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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, primary human fibroblast cells and primary human keratinocyte cells. The model has a fibroblast gradient mimicking papillary and reticular dermal compartments, with a higher fibroblast concentration in the papillary layer, see Figure 1. For a general protocol of bioprinting CELLINK SKIN see *Bioprinting Protocol CELLINK SKIN*.

Material needed

- CAD software
- Slic3r software
- USB Flash Drive
- Sterile conical bioprinting nozzles, 22G*
- 3 cartridges, 3cc*
- BIO X*
- CELLINK SKIN*
- Primary human fibroblasts, HDF
- Primary human keratinocytes, HEK
- Cell culture medium
- Syringes and female/female Luer lock adaptor
- Empty cartridges with end and tip cap, 3cc*
- (CELLMIXER*)
- 24-well plate
- Transwell inserts
- Crosslinking Agent (included with the bioink purchase)
- Thrombin, 100 U (included with the bioink purchase)

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

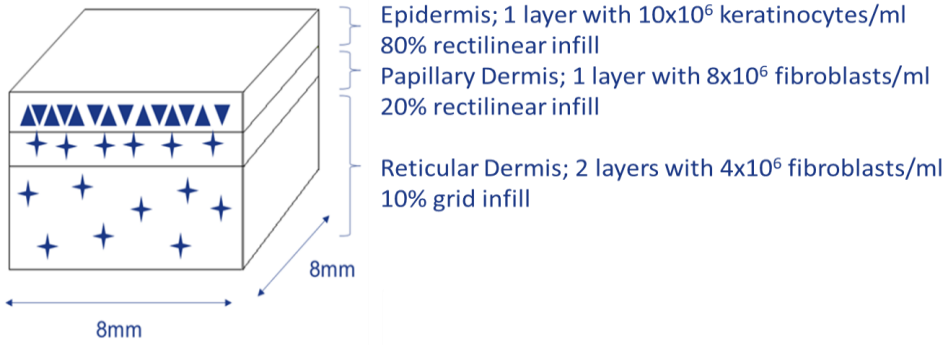

Figure 1. Blueprint of Skin model.

Protocol

This protocol can be performed with printheads and print bed at room temperature, where room temperature is between 20-25°C.

Step	Title	Material	Description
1	Creation of stl files needed for the model	- CAD software	Open the CAD software and create following 3 stl files, each representing a layer of the blueprint in Figure 1: - Top part (reticular dermis): • Shape: Rectangular • Size = 8 x 8 x 0.8 mm (2 layers of 0.4 mm) - Middle part (papillary dermis) • Shape: Rectangular • Size = 8 x 8 x 0.4 mm (1 layers of 0.4 mm) - Bottom part (epidermis): • Size = 8 x 8 x 0.4 mm • Layer height = 0.4 mm (1 layers of 0.4 mm) Save the parts as three separate stl files. Note: The model will be printed upside down and then flipped after crosslinking to maintain grid structure. Thereof the inverted order of the objects.

2	Creation of G-code	- Slic3r - USB	- Open Slic3r. - Add the bottom part into Slic3r and open the object settings for the bottom part. In the object settings add the middle and top part in named order. This is to have all three stl objects in the same model. Opening all three files directly will generate 3 different models. - Make sure the parts are aligned and on top of each other and adjust the slicing parameters to following: - Bottom part (epidermis): • Printhead = 1 • Infill pattern = rectilinear • Infill density = 80% • Printing speed, F = 10 mm/s - Middle part (papillary dermis): • Printhead = 2 • Infill pattern = rectilinear • Infill density = 20% • Printing speed, F = 10 mm/s - Top part (reticular dermis): • Printhead = 3 • Infill pattern = grid • Infill density = 10% • Printing speed, F = 10 mm/s - Export G-code and save to a USB flash drive.
3	Prepare printing	- Sterile conical bioprinting nozzles, 22G - BIO X	- Make sure G-code is working and that sterile nozzles, 22G, sterile cartridges, BIO X and other equipment needed for printing are in place before proceeding to next step.
4	Prepare bioink	- CELLINK SKIN bioink	- Prepare and pre-warm at least 3 mL of CELLINK SKIN bioink according to the *Mixing Cells Protocol CELLINK Series*. Note: Distribute the bioink into three syringes with at least 1 mL in each syringe. To maintain a full 24 well plate it is recommended to use 1.5 mL bioink for the reticular dermis layer.
5	Prepare cell suspension	- HDF - HEK - Nutritious solution for	- Detach the cells according to protocol for your cells. - Prepare three cell suspensions with following quantities of cells:

		respectively cell type - 3 pcs 3 mL syringes with Luer lock connection	• HEK 10 x10^6 cells/mL bioink for the epidermis. • HDF 8 x10^6 cells/mL bioink for the papillary dermis. • HDF 4 x10^6 cells/ml bioink for the reticular dermis. 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 nutritious solution 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. As nutritious solution complete cell culture medium usually is used.
6	Mix the CELLINK SKIN bioink with the cells	- Cell suspensions - Syringes with pre-warmed CELLINK SKIN bioink - 3 mL syringes with Luer lock connections - (CELLMIXER) - Female/female Luer lock adaptor - Empty cartridges	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 the Mixing Cells Protocol CELLINK Series. - Take the syringes with pre-warmed CELLINK SKIN bioink and attach it to an empty 3 mL syringe using a female/female Luer lock adaptor. Transfer half of the bioink to each syringe. 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 gently mix the bioink back and forth between the syringes until the cell suspension is homogeneously incorporated. Note: Make sure to add epidermal cell suspension to the CELLINK SKIN bioink prepared for the epidermis etc.

			- Transfer the CELLINK SKIN bioink with cells to an empty cartridge and cap the cartridge. - Repeat for all three cell suspensions. Note: If there are bubbles in the bioink, make a quick centrifugation for 1.5 min at 1600 rpm. - Place cartridge on counter to reach room temperature. If the bioink has been pre-heated, it can instead be placed in a fridge for 3-5 min. Do not attach a nozzle to the cartridge until the start of the print session. Note: If preparing bioink quantities of >2 mL of CELLINK SKIN it is recommended to use a CELLMIXER to mix in the cell suspension into the bioink. For instructions see the *Mixing Cells Protocol CELLINK Series*.
7	Set up of the Bioprinter	- BIO X - USB with G-code	- Open the G-code on the BIO X and set the bioprinting parameters as shown below for printhead 1, 2 and 3: • Nozzle type and size: Conical tip, 410 µm (22G) • 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. Note: Since layer parameters is set in the G-code, this function is disabled on the BIO X.
8	Print-session	- The three cartridges of CELLINK SKIN with cells - Sterile conical bioprinting nozzles 22G	- Make sure the cartridges are room temperature. - Attach a 22G nozzle to each cartridge and mount cartridges with keratinocytes into printhead 1, the fibroblasts of 8 x10^6 cells/mL into printhead

			2 and fibroblast of 4x 10^6 cells/mL into printhead 3. - Calibrate the printheads and start 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.
9	Crosslinking	- Crosslinking Agent - Thrombin	CELLINK SKIN is crosslinked with ions using the $CaCl_2$ 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 in 10 mL of cell culture medium by adding 1 mL of the medium to the thrombin vial. Then transfer the 1 mL of thrombin solution back to the 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.
10	Incubation	- Cell culture medium	- 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; flip the constructs around so the top part (reticular dermis) becomes the base and transfer the constructs to transwell inserts. Adjust the cell culture medium so the epidermis is exposed to air. 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. - 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.

Want to see our talented Biologist proceed to this protocol? Feel free to find the video here:

Want to see our existing tissue model?
Just go to http://bioverse.co/ and discover a whole library of CAD files especially created for sharing 3D Bioprinting models.

This protocol is compatible with the use of other bioprinters. For more information, please contact: info@cellink.com

References:

N/A

Applications:

Image example of result you can obtain in Figure 2 and 3 below.

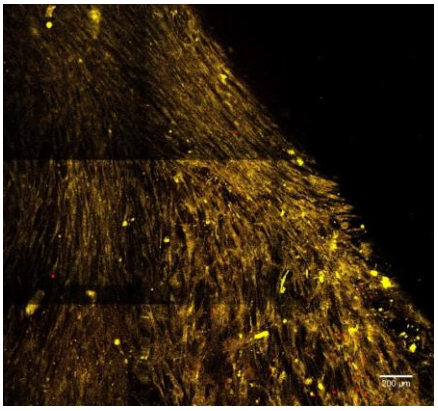

Figure 2. Multiphoton image of skin tissue model, dermal compartment, 14 days from print. Yellow = cells, Red = ECM. 20x magnification.

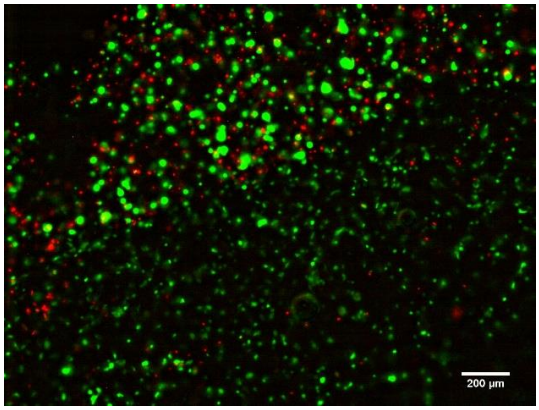

Figure 3. Viability stain of skin tissue model day 7 from print. Epidermis in upper left of image and dermis in lower, right part. Green = live cells, red = dead cells. 4x magnification.