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"World's first computer-connected lab grown human bi-oprinted skin with a sensory nervous system for instant-neous cosmetics and fragrance testing."

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1. Introduction

Sensory perception - tingling, soothing effects, and emotional responses, plays a fundamental role in how consumers perceive cosmetics [1]. However, even with its importance, the integration of a functional sensory nervous system into in vitro models has been widely unexplored. To date, the cosmetics industry has largely relied on basic human models to assess sensory and emotional impact, due to the lack of biologic and sophisticated relevant models able of measuring these complex responses. It is only in the last few years that the industry has begun to recognize the need for more predictive, physiologically accurate systems that take neurosensory data into account during product development.

Cutaneous sensory neurons are primary afferent neurons specialized in the transduction of mechanical, thermal, and nociceptive stimuli into action potentials [2]. Their nerve endings, either free or encapsulated allow for specific detection depending on the nature of the stimulus. These action potentials are transmitted via different types of nerve fibers to the spinal cord and subsequently to the somatosensory cortex. They are involved in proprioception, nociception, and homeostasis. Their function is essential not only for sensory integration, but also for coordinating responses through interactions with other resident skin cells. These neurons form a functional interface with keratinocytes [3], but also with Langerhans cells, fibroblasts, and immune cells, enabling the transmission of outside signal information into inside biochemical and

cellular responses. This neurocutaneous crosstalk is essential for modulating inflammation, barrier function and even wound healing.

In response, we have developed a novel full-thickness human skin model incorporating differentiated sensory neurons from Human Induced Pluripotent Stem Cells (iPSC), primary human keratinocytes and fibroblasts using 3D bioprinting technology.

Innervated skin tissue model is connected a multi-electrode array (MEA) chip, enabling real-time recording of neuronal activity in response to various stimuli. By computerizing skin sensory responses, our approach represents a key step towards bridging the gap between biological sensation and digital evaluation. This model not only introduces a new way of performing sensory tests in cosmetics, but also opens the door for earlier and more accurate ingredient screening. Therefore, we have combined 3D bioprinted technology to produce sophisticated models, with electrophysiology to produce rapid laboratory data.

2. Materials and Methods

2.1 Cell populations

Fibroblasts and keratinocytes were isolated from donated human skin samples after informed consent. Sample collection was performed in accordance with French and European ethical guidelines and regulations applicable in local hospitals in Lyon, France. Optimal transport conditions were used to ensure the best viability of the samples. After enzymatic dissociation, cells were amplified in 225 cm² flasks using media (CTIGM.Fibro and CTIGM.Kerat, CTIBiotech, France).

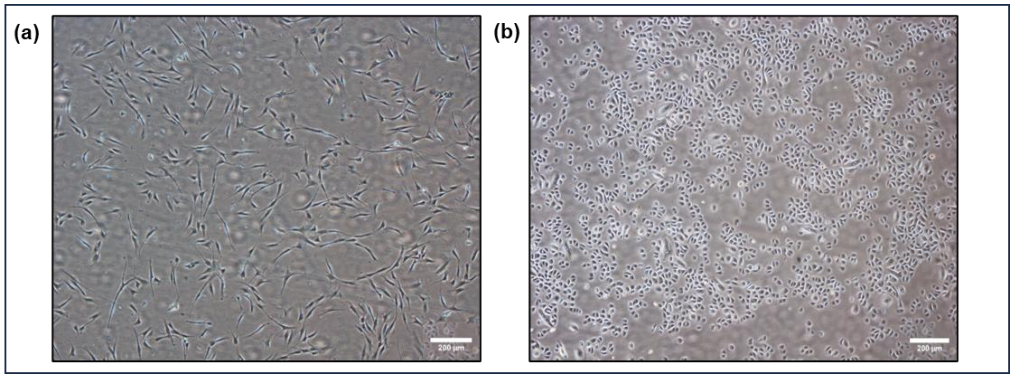

Figure 1. *2D culture of human juvenile primary cells:* (a) Fibroblasts; (b) Keratinocytes.

Human iPSC-Derived Sensory Neuron Progenitors (Axol Bioscience Ltd., Cambridge, United Kingdom) were generated from a newborn male donor's cord blood.

2.2 3D bioprinted skin with a sensory nervous system

After the amplification process, human primary keratinocytes and fibroblasts were harvested using Tryple enzyme (Tryple Select 1X, Gibco) and counted with a LUNA-FL Dual Fluorescence Cell Counter (Logos Biosystems, France).

Induced Pluripotent Stem Cell (iPSC)-derived neurons were thawed, counted, and directly used for bioprinting by mixing with a specialized bioink (Cellink, Gothenburg, Sweden), which supports cell viability, spreading, and skin layer formation. A similar approach was done separately for fibroblasts and keratinocytes. The cell-laden bioinks were then loaded into 3 mL cartridges (Optimum EFD, Nordson, USA) for subsequent printing and placed on the defined print heads.

The 3D skin construct was designed using the computer-aided design software PrusaSlicer (Prusa Research, Prague, Czech Republic). The digital model consisted of a stratified architecture comprising, from bottom to top: a basal layer of iPSC-derived neurons, a layer of fibroblasts (dermis-like), a layer of keratinocytes (epidermis-like) and vertical pillars of IPSC-derived neurons intended to promote interlayer connections and mimic neurocutaneous interactions.

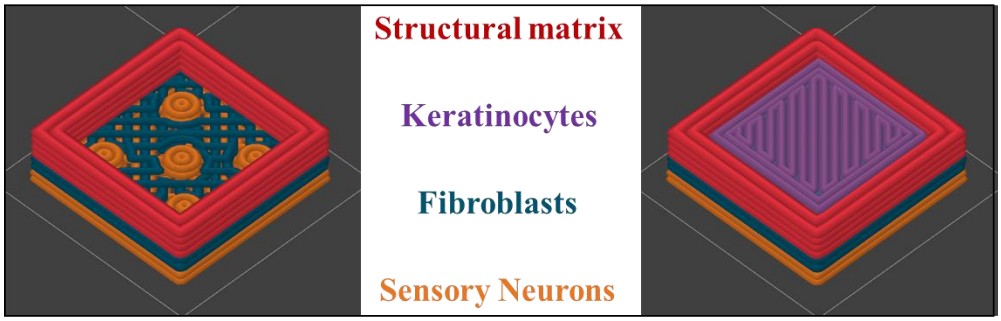

Figure 2. *Schematic representation of the printing layer procedure.*

The resulting G-code files were transferred to a Bio X 3D bioprinter (Cellink) and the bioprinting process was conducted in 12-well culture plates (TPP), under sterile conditions, following our optimized protocols.

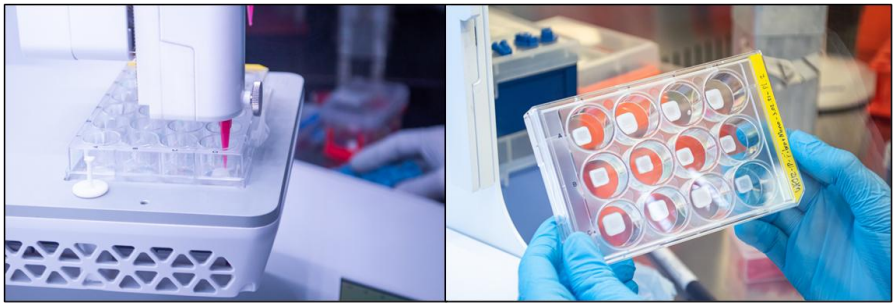

Figure 3. *3D Bioprinted skin models with a sensory nervous system.*

2.3. Maturation of 3D bioprinted models

3D printed models were grown over 3 weeks in Transwell culture inserts (VWR, USA), including the stages of dermal maturation, epidermal differentiation, air-liquid interface, and cornification of the bioprinted models. iPSC-derived sensory neuron progenitors were maturated directly within the 3D bioprinted model using successively several factors. The medium was supplemented with Glial Derived Neurotrophic Factor (GDNF), β-Nerve Growth Factor (β-NGF), Brain-Derived Neurotrophic Factor (BDNF), and Neurotrophin-3 (NT-3), in accordance with the protocol provided by Axol Bioscience. The timeline and concentration of these factors were applied as per the manufacturer's recommendations to promote maturation and sensory neuronal differentiation in the model.

2.4. Cell Viability analysis

3D models were analyzed to evaluate viability and morphology inside the bioink. Live/Dead kit reagents (Invitrogen, USA) were used for viability analysis. Calcein AM (live cells) and Ethidium homodimer-1 (dead cells) for 30 minutes before microscopic analysis. Live cells were analyzed at 494/517 nm and dead cells at 528/617 nm (excitation/emission) on a Nikon Ti-S Eclipse fluorescence microscope (NIKON, Japan).

2.5. On-Chip Multi-Electrode Array (MEA) measurements

The 3D Bioprinted models were placed onto a 6-well Cytoview MEA plate containing each 64 electrodes (Axion Biosystems, Atlanta, USA). Electric activities (sampling rate of 12,5 kHz) were measured by Axion Maestro Pro system in a 37 °C and 5% CO_2 chamber.

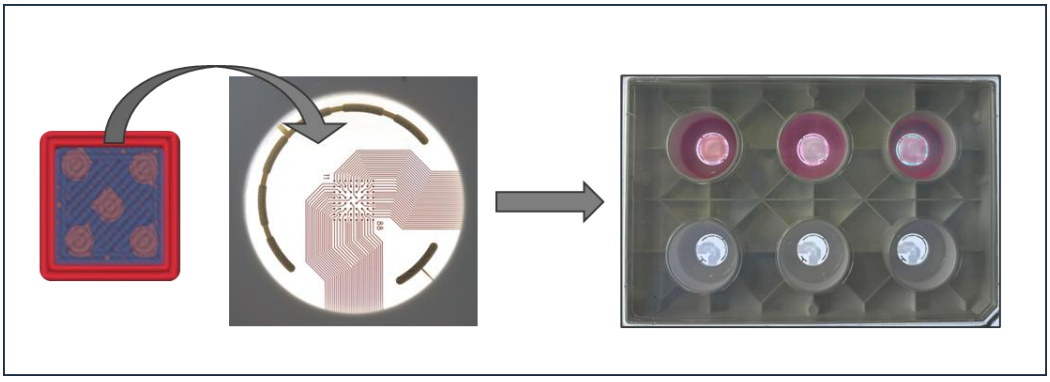

Figure 4. *On-Chip Multi-Electrode Array (MEA) plate.*

Manufacturer's software (Axion BioSystems IntegratedStudio (AxIS) Navigator and all the integrated tools were used to record and analyze. The test molecules (purchased from Medche

and Merck) were applied and measurements were conducted within 30 minutes following application:

Table 1. *Reagents used on 3D skin models*

Reagents	Concentration used
A803467	5 µM
Capsaicin	10 µM
Menthol	30 µM
Cortisol	10 µM
Inexpensive Parfum	/
Expensive Parfum	/

3. Results

3.1 Design and bioprinting of the model

Reproducible replicates of the models were successfully achieved following 3D bioprinting of the three cell types used in the study: keratinocytes for epidermis, fibroblasts for dermis and Human iPSC-Derived Sensory Neuron Progenitors for creation of sensorial innervated dermis. Generally, on this pneumatic system many replicates could be made per hour and was only limited by cell availability.

All the layers printed correctly in their positions, and we succeeded in forming the structure of an innervated skin model (Figure 2).

3.2 Viability and neuronal development in 3D skin model

Viability of models was assessed using Live/Dead reagents and fluorescence microscopical analysis. We observed an overall very good viability of the Human iPSC-Derived Sensory Neuron Progenitors in the models (Figure 5). We also confirmed a clear 3D morphology of the iPS-derived cells in the bioink. Their morphology indicated good differentiation and maturation into sensory neurons.

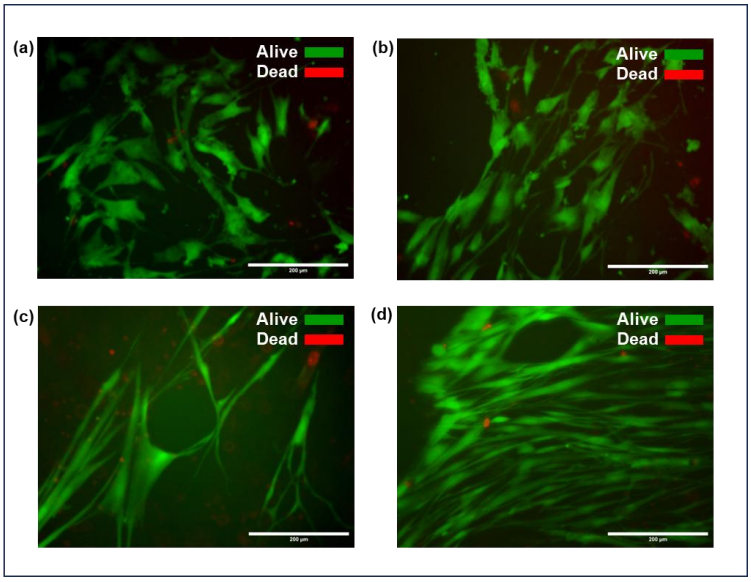

Figure 5. *Viability analysis of 3D Bioprinted models after Live/Dead staining and fluorescence microscopy analysis, after 28 days of maturation.*

3.3. Functional validation of model: Response to electrical stimulation

Neural activity in 3D skin models was recorded using MEA technology after electrical stimulation.

In the control model, containing no sensory neurons Figure 6 (a), no neuronal signal was detected, after the stimulation artifact visible in the white square. This suggests the absence of a functional electrical response to stimulation of other cell types. In contrast, in the 3D skin model incorporating sensory neurons Figure 6 (b), an increase in activity was observed after stimulation, reflecting an electrophysiological response.

The absence of response in the control condition highlights the role of sensory neurons in generating the signal. The artefact of stimulation, present identically in both conditions, confirms that it is of technical rather than biological origin.

These data show bioelectrical activity specific to the sensory model after stimulation.

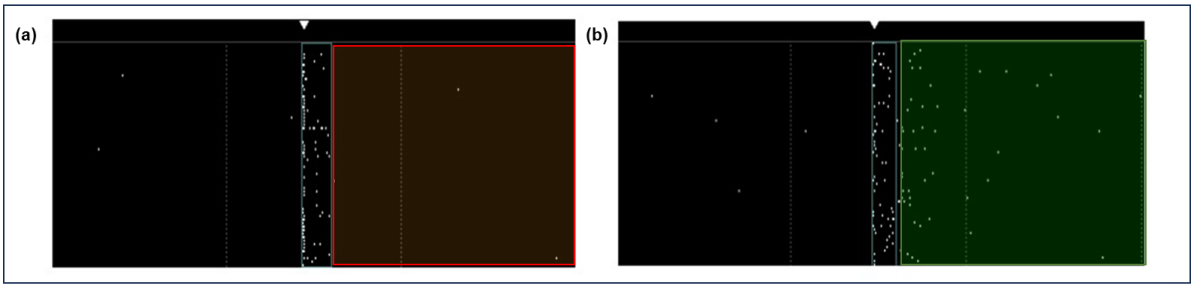

Figure 6. *MEA-based electrophysiological recordings of 3D skin model responses following electrical stimulation: (a) Control, 3D Skin model without sensory neurons; (b) 3D Skin model with sensory neurons. The white arrow indicates the site of electrical stimulation. The white square highlights the stimulation-induced artifact on the MEA signal.*

3.4 Quantification of neuronal spike activity following molecules application

To evaluate the responsiveness of the sensory neurons to chemical stimulation, spike activity was recorded using MEA following application of capsaicin or menthol.

As seen in Figure 7 (a), capsaicin (10 µM), a TRPV1 agonist, induced a slight increase of spike number compared to the baseline, suggesting a neuronal activation. The spikes number increased from around 11 for baseline to 13 and then 15 at successive post-treatment time points. Menthol (30 µM), a TRPM8 agonist, also increased neuronal activity Figure 7 (b), with a number of spikes increasing from around 1 at baseline to 5 after addition of the compound.

These results confirm that sensory neurons embedded in the 3D skin model are functionally sensitive to thermosensory ingredients.

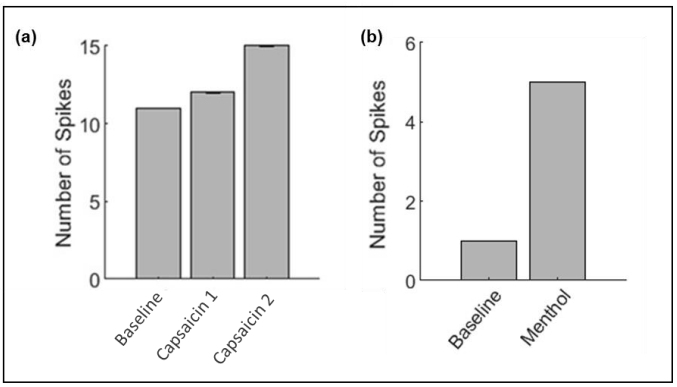

Figure 7. *Relative spike count recordings from 3D skin models using MEA following two different chemical stimulations. (a)* ***Capsaicin (10 µM)*** *– Baseline: recording prior to compound addition; Capsaicin 1: first post-treatment time point; Capsaicin 2: second post-treatment time point. (b)* ***Menthol (30 µM)*** *– Baseline: recording prior to compound addition; Menthol: recording after compound addition.*

The impact of different cosmetic formulations on the activity of the 3D skin model was assessed by measuring the number of spikes before and after the application of two different fragrances.

As shown in Figure 8 (a), the application of an inexpensive perfume resulted in a decrease in spike activity from 5 at baseline to 2 after application. This suggests a possible moderating effect on neuronal activity. In comparison, application of a more expensive perfume showed no reduction in neuronal activity. The number of spikes is stable, with values around 6 before and after treatment.

These preliminary results suggest that the 3D skin model with sensory neurons can detect differential neuronal responses after the application of cosmetics.

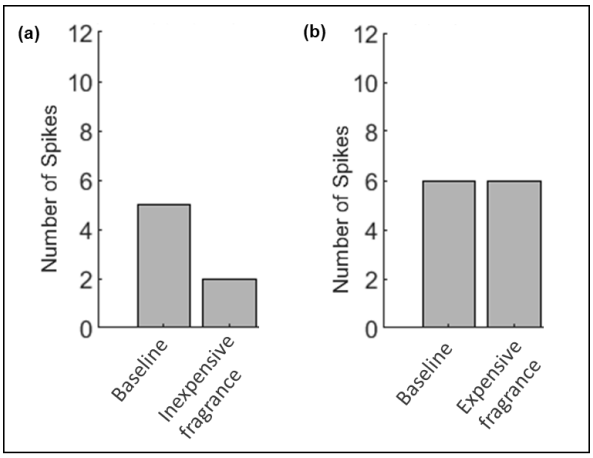

Figure 8. *Spike count recordings from 3D skin models using MEA following two different cosmetics application. (a) Inexpensive fragrance – Baseline: recording prior to compound addition; Inexpensive fragrance: recording after cosmetic application. (b) Expensive fragrance– Baseline: recording prior to compound addition; Inexpensive fragrance: recording after cosmetic application.*

To evaluate the sensitivity of the 3D skin model to neuromodulatory compounds, spike counts were recorded after application of cortisol (10 µM) and a sodium channel antagonist (A803467, 5 µM).

As shown in Figure 9, cortisol increased spike activity, with the number of spikes increasing from 4 at baseline to 11 after treatment. This suggests an increase in neuronal excitability. Later application of the sodium channel antagonist partially reduced this activity to 9 spikes, confirming the neuronal origin of the recorded signals.

These data demonstrate the responsiveness of the model to excitatory and inhibitory compounds.

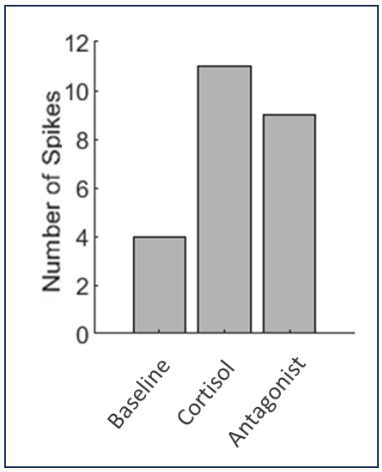

Figure 9. *Spike count recordings from 3D skin models using MEA following two different chemical applications. Baseline: recording prior to compound addition; Cortisol 10µM 1: recording after compound addition; Sodium channel Antagonist (A803467) 5µM: recording after compound addition.*

4. Discussion

The development of a full-thickness 3D bioprinted skin model innervated by a sensory neurons system represents a major advance in the field of cosmetic ingredient and product testing. The morphology and the viability of the sensory neuron's cells observed in the model suggest that the cells have the capacity to have a good development, differentiation and maturation in a 3D environment within the bioink. Moreover, the culture medium supplemented with growth factors allows a good co-culture of all the cell types within the 3D skin model. These results confirm the bio compatibility of these cell types in a bioprinting process and highlight the help of 3D environment to allow their development [4].

The ability of the models to have a measurable neuronal activity response is a strong indicator of the development and functionality of the integrated cells. Indeed, the basic 3D skin model (without neurons) showed no activity, which confirms that the responses recorded were due to the presence of the sensory neuron's cells. Our models have the ability to respond to known neurosensory stimuli [5] like capsaicin (TRPV1 agonist) and menthol (TRPM8 agonist) showing that the neurons integrated their functional receptor activity in the model.

In addition, the skin models were able to detect different responses to cosmetics fragrances and neuromodulator molecules [6] such as cortisol and sodium channel antagonist. These results confirm the hypothesis that the model could be a predictive tool for the selection of ingredients based on their potential to activate or inhibit cutaneous sensory pathways. The increase in activity observed with the application of cortisol and its reduction with A803467 confirm the neuronal origin of the spikes measured, highlighting the sensitivity of the system.

In future work, the integration of additional sensory modalities or immune components [7] could further improve its relevance for more complex applications, such as neurogenic inflammation studies.

5. Conclusion

We have developed a 3D human skin model, innervated by iPSC-derived sensory neurons, capable of generating a measurable electrophysiological response via MEA from both skin epidermal stimulation and systemic throughput. This model stands out for its viability, the maturation of neuronal cells, and its responsiveness to different types of stimulation. This offers an innovative way for assessing the sensory impact of ingredients and molecules in a reproducible process.

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Conflict of Interest Statement.

NONE.

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