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BIOPRINTING SKIN TISSUE MODEL PROTOCOL

CELLINK SKIN

This is a suggested procedure, please adjust according to your experimental needs. To maintain the sterility of the product, work under sterile conditions.

Protocol aim

The aim of this protocol is to provide instructions for printing a skin tissue model using CELLINK SKIN bioink, together with primary human fibroblast cells and primary human keratinocyte cells. The model is a suggested way to bioprint a simple, full thickness skin model with a fibroblast gradient mimicking papillary and reticular dermal compartments, see Figure 1.

Materials needed

- DNA Studio 4*
- USB Flash Drive
- Sterile conical bioprinting nozzles, 22G*
- 3 cartridges, 3cc*
- BIO X or BIO X6*
- Training ink like CELLINK START (optional)
- CELLINK SKIN*
- Primary human fibroblasts (HDF)*
- Primary human keratinocytes (HEK)
- Cell culture medium*
- Falcon tubes
- Syringes and female/female Luer lock adaptor
- Empty cartridges with end and tip cap, 3cc*
- 24-well plate
- Crosslinking Agent* (included with the bioink purchase)
- Thrombin, vial with 100 U (included with the bioink purchase)
- Transwell inserts

*The product can be purchased in the CELLINK shop at www.cellink.com/shop.

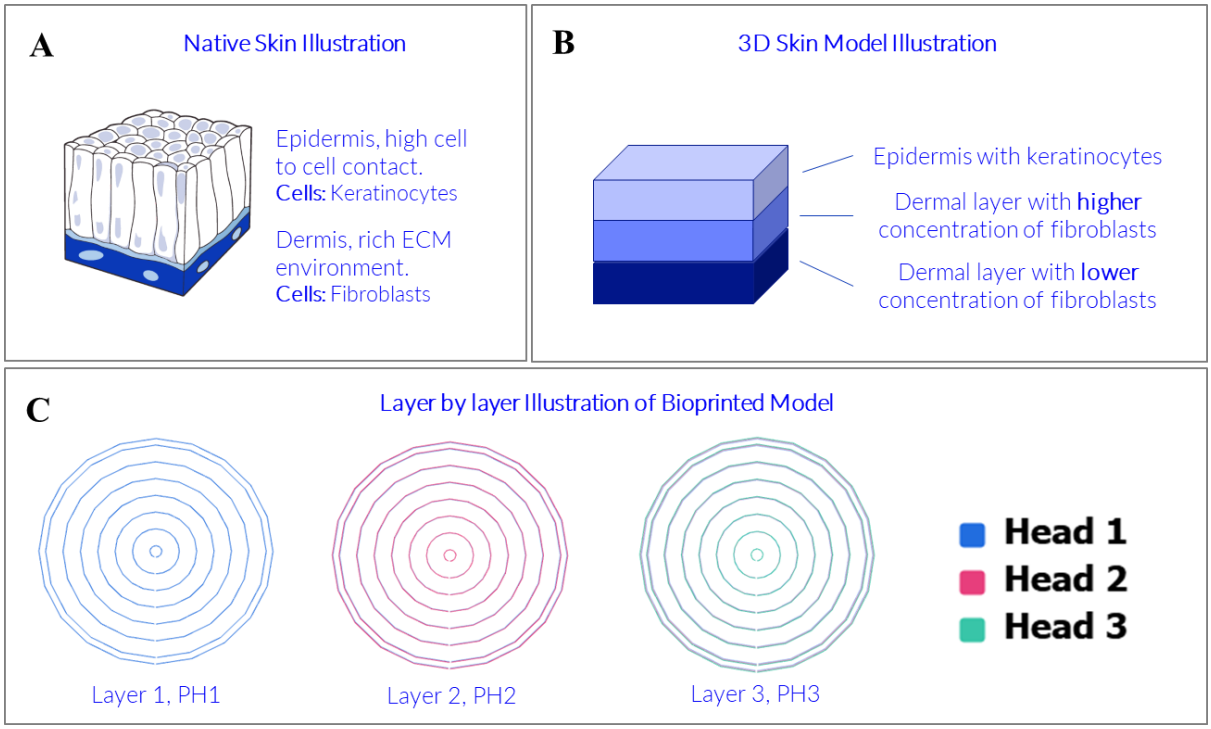

Figure 1. *Blueprint and illustration of skin model.* ***(A) Native Skin Illustration****: Sketch of native skin cellular organization, with the epidermal and dermal arrangement highlighted.* ***(B) 3D Skin Model Illustration****: A simplified view of the different sections included in the bioprinted 3D skin model. The two bottom compartments represent the reticular respectively papillary dermal layers with higher respectively higher fibroblast concentration. The top compartment represents the epidermal layer.* ***(C) Layer by layer Illustration of Bioprinted Model****: Layer by layer print pathway of a cylindrical skin patch where each compartment in the "3D Skin Model Illustration" is printed with 1 layer and concentric print pattern. PH = Printhead.*

Protocol

This protocol can be performed with printheads and print bed at room temperature, where room temperature is between 20-25°C. The skin model bioprinted in this protocol is the cylindrical skin patch exemplified in Figure 1C, the user is encouraged to change the shape and dimensions to fit experimental needs.

Example of alterations that can be incorporated if the protocol is adjusted accordingly:

- **Shape and dimensions:** Change the shape from cylindrical to rectangular or square if this format suits your experimental needs better. Adjust the dimensions to achieve smaller or larger skin patches.
- **Transwells:** In this protocol it is suggested to transfer the skin models from submerged culture into transwells to initiate air-liquid interface culture. If desired, it is also possible to bioprint the skin model directly onto transwell inserts by optimizing calibration, nozzle, and model size.
- **Embedded epidermis:** In the current protocol the epidermis is embedded into CELLINK SKIN bioink. To achieve an epidermis with less ECM around the keratinocytes and higher cell-to-cell contact, the epidermal bioink formulation can be changed to a high concentration cell suspension. Dispensing of cell suspensions however requires the use of a Syringe Pump printhead or EMD printhead, as the pneumatic printheads cannot accurately dispense liquid formulations. Here it is also recommended to print the perimeter in the epidermal layer with a bioink to form a barrier/edge that can keep the cell suspension on top of the model until the epidermal cells have had the possibility to adhere to the dermis.

1. Creation of Model

MATERIAL

DNA Studio 4 (alternatively other CAD software, Slicer software and g-code editor program)

USB Flash Drive, if DNA Studio 4 is not connected directly to the BIO X or BIO X6

DESCRIPTION

- At the start page of DNA Studio 4, create a new print protocol using the “Model” option.
- Select Petri dish as the desired print layout.
    - When working with a petri dish there is only one model created in the centre of the print bed. To work with only one model is usually easier when optimising a print protocol as it is quick and easy to identify potential errors in the modelling.
    - In Step 2 it is recommended to switch from using petri dish to using the well plate option to bioprint multiple replicates of the skin patches.
- In the “Model” tab create a new shape of desired dimensions. For example, a cylinder of 10 mm in diameter and 1.2 mm height to have the 3 layered model.
    - If not using DNA Studio 4, open a STL file created in other CAD software with desired dimensions and shape.
- In the “Layer” tab, change the infill pattern to “concentric” and increase the infill density to 42%. Also, add additional “Layer groups” to assign different printheads to different layers:
    - Layer group 1: Assign to layer 1 and PH1.
    - Layer group 2: Assign to layer 2 and PH2.
    - Layer group 3: Assign to layer 3 and PH3.

At this stage the modelling part of the protocol set up is complete. To transport the model to a BIO X bioprinter, click on the option “open copy in g-code editor” to save the g-code. To save the protocol, go to the next tab “Printheads” and use the top left saving options to save the partially set up protocol. If not using DNA Studio 4, continue to the last tab “Summary” to export the g-code.

2. Preparation for printing

MATERIAL

Training ink, example CELLINK START (optional)

Conical bioprinting nozzles, 22G

BIO X or BIO X6

24-well plate

DESCRIPTION

- To test the model, equip the BIO X or BIO X6 with three cartridges filled with an ink for training purposes, for example CELLINK START, and capped with 22G nozzles.
- Open the saved protocol or g-code on the BIO X/BIO X6.
- Go through the setup of the protocol to start the print.
    - Use 20-25 kPa and 10 mm/s for all printheads.
    - If more guidance is needed on the set up and printing of a protocol, please refer to the instrument manual.
- Utilize automatic or manual calibration and test the print protocol. Ensure all printheads move as intended and that the final model have the desired characteristics.

Before proceeding to the next step, make any changes needed in the model. It is also recommended to repeat this procedure with CELLINK SKIN diluted 10+1 with PBS to simulate cell embedded bioink utilizing recommended start parameters in Step 6. To bioprint acellular with the correct bioink allows for practicing and optimization of print parameters before proceeding to the cell embedded printing. It also allows for testing calibration, if printing into transwell inserts is intended utilize this opportunity to validate the procedure.

3. Preparation of bioink

MATERIAL

CELLINK SKIN bioink

3 x 3 mL syringes with Luer Lock connection

DESCRIPTION

- Make sure that sterile 22G nozzles, the BIO X/BIO X6 and other equipment needed for printing are in place before proceeding with the next steps.
- Pre-warm at least 3 mL of CELLINK SKIN bioink to room temperature.

Note: Distribute the bioink into three syringes with at least 1 mL in each syringe. If it is desired to bioprint the epidermal layer with a high-density cell suspension instead of keratinocytes embedded in bioink distribute the bioink between two syringes instead.

4. Preparation of cell suspension

MATERIAL

HDF

HEK

Cell culture medium

3 Falcon tubes or similar

DESCRIPTION

- Prepare three cell suspensions with following quantities of cells:
    - HDF 4 x10^6 cells/mL bioink for the reticular dermis.
    - HDF 8 x10^6 cells/mL bioink for the papillary dermis.
    - HEK 10 x10^6 cells/mL bioink for the epidermis.

Example: If you have prepared 1 mL of bioink for the epidermal layer the number of HEK needed is 10 x10^6. If you have prepared 1.5 mL of bioink for the epidermal layer the number of HEK needed is 15 x10^6.

- Spin down the cell suspensions and reconstitute the cell pellet in cell culture medium so that the total volume is 100 µL per 1 mL bioink.

Note: For 1 mL bioink make the total volume of cell suspension to 100 µL, for 1.5 mL bioink make the total volume of cell suspension to 150 µL.

5. Mixing CELLINK SKIN bioink with the cells

MATERIAL

Cell suspensions

Syringes with pre-warmed CELLINK SKIN bioink

3 mL syringes with Luer lock connections

Female/female Luer lock adaptor

Empty cartridges

DESCRIPTION

- At this point, mix the ten parts of bioink with the one part of cell suspension, taking care not to introduce air bubbles to the mixture. For detailed instructions see *Mixing cells and bioink Protocol.*
    - Take the syringes with pre-warmed CELLINK SKIN bioink and attach each of them to an empty 3 mL syringe using a female/female Luer lock adaptor. Push half of the bioink over to the syringe attached.

Note: To avoid introducing air into the bioink when mixing, carefully pre-fill the Luer lock adaptor with CELLINK SKIN before attaching the empty syringe.

    - Detach the syringes and pipette the desired cell suspension into one of the CELLINK SKIN containing syringes. Attach the syringes again and gently mix the bioink back and forth between the syringes until the cell suspension is homogeneously incorporated.

- Transfer each of the CELLINK SKIN bioink with cells to empty cartridges and cap the cartridge with a tip cap.

Note: Keep track of which syringe that have which cell type and density.

Note: Do not attach a nozzle to the cartridge until the start of the print session.

6. Setting up the bioprinter

MATERIAL

BIO X or BIO X6

DESCRIPTION

- Open the protocol optimized in Step 2.
- If the start parameters have not been optimized for the CELLINK SKIN cartridges, use bioprinting parameters as shown below for printhead 1, 2 and 3 as a starting point:
    - Nozzle type and size: 22G conical tip
    - Printing pressure: 12 kPa**
    - Printing speed = 10 mm/s**
    - Printhead temperature: -
    - Print bed temperature: -

** This is recommended starting parameters for the print. The pressure needed and optimal speed depends on the temperature of the surroundings and preparation procedures (amount of bioink and actual temperature of the bioink). ALWAYS test the pressure before starting the print and be prepared to adjust the speed and pressure during the print, especially for long printing sessions.

7. Printing

MATERIAL

The three cartridges of CELLINK SKIN with cells

Sterile conical bioprinting nozzles, 22G

24-well plate

DESCRIPTION

- Make sure the cartridges are at room temperature.
- Attach a 22G nozzle to each cartridge and mount cartridge with fibroblast of 4 x10^6 cells/mL into printhead 1, the fibroblasts of 8 x10^6 cells/mL into printhead 2 and keratinocytes into printhead 3.

Note: It is not recommended to test the extrusion until after calibration to avoid clogging of the nozzle tip, simply place the cartridges in the printhead.

- Use the automatic calibration to align the printheads, or manually calibrate the printheads to the same point in the 24-well plate.
- Test the extrusion of all cartridges to fill the nozzle tip with bioink and start the bioprinting.
- If printability is not as desired, adjust the pressure up/down by 1 kPa to extrude more/less material.

Note: If waiting too long between extrusions the bioink can dry in the nozzle causing it to clog. If this occurs, replace with new nozzle.

8. Crosslinking

MATERIAL

Crosslinking Agent

Thrombin

Cell culture medium

DESCRIPTION

- CELLINK SKIN is crosslinked with ions using the $CaCl_2$-containing Crosslinking Agent. Thrombin is reconstituted in cell culture medium and incubated with the constructs overnight.
    - **Ionic crosslinking**: Submerge the cell-laden constructs in the Crosslinking Agent for 5 min. Remove the Crosslinking Agent and rinse the constructs with basal culture media once.
    - **Thrombin**: Reconstitute the thrombin by adding 1 mL of cell culture medium to the thrombin vial. Then transfer the 1 mL of thrombin solution to a tube with 9 mL of medium to receive a 10 U/mL dilution. Mix gently by pipetting up and down 2-3 times. Submerge the samples in the thrombin containing medium and incubate overnight in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to your application.

Note: The bioprinted CELLINK SKIN constructs require at least 0.3 mM calcium in the cell culture medium to not disintegrate. This since the crosslinking mechanism of the bioink is ion dependent. Be careful with keratinocyte expansion mediums since these mediums generally have a very low level of calcium.

9. Incubation

MATERIAL

Cell culture medium

DESCRIPTION

- The next day, switch the thrombin containing medium to regular cell culture medium and incubate in standard culture conditions or according to your application.
- When air-liquid interface culture is desired; transfer the constructs to transwell inserts. Adjust the cell culture medium so the epidermis is exposed to air.
- Time recommendations: Submerge constructs for 2-5 days before initiating air-liquid interface culture. Incubate for at least 14 days to analyse the cell viability and morphology.