Application Note

# Bioprinting full-thickness skin models with NGB-R™

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## Abstract

Turning bioprinted skin tissues into functional ones is a complex process that first requires to address critical issues of cell viability, proper tissue morphogenesis and post-printing biological functions emergence. With these in mind, Poietis have conceived NGB-R™, a multi-modal 4D biofabrication platform that serves a disruptive cytocentric approach to bioprinting in which living cells are incorporated into a bioprinted tissue – not mixed in a bioink. Combining several bioprinting technologies, including unique and exclusive laser-assisted bioprinting (LAB), we have established a reproducible process workflow for successful full-skin tissue model biofabrication.

The dermis was made by incorporating primary human fibroblasts with LAB into layers of collagen-I preliminary printed with ink-jet. Following a 6-day dermal maturation time and air-liquid interface, the epidermis was then bioprinted with primary human keratinocytes using the same principles as for the dermal construct. Post-printing cell viability tests showed a ~95% viability for both fibroblasts and keratinocytes. Masson Trichrome staining highlighted dermal equivalent structure. Staining with anti-collagen-I antibody highlighted secretion of human collagen-I by fibroblasts, while HES, anti-cytokeratine 5 and anti-filaggrin stainings of keratinocytes printed on cellularized dermis highlighted epidermal stratification.

These results demonstrate that LAB proves an ideal technology when bioprinting living cells and confirm that both Poietis' full-skin biofabrication process and the NGB-R™ platform are suitable for this tissue model.

## Introduction

In-vitro skin models are used for research purposes to investigate physiological and pathological processes of the skin, as well as skin metabolism. Bioprinting technologies enable by design the creation of fully-defined 3D cell structures. Among them, laser assisted bioprinting (LAB) combines a high cell viability with a high resolution which makes possible the creation of cell patterns at a biologically-relevant scale for morphogenesis processes.

The presented full-thickness skin model is composed of a multi-layer dermis, made of primary human fibroblast cells printed in-between collagen I layers, covered by a fully differentiated epidermis, made of bioprinted primary human keratinocytes. Skin biofabrication was performed using Poietis' original 4D multi-modal bioprinting platform NGB-R™ [see figure 3], commercially available since 2019, which combines three bioprinting technologies (LAB, bio-extrusion and micro-valve/inkjet printing heads) and relies on built-in robotics for automated and reproducible workflows. For this full-thickness skin model, LAB was used to print living cells while inkjet bioprinting was used to deposit collagen layers.

## Laser bioprinting & bioprinting performance

As a result of 10+ years' research, Poietis have developed a unique and exclusive bioprinting technology based on laser-assisted bioprinting (LAB) dedicated to translational research in tissue engineering and biology. Ideal for countless regenerative-medicine applications, LAB [see figure 1] makes it possible to bioprint living cells – as well as hydrogels and biomaterials, with cell-level resolution by depositing micro-droplets of 300µm to 50µm of cell bioink with a precision of a few microns only.

**Figure 1** – laser-assisted bioprinting head with metalized donor inside NGB-R™.

There are many advantages to printing living cells with LAB, starting with viability. While conventional nozzle-based bioprinting methods are usually synonym of harmful process-induced forces causing cell injury and damage, LAB is a nozzle-free technology which takes shear stress out of the bioprinting process, resulting in a much superior cell viability ratio of an average 95%. Cells are indeed transferred from a donor to a receiver with a pulsed laser beam as driving projection force, hence do not suffer from any pressure force whatsoever – a critical difference which helps conserve cells' functionality and vitality post-printing.

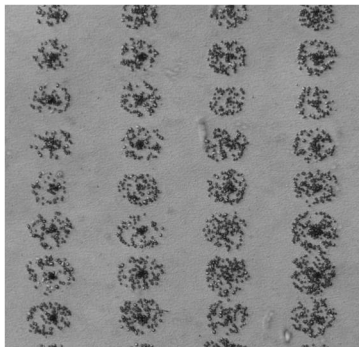

**Figure 2** – Pattern of fibroblast cells printed over a layer of Collagen-I

Another major interest of LAB lies in cell patterning and advanced tissue modeling, with the unprecedented capacity to spatially organize cells in 3D to mimic micro-patterns of tissue components and therefore replicate specific biological and cellular environments. With outstanding precision, living cells can easily be distributed within the extra-cellular matrix (ECM)/ bioprinted tissue itself according to appropriate, custom patterns designed beforehand [see figure 2]. This cell-patterning or guided self-organization approach is considered a key component of 4D bioprinting as it helps induce key biological processes [cell migration, proliferation, differentiation…] leading to more functional tissues. Overall, LAB favors better tissue morphogenesis control as the technology allows to precisely control cellular interactions and guide biological processes of organogenesis.

## A different, cytocentric approach to bioprinting

NGB-R™ is a multi-modal 4D bioprinting platform boasting the laser-assisted bioprinting (LAB) technology described above, along with complementary nozzle-based bio-extrusion and inkjet technologies. The instrument serves a new approach to bioprinting, which consists of printing cells separately from the ECM/biomaterials with distinct bioprinting techniques, thus taking advantage of what every technology offers best. Although NGB-R™ can work with commercial cell-loaded bioinks, the path described below allows to bypass the requirement to purchase and rely on a specific bioink along with numerous related issues, thus offering a greater level of versatility and convenience.


**Figure 3** – Poietis' NGB-R™ multi-modal 4D bioprinting platform

Here, living cells are not mixed with an "ink" (i.e. biomaterials and hydrogels) but rather incorporated into a bioprinted tissue. Cells therefore

remain in their medium and get printed as such, with LAB. Spreaded across a metalized donor placed over the laser-assisted bioprinting head [see figure 1] cells are getting projected in droplets onto a receiver with high precision and viability, according to a specific pattern [see figure 2].

On the other hand, layers of biomaterial such as collagen are printed in either filaments (bio-extrusion) or drops (inkjet) independently from living cells. The approach then consists of a layer-by-layer sequence, with cell layers and biomaterial layers superimposing [see figure 4]. An automated robotic arm operates the whole bioprinting process along with the predefined "printing jobs" sequence, ensuring high level of reproducibility and precision.

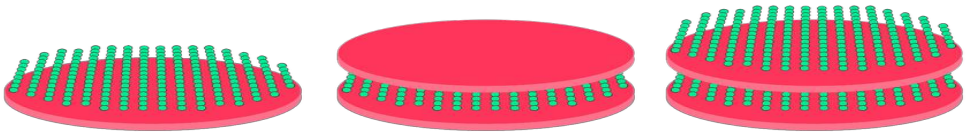

**Figure 4** – layer-by-layer tissue construct with living cells being incorporated in-between layers of biomaterial.

Before a first layer of cells is projected, a primary "bed" of soft biomaterial is printed at the bottom of the receiver so as to secure smooth cell landing and ensure better cell attachment/adherence. This prevents cell droplets from bursting against the receiver surface and constitutes the basis of the bioprinted tissue.

## Skin tissue biofabrication workflow

The biofabrication process for bioprinted full-thickness skin tissue models was based on the same cytocentric bioprinting approach as described above and followed a series of specific steps detailed below.

**1. Layer-by-layer tissue computer-assisted design**

Poietis' CAD [a proprietary cytocentric software designed to create the files that will then be loaded onto the platform for tissues to be bioprinted] was used to create the tissue construct and .bio files. Every single layer of the tissue were designed in a "2D-to-3D" approach, superimposing layers of living cells to be projected with LAB and layers of biomaterial to be printed with micro-valve (ink-jet bioprinting), in specific patterns and sequence.

**2. Cell patterning and laser parameters setup**

Ahead of the tissue biofabrication, exact cell patterns were defined. Those consist of a specific 3D spatial distribution and organization of cells incorporated within the extra-cellular matrix (ECM)/biomaterial, and a specific quantity of cells per droplet [see figure 5], which may easily be adjusted by playing on the cell concentration in the bioink (cells + medium only) and the laser beam energy level.

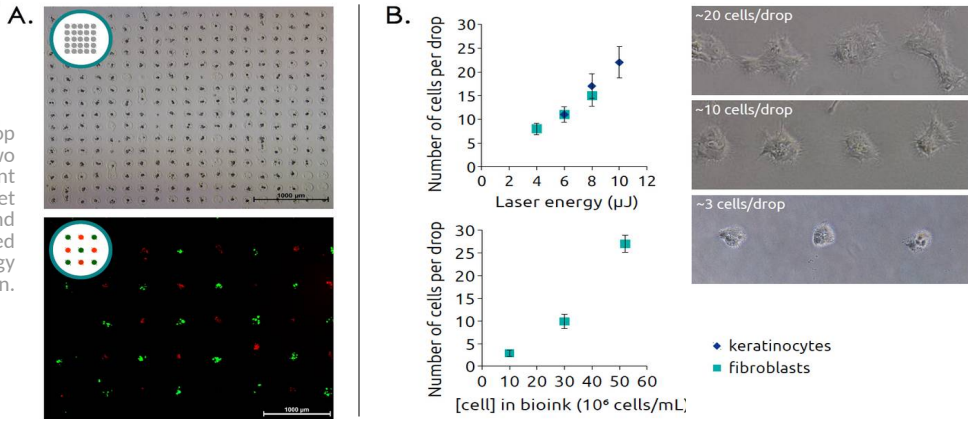


**Figure 5** – (A) Drop distribution with one or two cells types. (B) Different numbers of cell per droplet for keratinocytes and fibroblasts can be obtained by variation of laser energy or cell concentration.

### 3. Dermis biofabrication (D0)

To ensure further cell adhesion, an initial layer of collagen-I was printed with the NGB-R™ instrument's micro-valve head. Following gelification, a first layer of human primary fibroblasts was incorporated with LAB according to the specific pattern of cell distribution created above [see figure 5]. This layer-by-layer process was then repeated, superimposing layers of fibroblasts and layers of collagen-I [see figure 6].

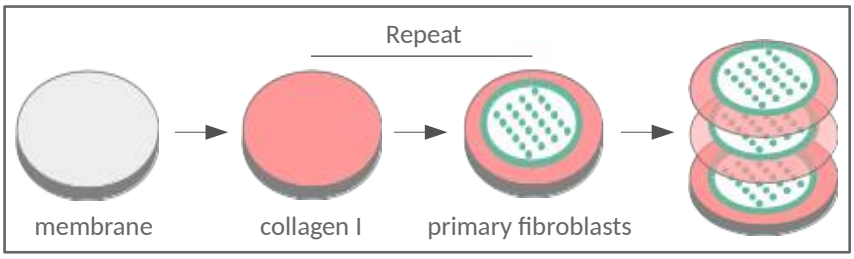


**Figure 6** – Dermis biofabrication process with patterned fibroblasts and collagen-I, designed for cell adherence, proper gelification and ideal and guided self-organization.

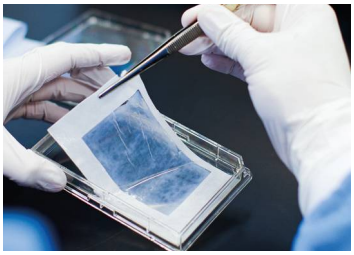

**Figure 7** – Bioprinted dermis.

### 4. Dermal equivalent maturation (D0-D6)

5 to 7 days were allocated to dermal maturation, time during which its structure and thickness evolution were closely monitored and controlled, as shown below [see figures 8-9]. Anti-collagen-I antibody staining also showed successful secretion of human collagen-I by bioprinted fibroblast cells, post-printing [see figure 10].

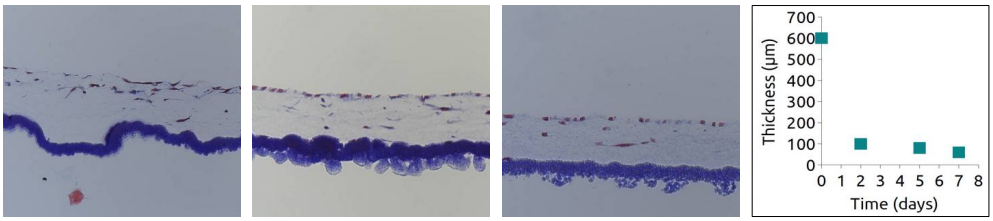


**Figure 8** – Masson Trichrome staining showing a decrease in thickness from 600µm at D0 to about 70µm within 7 days of maturation.

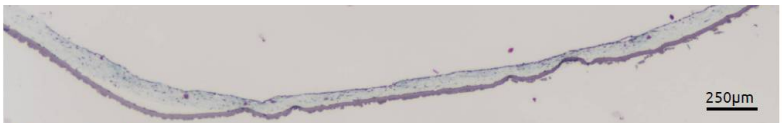


**Figure 9** – Masson Trichrome staining highlights dermal equivalent structure.

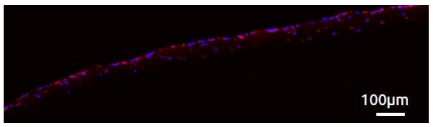


**Figure 10** – Anti-collagen I antibody staining highlights secretion of human collagen-I by fibroblasts.

### 5. Epidermis biofabrication (D9)

Following the key stages of dermal-equivalent maturation (D0-D6) and air-liquid interface (D6-D9), primary human keratinocytes were then incorporated into the dermal equivalent with LAB following a precise pattern [see figure 11] with one, unique homogeneous layer of cells.

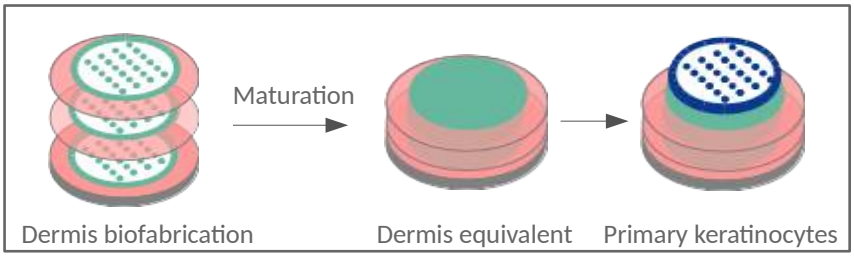


**Figure 11** – Epidermis biofabrication process with patterned primary human keratinocytes and collagen-I, following dermal maturation and air-liquid interface.

**Figure 12** – Bioprinted full-thickness skin tissues being controlled.

## Bioprinting process evaluation

Thorough control, monitoring and evaluation were carried out at every single stage of the full-skin tissue bio-fabrication process. Starting with preliminary tests for cell viability [see figure 13] as well as for key biological functions including cell proliferation, adhesion and ECM-protein synthesis [see figure 14].

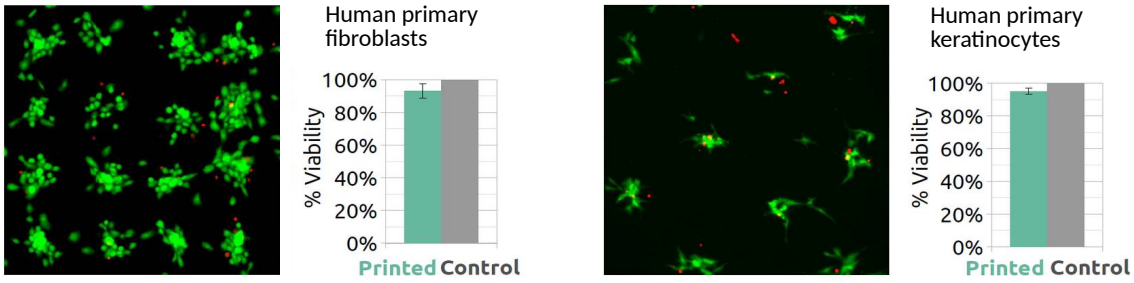


**Figure 13 – Cell viability test**. Live (green) and dead (red) cells are respectively stained with calcein and ethidium. Viability is ~95 % for human primary keratinocytes and fibroblasts.

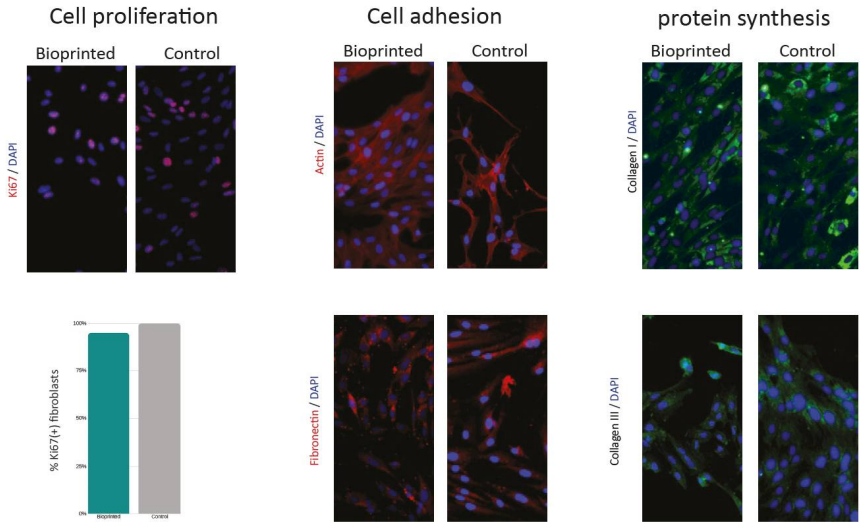


**Figure 14 – Cell proliferation/adhesion/ECM protein synthesis.** No difference between bioprinted cells and controls were observed, proving good bio-compatibility of the process.

The evolution of bioprinted keratinocytes patterns were also closely monitored and tracked over time [see figure 15] as yet another proof of cell viability and proliferation.

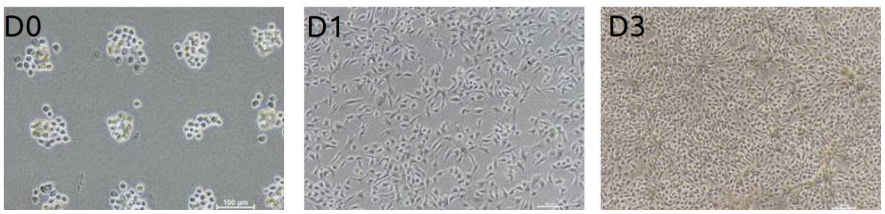


**Figure 15** – a pattern of primary human keratinocytes printed over collagen-I tracked over a 3-day time lapse.

Once key vitality and biological functions were confirmed, we proceeded to evaluating the 3D printing process itself with thorough control of the dermis bioprinting sequence, including proper maintenance of established cell patterns and accurate preservation of cell positioning across the numerous bioprinted layers, as well as proper gelation. These tests [see figure 16] confirmed cell pattern was properly maintained layer after layer and

that microfibers were homogeneously distributed.

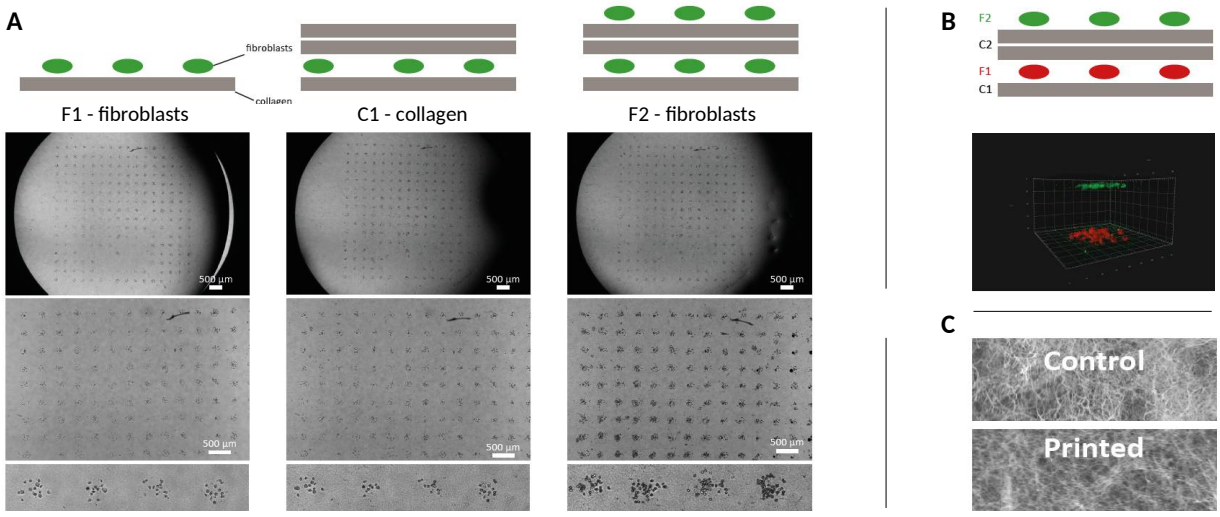


**Figure 16** – (A) pattern maintaining and cell positioning evaluation: control of pattern positioning in x y and z axis. (B) cell positioning in z: confocal microscopy reveals precise control of cell positioning as we observe 2 fibroblasts patterns (green and red) separated by a collagen-I layer. (C) gelation test: SEM microscopy revealing homogeneous distribution of microfibers after collagen-I bioprinting.

## Results and discussion

This disruptive cytocentric approach to bioprinting enabled by the NGB-R™ multi-modal 4D bioprinting platform proved very well-suited to creating functional full-thickness skin tissue models. The instrument's laser-assisted bioprinting technology allowed for a very high ~95% viability ratio for both primary human fibroblasts and keratinocytes, along with precise cell patterning layer after layer, successfully maintained throughout the entire process. Furthermore, bioprinted full-thickness skin tissues demonstrated good biological functions including cell proliferation, adhesion and ECM protein synthesis.

**Figure 17** – 6-well plate containing bioprinted human full-skin tissues, biofabricated with NGB-R™.

When it comes to evaluating bioprinted skin tissues, the control and assessment of epidermal stratification and maturation proves a critical phase. In these pictures [see figure 18] HES, anti-cytokeratine 5 and anti-filaggrin stainings of keratinocytes printed on cellularized dermis highlighted epidermal stratification.

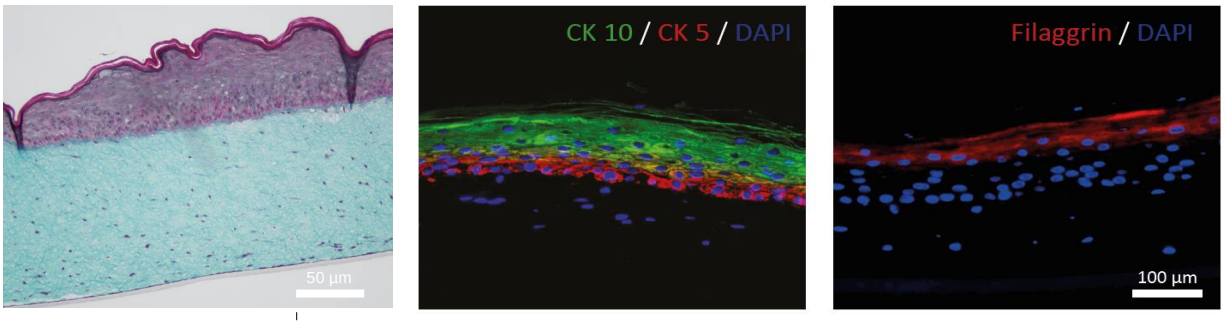


**Figure 18** – HES, anti-cytokeratine 5,10 and anti-filaggrin stainings of keratinocytes printed on decellularized dermis.

In the histology below [see figure 19] H&E staining shows that our model mimicked histological architecture of the native skin with the presence and proper organization of both dermal an epidermal compartment. A well-developed basement membrane is present at the interface between the cells of epidermis and dermal compartment. Epidermis is well stratified and differentiated with the presence of both stratum spinosum, stratum granulosum and stratum corneum.

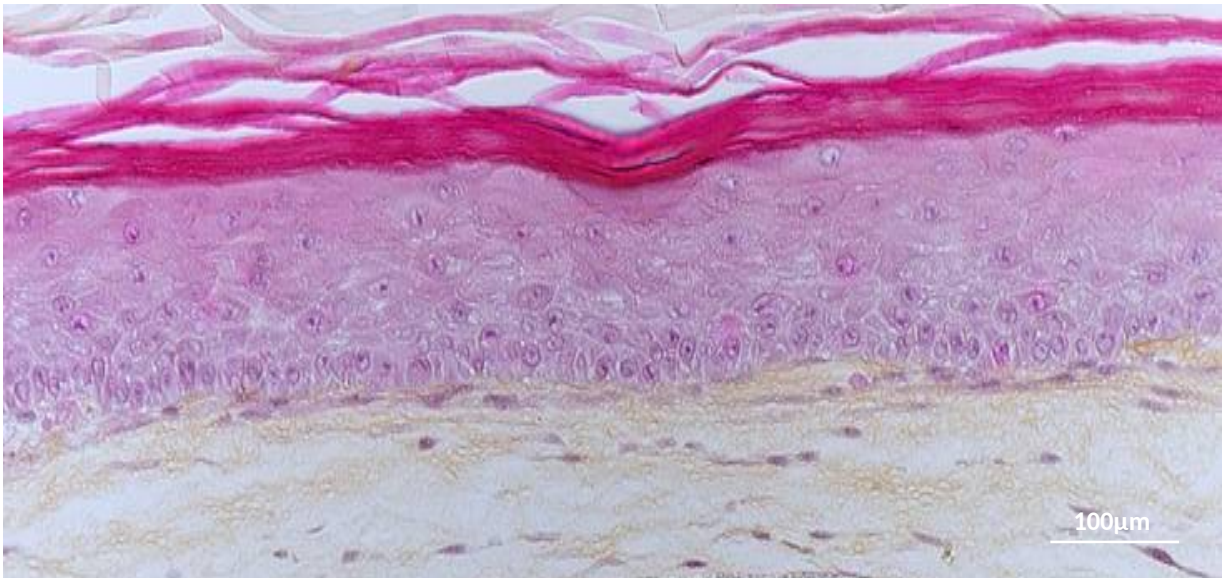


**Figure 19** – Bioprinted human full-skin tissue histology.

## Illustrated example of possible applications

In-vitro skin tissues can serve many different applications, including cosmetics and drug testing, cancer research and many other. BASF, a Poietis' early partner, relied on the skin models bioprinted with NGB-R™ to evaluate cosmetic firming actives and ingredients effectiveness for skin care applications.

Through key partnerships in 2015 and 2017, Poietis' bioprinted full-skin models helped BASF validate then commercialize a couple of active ingredients: Dermagenist™ and Replexium©. Dermagenist™ led the way being BASF's first active ingredient which efficacy has been confirmed by using laser-assisted bioprinted skin models. "A mature and thick dermis is an essential condition to obtain optimum results in epidermal printing", said Sebastien Cadau, responsible for tissue engineering development at BASF.

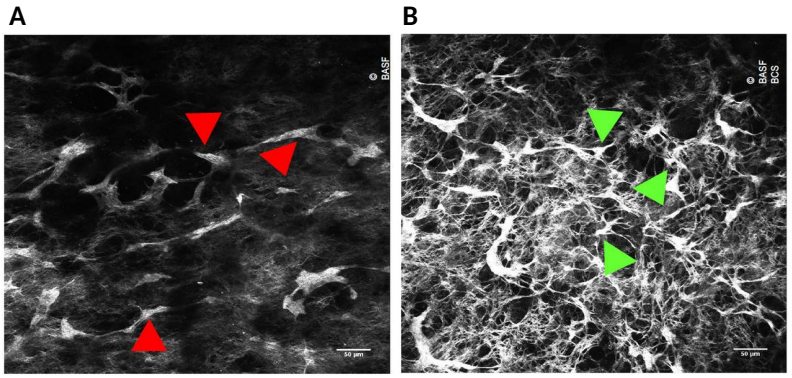


**Figure 20** – Visualization by second harmonic generation (SHG) of the effect of Acetyl Tetrapeptide-9 and Acetyl Tetrapeptide-11 complex on the collagenic network in 3D bioprinted skin. Collagen fibers are visualized in grey. Images show a 3D stack acquisition in depth of 10 planes of the upper part of the dermis. Scale bar: 50µm.

**A** – control
**B** - Acetyl Tetrapeptide-9 (7.3µg/ml) and Acetyl Tetrapeptide-11 (9.8µg/ml)

In *First time use of 3D micro-patterning screening and 3D skin printing to select firming actives* (S. Pain et al., IFSCC, 2017): "This bioprinted skin is suitable for dermo-cosmetic evaluations as it allows to observe some changes induced by a treatment with an Origanum majorana extract used at 0.04%: in the dermis LOXL1 elastin cross-linking enzyme seems increased and in the epidermis thickness and involucrin are modulated" [see figure 21]

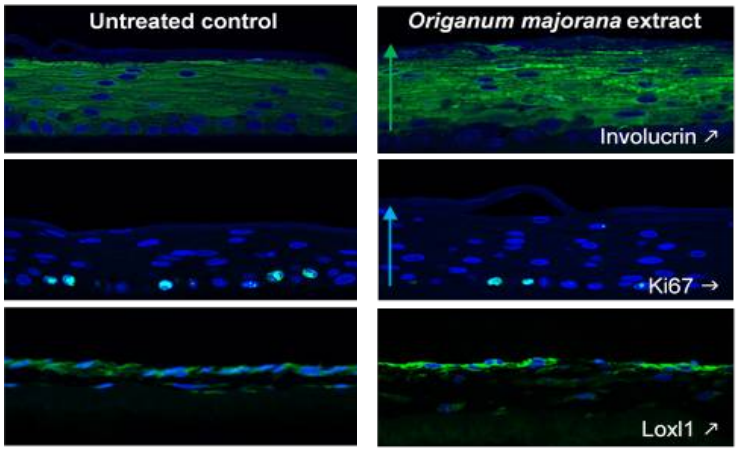


**Figure 21** – Immunostaining of Bioprinted skin treated or not by Origanum majorana extract at 0.04% after 14 days.

Bioprinted skin is formed by a condensed dermis with a suitable repartition of fibroblasts and a well anchored epidermis. Even if the epidermis is not fully mature due to a short differentiation period after printing (one-week air-liquid interface only), an accurate keratinocytes differentiation process is observed.

## Conclusions

This application notes demonstrates how powerful and efficient Poietis' NGB-R™ bioprinter can be at making functional tissues involving several cell types. Although compatible with the conventional use of commercial cell-loaded bioinks, the platform boasts a different 4D-bioprinting approach based on advanced patterning and precise spatial cell distribution. This process, consisting in incorporating multiple layers of patterned cells into ECM/biomaterial with very high resolution and precision – instead of extruding them all-together at once, promotes and leads to the emergence of key biological functions in the bioprinted model, therefore proving more suitable for tissue morphogenesis. Here, both primary human fibroblasts and keratinocytes were successfully bioprinted with very high viability level, then proliferated, migrated and self-organized. Dermal and epidermal constructs maturated and strengthened, ensuring proper development.

## References

**New bioprinted skin, cosmetic in vitro model,** S. Cadau et al., *Journal of Cosmetic Science (2017)* **https://pubmed.ncbi.nlm.nih.gov/29465388**

**Cell patterning by laser-assisted bioprinting**, R. Devillard et al., *Methods in Cell Biology (2014)* **https://pubmed.ncbi.nlm.nih.gov/24439284**

**Cell patterning technologies for organotypic tissue fabrication**, B. Guillotin et al., *Trends in Biotechnology (2011)* **https://pubmed.ncbi.nlm.nih.gov/21256609**

**Laser-assisted cell printing: principle, physical parameters versus cell fate and perspectives in Tissue Engineering**, F. Guillemot et al., *Nanomedicine (2010)* **https://pubmed.ncbi.nlm.nih.gov/20394540**

**Laser-assisted bioprinting of engineered tissue with high cell density and microscale organization**, B. Guillotin et al., *Biomaterials (2010)* **https://www.sciencedirect.com/science/article/pii/S0142961210006927**

**Patterning of Endothelial Cells and Mesenchymal Stem Cells by Laser-Assisted Bioprinting to Study Cell Migration** , JM. Bourget et al., *BioMed Res Int (2016)* **https://pubmed.ncbi.nlm.nih.gov/27833916**

**BASF & Poietis' Joint Press Release,** BASF.com (2017) **https://www.basf.com/global/en/media/news-releases/2017/10/p-17-352.html**

**First time use of 3D micro-patterning screening and 3D skin printing to select firming actives**, S. Pain et al., IFSCC (2017)

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