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BIOPRINTING SKIN TISSUE MODEL PROTOCOL

General

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 bioink of choice. Skin is a tissue with many niches and bioprinting of skin can be done with a wide variety of bioinks. Following this protocol gives a generic approach to bioprinting a skin tissue model with primary human fibroblast cells and primary human keratinocyte cells using any type of bioink.

Materials needed

- DNA Studio 4*
- USB Flash Drive
- Sterile conical bioprinting nozzles*
- 3 cartridges, 3cc*
- BIO X or BIO X6*
- Training ink, example CELLINK START (optional)
- Bioink of choice*
- Primary human fibroblasts (HDF)
- Primary human keratinocytes (HEK)
- Cell culture medium*
- Falcon tubes
- Empty cartridges with end and tip cap, 3cc*
- Syringes and female/female Luer lock adaptor
- Empty cartridges with end and tip cap, 3cc*
- Well plate
- Transwell inserts

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

IF USING A LIGHT SENSITIVE BIOINK, ENSURE TO KEEP THE BIOINK PROTECTED FROM LIGHT WHENEVER TRANSFERRED FROM THE ORANGE UV PROTECTED CARTRIDGES TO AVOID CROSSLINKING BEFORE PRINTING. WORK WITH 3D PRINTERS IN DARK. THE PHOTOINITIATOR IS SENSITIVE TO REPEATED OR PROLONGED EXPOSURE TO HEAT.

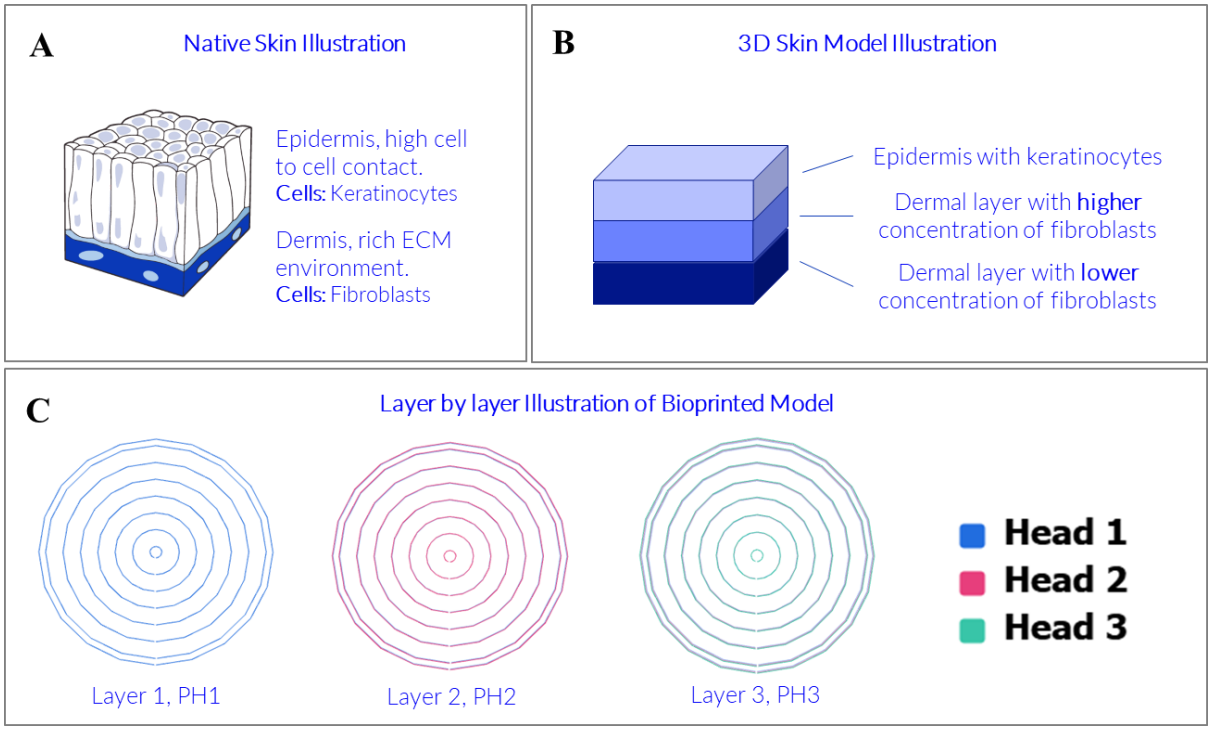

***Figure 1**. Blueprint and illustration of skin model. **(A) Native Skin Illustration**: Sketch of native skin cellular organization, with the epidermal and dermal arrangement highlighted. **(B) 3D Skin Model Illustration**: A simplified view of the different sections included in the bioprinted 3D 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 a 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 gives a general introduction how to bioprint a skin tissue model. Figure 1 exemplifies how to translate the native cellular arrangements into a bioprintable model, taking into account three components of native skin (reticular dermis, papillary dermis and epidermis). There are however more components in native skin and the following model can be both more complex, by adding additional components, or less complex by reducing the number of objects. A good starting point can be to only focus on bioprinting a dermis with an epidermis, without adding a fibroblast gradient.

How to bioprint the skin model exemplified in Figure 1C with CELLINK SKIN can be found in the *Bioprinting Skin Tissue Model Protocol CELLINK SKIN*, it is however encouraged to change the shape and dimensions to fit experimental needs. Example of additional alterations that can be incorporated into skin bioprinting:

- **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 the 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 STL files needed for the 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

The following description outline how to create the model in Figure 1C, adjust to experimental needs.

- 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 with 22G nozzles.
    - If not using DNA Studio 4, open a STL file created in other CAD software with desired dimensions and shape.
    - Adjust layer height to match selected nozzle or needle dimensions.
- 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

Well plate

DESCRIPTION

- To test the model, equip the BIO X or BIO X6 with as many cartridges filled with an ink for training purposes as needed to test print the model created in Step 1. For example, three cartridges filled with 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 the bioink that will be used with cells, diluted 10+1 with PBS to simulate cell embedded bioink. Utilizing recommended start parameters outlined in the selected bioink' s *Bioprinting Protocol*. 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

Selected bioink

3 mL syringes with Luer Lock connection

Required printheads, for example Temperature-controlled printhead or Syringe Pump printhead

DESCRIPTION

- Make sure that sterile nozzles, the BIO X/BIO X6 and other equipment needed for printing are in place before proceeding with the next steps.
- Pre-warm required amount of bioink to room temperature or other temperature, see *Bioprinting Protocol* for selected bioink for directives on how to prepare bioink for cell embedding.
    - If working with a gelatin based bioink, like GelXA SKIN or GelMA pre-warm required volume of bioink in an incubator or printhead to 37°C until it's liquid, this usually requires 30-60 min.
- Pre-heat and/or cool printheads and print bed to temperatures outlined in the *Bioprinting Protocol* for the selected bioink.

Note: Distribute the bioink into the 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 do not prepare any syringe of bioink for the epidermis.

4. Preparation of cell suspension

MATERIAL

HDF

HEK

Cell culture medium

Falcon tubes or similar

DESCRIPTION

- Prepare cell suspensions with desired cell number to be mixed with the bioink, for example:
    - 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. For exact ratio of cell suspension to bioink check the bioprinting protocol for selected bioink.

5. Mixing bioink with the cells

MATERIAL

Cell suspensions

Syringes with prepared bioink

3 mL syringes with Luer lock connections

Female/female Luer lock adaptor

Empty cartridges, amber if the bioink is light sensitive

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 prepared 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 bioink before attaching the empty syringe.

    - Detach the syringes and pipette the desired cell suspension into one of the 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 bioinks with cells to empty cartridges and cap the cartridges with a tip cap.

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

- Allow the cell laden bioink to reach printability temperature before starting the printing. Set up the BIO X/X6 while the bioink is reaching printability temperature.

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 bioink cartridges, use bioprinting parameters as recommended in the *Bioprinting Protocol* for the selected bioink.

Note: This print parameters in the *Bioprinting Protocol* are recommended starting parameters for the bioink. 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 cartridges of bioink with cells

Sterile nozzles

Well plate

DESCRIPTION

- Make sure the cartridges are at an optimal temperature for printing.
- Attach nozzles to each cartridge and mount the cartridges into respectively printhead, ensure the desired blend is assigned to correct printhead.

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 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

And/or

405/365 nm LED modules for photocuring

Thrombin (if applicable with bioink)

Cell culture medium

DESCRIPTION

- Use the correct crosslinking method outlined in the *Bioprinting Protocol* for selected bioink.
    - See example of crosslinking for CELLINK SKIN in the *Bioprinting Skin Tissue Model Protocol CELLINK SKIN*.

Note: To verify that the crosslinking is sufficient, add 37°C medium to one printed well and observe that it does not dissolve.

- Add cell culture medium, potentially supplemented with thrombin if applicable.

Note: For alginate containing bioinks that are depending on ionic crosslinking, like CELLINK bioinks and GelXA bioinks, it is recommended to have at least 0.3 mM calcium in the cell culture medium. Be careful with keratinocyte expansion mediums since these generally have a very low level of calcium. If using GelMA based bioink like GelMA and GelXA, it is required to ensure that the constructs have been properly photocured before adding the cell culture medium to not disintegrate.

9. Incubation

MATERIAL

Cell culture medium

DESCRIPTION

- Incubate samples in standard culture conditions or according to your application, preform regular medium changes according to your cell culture protocols.
    - If using thrombin containing medium for crosslinking, switch the thrombin containing medium the next day to regular cell culture medium.
- 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.