin three-dimensional structure than on the tissue itself. Our work is based on the evidence reported that microfluidic cultures on a chip mimic the cell microenvironment in a more reliable manner (Ref1). As the reviewer suggests, this kind of static cultures can be maintained in a dynamic environment but our main goal is to develop a functional skin directly inside the microfluidic device.
For this first approach we wanted to show the procedure in order to obtain the three dimensional construct by means of microfluidics. We are aware that further studies should be done to develop a fully differentiated skin. Indeed, this type of work is currently ongoing. As is explained in the revised version of the manuscript, our long-term aim is to apply this technique to a fully developed skin tissue, properly characterized it and demonstrate its viability and function.

References:

Ref1: Sung JH, Esch MB, Prot JM, Long CJ, Smith A, Hickman JJ, Shuler ML. Microfabricated mammalian organ systems and their integration into models of whole animals and humans. Lab Chip. 2013 Apr 7;13(7):1201-12.

Line 79: What authors mean by "as they are classically defined in the field"?

Answer: We apologize that the expression cannot be clearly understood. For that reason we have introduced new references and a better explanation of organ-on-a-chip definition.

Although works regarding organ-on-a-chip are diverse and model tissue in different manners, the vast majority do not adjust to the classical definition of organ-on-a-chip. Donald Ingber, who can be considered as the father of the organ-on-a-chip, and Sangeeta Bhatia define this technologies in the journal Nature Biotechnology as "microfluidic devices for culturing living cells in continuously perfused, micrometer-sized chambers in order to model physiological functions of tissues and organs" (Ref1). Most of the so-called skin-on-a-chip devices in literature do not fulfil this definition; they use microfluidic channels for nurturing cells and place human skin equivalents on top of them, but the tissue is not inside these channels. Regarding this definition, they could be considered as artificially perfused tissues, but not organ-on-a-chip. This requirement for the organs-on-a-chip has been added to the text: "Ingber's definition for organs-on-a-chip states that the organ must be placed inside the microfluidic channels, which is a condition that only a few devices fulfil". Ingber review has been also added to that line as a reference.

References:

Ref1: Bhatia SN, Ingber DE. Microfluidic organs-on-chips. Nat Biotechnol. 2014;32(8):760-772

Line 81-83: "To our knowledge, there is currently no literature showing a microfluidic chip system capable of producing a multilayered skin in situ, including both epithelial and a stromal components." Authors may look at literature carefully. See for example, Sriram et al. Materials Today, Volume 21, Number 4, 2018, 327-340.

Answer: Indeed, there are currently on the literature, several microfluidic devices used for modelling skin (as the one mentioned by the reviewer and others such as Kim et al. Int J Mol Sci. 2020;21(11):1- 15) that subject the 3D culture to dynamic conditions. However, these systems cannot be considered *stricto sensu* as microfluidic organ-on-chips regarding Ingber's definition ((Bhatia SN, Ingber DE. Microfluidic organs-on-chips. Nat Biotechnol. 2014;32(8):760-772. doi:10.1038/nbt.2989). Although it is true that keratinocytes are perfused through a microfluidic channel on top of the dermal compartment in Sriram publication, the dermal compartment is actually pipetted by hand in the chip in a well-like structure between the bottom and the top channel, before closing the device.
We have included these two references in the manuscript and the sentence has been modified to avoid concept misunderstanding.

Major Concerns:

Line 97-107: 1. Chips design and micromachining parameters. This section doesn't show how the chip looks like, fabricated or works. The authors need to

describe in detail the fabrication process and provide a descriptive figure of the fabrication process, such as a cross-sectional view of every step.
What is the function of the vinyl part and PDMS part?

Answer: Regarding this statement we have extended the section where the micromachining process is described with a deeper explanation of each step. Also, an extra figure showing this procedure has been included in the manuscript as Figure 1.
The microfluidic channels are found in the vinyl part, which is the physical support of the device. PDMS layer is only used as a top lid that assures correct anchorage of tubing. We have included this explanation in the revised manuscript.

Line 135: Why ten vinyl layers?

Answer: According to the literature review, we found that the skin thickness can vary depending on the part of the body where it is found. The thinnest epidermis is found at the eyelid and the thickest at the sole of the feet, varying from 0.1 to 1.5 mm respectively (Ref1). On the contrary, the dermis can be 30-40 times thicker than the epidermis (Ref2). Since we wanted to miniaturize the organ, we decided to generate a dermal compartment of 500 µm. To do that, we defined an upper chamber of 950 µm high that would allow the epidermis differentiation and cornification through air pumping. This upper space would also permit drug or cosmetics perfusion for testing purposes.

References:

Ref1: Kolarsick PAJ, Kolarsick MA, Goodwin C. Anatomy and Physiology of the Skin. J Dermatol Nurses Assoc. 2011;3(4):203-213. doi:10.1097/JDN.0b013e3182274a98

Ref2: de-Souza IMF, Vitral GLN, Reis ZSN. Skin thickness dimensions in histological section measurement during late-fetal and neonatal developmental period: A systematic review. Ski Res Technol. 2019;25(6):793-800. doi:10.1111/srt.12719

The chip structure is NOT CLEAR at all. The authors need to re-write the protocol properly assisted with drawing. Chip images need also to be provided.

Answer: We have included new pictures of the chip and modified figures in the manuscript to enhance the comprehension of the chip structure, fabrication, assembly and functioning. The protocol has been extended to include more steps in the fabrication process.

The authors claim that their device would overcome the drawback of PDMS, but they are using PDMS too.

Answer: It is true that we are still using PDMS but the main structure of the chip, holding the microfluidic channels, is on the vinyl part. PDMS at the topmost of the chip is used to better anchor the tubing. However, it could be replaced by vinyl layers that would provide the same functionality. We have extended the PDMS drawbacks in the introduction and the advantages of using vinyl over PDMS as the main structure of the chip.

Figure 1 highlight the pumps (which is not necessary) but show no details of the chip. The figure needs to be reproduced to show the chip structure including a cross-sectional view.

Answer: Figure 1 (now Figure 3) has been modified in order to provide a better understanding of the chip, its geometry and structure. We think new figures 1 and 2 will also help to better visualize the chip and its structure.

How the fibroblasts were embedded in the gel? What ration is used? Number of cells?The authors need to explain the concept of parallel flow and its advantage before jumping to experimental description.

Answer: Fibroblasts are embedded in the gel by directly introducing them in the pre-gel prior to gelification. Once the hydrogel is crosslinked, fibroblasts anchor to the matrix fibers developing three dimensional spindle shape as found in real human skin. Fibroblasts are added to a final concentration of 50,000 cells/mL according to the literature (Ref1).

We apologize for the lack of explanation about the parallel flow method used. We now include in the Introduction a brief description of the concept of what a parallel flow is and the conditions to obtain it in a microfluidic channel.

References:

Ref1: F. Larcher et al., "*Large surface of cultured human epithelium obtained on a dermal matrix based on live fibroblast-containing fibrin gels*" Burns, vol. 24, no. 7, pp. 621–630, 2002

In Fig.2, what is the optimum flow rate of PBS and Pre-gel+FB? Despite they are mentioned in the Results section, but the optimization of the flow rate need to be presented in the experimental section in more detail.

Answer: The solution provides infinite combinations of flow rates between the two fluids depending on the desired hydrogel height but, there are some aspects that must be taken into consideration when designing the experimental set-up. If the flow rate of the pre-gel is too high, the shear stresses will increase and cells can be damaged. If the flow rate of the pre-gel is too low, shear thinning behavior is not reached and cross-linking can take place during pumping. This would block the tubing and parallel flow will not be developed. Taking these two concepts into consideration, we set the flow rate limits (below and over) for establishing the parallel flow; these flow limits are described in the Results section. We have rheological studies previously performed in our facilities characterizing the shear thinning behavior of these fibrinogen pre-gels. Besides, we also developed a mathematical model that allowed us to relate the desired height of the hydrogel with the flow rates. These two studies are under revision in another publication. For this reason we could not cite nor include it in this protocol.

Fig. 4 should be placed in the very beginning of the paper.

Answer: We appreciate this comment and realized that the order of the figures may difficult the correct comprehension of the device and how it works. Figure 4 has already been moved to the beginning of the manuscript and modified (now Figure 2).

How Fig. 5 was obtained? What are the boundary conditions? What the colour gradient means? What x and y represent? The figure quality is very low and need to be enhanced. A cross-sectional view of the channel (with parallel flow) need to be added to indicate where is the fluid flow take place in the simulated model.

Answer: As mentioned before, we developed the mathematical solution for the parallel flow developed in the microfluidic channel. It is now under revision for publication.

Parallel flows are found in several engineering applications, primarily in heat exchangers, oil-water flows in petroleum industry or steam-water flows in nuclear reactors. Phase holdup of two-fluids occurs at low flow rates in horizontal channels where a fraction of the channel is occupied by a fluid phase. They have a well-defined interface and their viscosities largely affect the velocity. In our case, two-phase flow is developed between the fibrin pre-gel (fluid 2) and a sacrificial fluid (PBS, fluid 1). In order to solve this problem, we used Navier-Stokes equations and the continuity equation with their corresponding boundary conditions.

$$\frac{\partial v_x}{\partial x} + \frac{\partial v_y}{\partial y} = 0$$

$$\frac{\partial(\rho v_y)}{\partial t} + \frac{\partial(\rho v_x v_y)}{\partial x} + \frac{\partial(\rho {v_y}^2)}{\partial y} = -\frac{\partial p}{\partial y} + \mu \frac{\partial^2 v_y}{\partial y^2}$$

$$\frac{\partial(\rho v_x)}{\partial t} + \frac{\partial(\rho {v_x}^2)}{\partial x} + \frac{\partial(\rho v_x v_y)}{\partial y} = -\frac{\partial p}{\partial x} + \mu \frac{\partial^2 v_x}{\partial x^2}$$

The main difficulty in the solution of the Navier Stokes equations arises from their nonlinearity but, applying some assumptions such as stationary state, convection term neglect or considering just transversal velocity; vanish nonlinear terms.

The flow rates obtained by solving these equations are:

$$Q_1 = w\left(-\frac{P_l}{6\mu_1}(H^3 - h^3) + \frac{A_1}{2}(H^2 - h^2) + B_1(H - h)\right)$$

$$Q_2 = w\left(-\frac{P_l}{6\mu_2}h^3 - \frac{A_2}{2\mu_2}h^2 + \frac{B_2}{\mu_2}h\right)$$

Where A_1, A_2, B_1 and B_2 are the integration constants and *w* is the width of the channel.

These are subjected to boundary conditions:

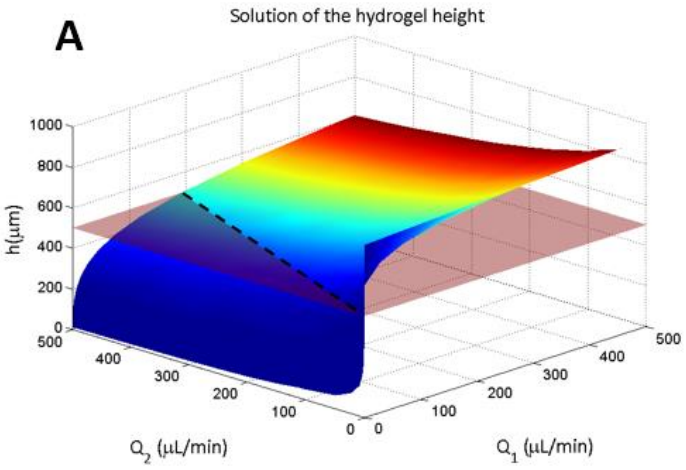
- No-slip conditions at the walls:
  - At $y = 0 \rightarrow \quad v_2 = 0$
  - At $y = H \rightarrow \quad v_1 = 0$
- At the interface ($y = h$) we impose:
  - Continuity of velocity: $\quad v_1 = v_2$
  - Continuity of shear stress: $\mu_1 \frac{\partial v_1}{\partial y} = \mu_2 \frac{\partial v_2}{\partial y}$

By imposing boundary conditions and the desired height of the hydrogel the equations can be solved providing a set of solutions or a possible combination of flow rates that lead to that height.

Shading in the image corresponds to the “surface of solutions” for the dermal compartment height that the different flow rates of both, pre-gel and sacrificial fluid, provide. When imposing the desired height (reddish plane at h= 500 μm)

one can obtain the black dashed line observed in the figure below. In the manuscript it is shown as a top view with three different imposed heights (h= 300, 500 and 700 μm).

Here we show a three dimensional view of the solution with one cutting height.



We apologize for the quality of the figures. We realized that the quality of the figures when converted to a PDF file was strongly decreased. However, we now uploaded them at a higher resolution.

In general, there are no sufficient description of the results. Figure captions are not enough. More description MUST be provided.

Answer: According to the journal guidelines for the Representative Results section we tried to be very concise just providing an overview of the success of the protocol. We also included some suboptimal experiments to demonstrate the range of outcomes possible, as nonconfluent epidermal layer on top of the dermal compartment or fibroblasts that sediment to the lower part of the hydrogel (figures 8 and 10 of the revised version of the manuscript).

Regarding figure captions, JoVE specified that they should not be very large and include results discussion or methods: *"Each figure or table, including supplemental figures/tables, must have an accompanying legend consisting of a short title and a short description of each panel or a general description. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.".*

HaCaT cell will not grow as multilayers epidermis. There will not be any Stratum Coronium layer for example. Therefore, the model won't represent a full thickness even though a dermis layer is included.

Answer: According to the literature, HaCaT cells can develop a well differentiated epidermis with a functional *stratum corneum* and basal lamina. We recommend the reviewer these two articles where HaCaT functionality has been deeply analysed (Ref1 and Ref2). For this reason, we believe that a preliminary assessment of the microfluidic device can be carried out using this cell line. However, we believe that a fully functional skin equivalent for testing purposes should be done with primary keratinocytes which is the final objective of our research line.

References:

Ref1: Veronika M. Schoop, Norbert E. Fusenig, Nicolae Mirancea, Epidermal Organization and Differentiation of HaCaT Keratinocytes in Organotypic Coculture with Human Dermal Fibroblasts, Journal of Investigative Dermatology, Volume 112, Issue 3,1999.

Ref2: Dirk Breitkreutz, Veronika M. Schoop, Nicolae Mirancea, Markus Baur, Hans-Jürgen Stark, Norbert E. Fusenig, Epidermal differentiation and basement membrane formation by HaCaT cells in surface transplants, European Journal of Cell Biology, Volume 75, Issue 3, 1998.

In general, the manuscript is very brief, and reader may not find the necessary information to follow despite the fact that it will be accompanied with a video (which is not available yet). However, I feel more information and details are required.

Answer: We have included more pictures and a more detailed description of the processes hoping to improve the quality and comprehension of the protocol and results for the reader.

We expect that the video (when available) will complement this work for a better understanding.

Minor Concerns:

- **Line 104: 1.3. Vinyl sheets are micromachined with an edge plotter. Why Vinyl is selected? What is the advantage as a material? What are the attractive properties?**

  Answer: We appreciate the reviewer's question and we have included in the Discussion section the reason for the selection of this material, its properties, and advantages over PDMS.

Monomeric Polyvinyl Chloride (PVC) is an economical material and it has been widely used in biomedical applications due to its biocompatibility (Ref1). Besides, many suppliers can be found in the market.

Adhesive sheets are very attractive for microfluidic purposes (Ref2, Ref3) because they do not require additional treatments for its assembly, as it has been reported in previous questions.

References:

Ref1: Lungu, Maria & Moldovan, Lucia & Craciunescu, Oana & Doicin, Cristian. (2010). Biocompatible Blends Based on Polyvinyl Chloride and Natural Polymers for Medical Device Fabrication. Materiale Plastice. 47. 278-281.

Ref2: S. R.A. Kratz, C. Eilenberger, P. Schuller, B. Bachmann, S. Spitz, P. Ertl & M. Rothbauer. Characterization of four functional biocompatible pressure-sensitive adhesives for rapid prototyping of cell-based lab-on-a-chip and organ-on-a-chip systems. Scientific Reports (2019) 9:9287.

Ref3: Sanjin Hosic, Adam Bindas, Marissa Puzan, Will Lake, Jonathan Robert Soucy,
Fanny Zhou, Ryan Alan Koppes, David Breault, Shashi Murthy, and Abigail N Koppes. Rapid prototyping of multilayer microphysiological systems. ACS Biomater. Sci. Eng.(2020)

- **Through the protocol, the description is written in instruction language using (e.g. add, pour, cure,..). Please check the style of writing the protocol in JoVE if this suitable.**

  Answer: JoVE asks for the authors to include the protocol section as a list of steps, writing them in an imperative language. Some of the protocol items were not written in this style and the editor asked us to modify them.

- **Cell Culture: Which cell lines (keratinocytes and Fibroblasts) were used? From which supplier or source?**

  Answer: Keratinocytes are an immortalized cell line called HaCaT while human fibroblasts are primary cells. The name of the HaCaT cell line has been added to the protocol in 5.2. Both cells are modified to express green fluorescence and were kindly donated by Dr. Marta García. HaCaT cells are commercial and were bought to ATCC. Primary fibroblasts come from healthy donors and were obtained from the collection of biological samples of human origin registered in "Registro Nacional de Biobancos para Investigación Biomédica del Instituto de

Salud Carlos III'. We have added the origin of the cells as a NOTE in the cell culture section of the protocol.

- **Line 171: "5.3. Cells are cultured in DMEM 1x supplemented with 10% of FBS and 1% of Antibiotic/Antimycotic." Which cells? Need to be mentioned even though they are mentioned in the table in the end of the paper.**

  Answer: Both keratinocytes and fibroblasts are cultured using the same culture medium. This specification has been added to the cell culture section.

- **Line 242: What authors mean by "hKCs and hFBs must not be fluorescent"?**

  Answer: An explanation of why hKCs and hFBs must not be fluorescent has been added as a note in "9. Cell viability assay". Live/dead cell kit uses green/red fluorescence as marker for live and dead cells respectively. For avoiding confusions between fluorescent cells and the used kit, cells used for this specific experiment must not be fluorescent.

The authors need to mention the source of materials such as the Vinyl sheets, Live/dead assay reagents, etc. Need to be mentioned in the text even though they are mentioned in the table in the end of the paper.

Answer: Following JoVE's author guidelines: *"Avoid the use of commercial language, including ™/®/© symbols or company brand names before/after an instrument or reagent. Cite these in the Table of Materials instead."* We just include the source of materials in the table at the end of the document.

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Reviewer #2:

The authors thank the referee for the positive comments on our manuscript, which have made us produce a revised version which, we believe, improve its quality. The modifications to the original manuscript are highlighted in **GREEN**.

This is an excellent initiative-and the authors lauded for their efforts.

Major Concerns:

1. Unclear how troubleshooting of problems encountered was done--will be good to have a table with issues encountered and solutions.

Answer: We thank the reviewer for this pertinent point, and we have included a table summarizing troubleshooting of problems as suggested in the Results section of the revised manuscript.

Troubles	**Solutions**
Introducing a 3D hydrogel in the upper chamber leaving a free space above to seed keratinocytes on top of it after dermal generation	Apply a parallel flow of two fluids (PBS and pre-gel) with different viscosities to create a dermal compartment with a controlled height.
Channel misalignment during vinyl sheet stacking	Use of a custom-made aligner to pile up all the sheets in the correct place
Achieve a confluent keratinocyte monolayer on top of the fibrin gel to simulate the epidermis	Allow cell attachment to the gel prior removing the tubes from the chip, avoiding air bubbles to enter the channel and displace the cells.
Hydrogel height loss along the channel due to pre-gel leaking to the lower channel during parallel flow protocol through the porous membrane.	Pump PBS through the lower channel during parallel flow establishment.
Fibroblasts sedimentation inside the gel	Thrombin concentration was slightly increased to accelerate fibrin gelation

2. How are results presented?

Answer: The results section comprises a brief description of the microfluidic device and its suitability for skin-on-a-chip applications. The use of adhesive vinyl layers is one of the main novelties in this article which turns to be an economical and easy-to-use material for microfluidic platforms, compared to traditionally used materials as PDMS. We have added new figures and modified previous

ones to clarify the chip fabrication protocol, and to give a better understanding of how it is and works.
We also included confocal images to visualize the spatial distribution of the cells inside the dermal and epidermal compartments. Cells seeded as epidermis allowed us also to confirm that the selected height for the dermal compartment applied in the mathematical model was obtained experimentally.

Results are presented providing an overview of the success of the protocol proposed by the authors. Besides, we included some suboptimal experiments to demonstrate the range of outcomes possible and how we solved them (Figures 8 and 10 of the revised manuscript).

3. What is the interpretation of the data?

Answer: We present a new methodology based on the parallel flow of two fluids that allows the *in-situ* generation of a bilayered skin construct with a lower dermal compartment containing human primary fibroblasts and an upper epidermal compartment composed of a single layer of HaCaT human epidermal cells.

The chip is composed of two chambers: the upper one contains the skin construct and leaves free space to allow HaCaT differentiation and stratification and/or perfuse culture medium, air or even drugs in the future. The lower chamber is continuously perfused with a culture medium simulating a blood vessel.

Parallel flow method provides two main advantages: firstly the use of a single channel for the whole tissue construct and secondly the capability to regulate the height of the gel according to the design of the experiment. Although we have established a standard protocol for h = 500 μm the method enables us to modify it at will.

Life/Dead assay confirmed that the microfluidic vinyl chip is biocompatible and suitable for cell culture. The seeding process using syringe pumps, tubing and needles is not harmful for the cells. It also allows us to introduce a fibroblast containing hydrogel in the channel and a later seeding of a confluent keratinocytes monolayer on top.

Non-satisfactory results were successfully addressed and overcome improving the reproducibility of the experiments. This troubleshooting has been added in a table to the Results section as the reviewer suggested.

4. How can the results be presented with extrapolation?

Answer: The work presented in this article is at an early development stage and it is more focused on the procedure of fabricating the three-dimensional structure of the skin than in the tissue itself. Our work is based on the evidence reported that microfluidic cultures on a chip mimik the cell microenvironment in a more reliable manner.
For this first approach we wanted to show the procedure in order to obtain the three dimensional construct by means of microfluidics. We are aware that further studies should be done to develop a fully differentiated skin. Indeed, this type of work is currently being done. As is explained in the revised version of the manuscript, our long-term aim is to apply this technique to fully developed skin tissue, properly characterized it and demonstrate its

These chips can be used to model skin and also they could be suitable for incorporating biosensors for high throughput drug and cosmetic testing (Ref1 and Ref2).

This method could be used, not only to model skin tissue, but to any other complex and multilayered tissue with a similar three-dimensional structure such as cornea or intestine.

References:

Ref1: Liu MC, Shih HC, Wu JG, Weng TW, Wu CY, Lu JC, Tung YC. Electrofluidic pressure sensor embedded microfluidic device: a study of endothelial cells under hydrostatic pressure and shear stress combinations. Lab Chip. 2013 May 7;13(9):1743-53.

Ref2: Kilic, T., Navaee, F., Stradolini, F., Renaud, P., & Carrara, S. (2018). Organs-on-chip monitoring: sensors and other strategies. Microphysiological Systems, 2.