Ref No: BPR-KT-01123 1002J/2002J
Date: 18-JAN-2023
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PROTOCOL

SKIN TISSUE MODEL KIT

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 how to use the Skin Tissue Model Kit A and B. It includes creating the G-code and instructions of bioprinting a skin tissue model using CELLINK SKIN (Kit A) or GelXA SKIN (Kit B) bioink, together with 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. The protocol also includes directions of use for the three antibodies included in the kit.

Materials needed

- Skin Tissue Model Kit A or B*
- CAD software
- Slic3r software
- USB Flash Drive
- Conical bioprinting nozzles, 22G*
- 3 cartridges, 3cc*
- BIO X*
- 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*
- 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.

Protocol

This protocol can be performed with standard pneumatic printheads at room temperature if using the Skin Tissue Model Kit A. If using Skin Tissue Model Kit B with GelXA SKIN, it is recommended to use the Temperature-controlled printheads at 24°C and print bed cooled to 15°C for best shape fidelity . Make sure to protect GelXA SKIN from light.

1. Creation of STL files needed for the model

MATERIAL

CAD software

DESCRIPTION

- Open the CAD software and create following three 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.

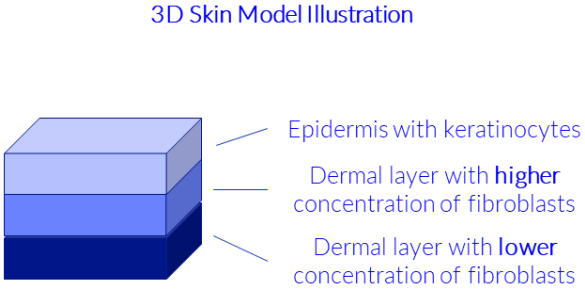

Figure 1. *Blueprint of skin model.*

2. Creation of G-code

MATERIAL

Slic3r

USB

DESCRIPTION

- 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 three 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. Preparation for printing

MATERIAL

Conical bioprinting nozzles, 22G

BIO X

Temperature-controlled printheads (optional)

DESCRIPTION

- Make sure G-code is working and that sterile nozzles, 22G, the BIO X and other equipment needed for printing are in place before proceeding to next step.

4. Preparation of bioink

MATERIAL

CELLINK SKIN/GelXA SKIN

3 mL syringe with Luer lock connections

Female/female Luer lock adaptor

Pipette tip or spatula

DESCRIPTION

- Kit A: Prepare and pre-warm at least 3 mL of CELLINK SKIN to room temperature.
- Kit B: Heat up GelXA SKIN at 37°C until it becomes liquid. The heating of the GelXA SKIN can be performed in a printhead or incubator and usually requires 30-60 min. When GelXA SKIN is warm, keep the cartridge horizontal when tip and end cap are removed. This is to prevent air from entering the cartridge or bioink from dripping out.

- For both: Connect the cartridge to a 3 mL syringe using a Luer lock connector and remove the end cap. Push approximately half of the bioink from the cartridge into the syringe by gently pushing the cartridge piston using a pipette tip or small spatula while simultaneously pulling the syringe plunger. To remove any air bubble derived from the dead volume in the syringe, separate the syringe and cartridge maintaining the Luer lock on the syringe. Hold the syringe with the tip facing upwards and gently tap the syringe to move air bubbles towards the tip. Carefully extrude air and pre-fill the Luer lock adaptor with the bioink before re-attaching the cartridge. Gently mix the bioink back and forth between the cartridge and syringe to homogenize the bioink.

Note: If there are bubbles in the bioink, make a quick centrifugation for 30 s at 600 x *g*.
Note: Distribute the bioink into three syringes with at least 1 mL in each syringe. To bioprint a full 24 well plate it is recommended to use 1.5 mL bioink for the reticular dermis layer.

5. Preparation of cell suspension

MATERIAL

HDF

HEK

Cell culture medium

3 pcs 3 mL syringes with Luer lock connection

DESCRIPTION

- Prepare three cell suspensions with following quantities of cells:
    - 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 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.

6. Mixing bioink with the cells

MATERIAL

Cell suspensions

Syringes with pre-warmed CELLINK SKIN/GelXA SKIN

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 with bioink Protocol*.
    - Take the syringes with pre-warmed 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 bionk 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 bioink syringe prepared for the epidermis etc.

- Transfer each of the cell laden bioink mixtures to empty cartridges and cap the cartridge with a tip cap.

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

7. Setting up the bioprinter

MATERIAL

BIO X

USB with G-code

Temperature-controlled printheads (optional)

DESCRIPTION

- Open the G-code on the BIO X and set the bioprinting parameters as shown below for printhead 1, 2 and 3:

  If using the Skin Tissue Model Kit A:

  - Nozzle type and size: Conical tip, 410 µm (22G)
  - Printing pressure: 12 kPa**
  - Printing speed = 10 mm/s**
  - Printhead temperature: -
  - Print bed temperature: -

  If using the Skin Tissue Model Kit B:

  - Nozzle type and size: Conical tip, 410 µm (22G)
  - Printing pressure: 10 kPa**
  - Printing speed = 10 mm/s**
  - Printhead temperature: 24°C
  - Print bed temperature: 15°C

** 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.

- *If using the Skin Tissue Model Kit A, go to 8a. If using the Skin Tissue Model Kit B, go to 8b.*

8a. Preparation for printing CELLINK SKIN

MATERIAL

The three cartridges of CELLINK SKIN with cells

Conical bioprinting nozzles, 22G

DESCRIPTION

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

Note: Be careful not to touch the printhead with the nozzle tip when inserting the cartridge. Alternatively, the cartridge can be placed in the printhead with the tip cap on and when in place switched to a nozzle.

- *Move to Step 9.*

8b. Preparation for printing GelXA SKIN

MATERIAL

The three cartridges of GelXA SKIN with cells

Temperature-controlled printhead (optional)

Conical bioprinting nozzles, 22G

DESCRIPTION

- If the cartridge is just taken from the heat or if the cartridge still feels warm after mixing in the cells, place it in the pre-heated Temperature-controlled printhead at 24°C for 5 minutes. If not using the Temperature-controlled printhead, place the cartridge on counter for 5-10 minutes to reach approximately 24°C.
- If the cartridge has cooled down below 22°C, re-heat the cartridge at 37°C for 5 min to reset.
- If using the BIO X or BIO X6, pre-cool the print bed to 15°C.
- Attach a 22G nozzle to each cartridge and mount cartridge with keratinocytes into printhead 1, the fibroblasts of 8 x10^6 cells/mL into printhead 2 and fibroblast of 4 x10^6 cells/mL into printhead 3.

Note: When printing with GelXA SKIN, the recommended printhead temperature for the highest printing fidelity is 23°C, though the bioink can be dispensed up to 32°C. Below 22°C the bioink can become too viscous resulting in chunky filaments and too high extrusion pressures needed.

Note: Be careful not to touch the printhead with the nozzle tip and if using very liquid materials, make sure that the bioink does not drip through the nozzle especially when attaching the air adapter. Alternatively, the cartridge can be placed in the printhead with the tip cap on and when in place switched to a nozzle.

9. Printing

MATERIAL

BIO X

24-well plate

DESCRIPTION

- Calibrate the printheads to the same point in the 24-well plate 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.

Note: Test the flow of the bioink after the calibration is performed and start with a low pressure and increase stepwise.

- If printing with GelXA SKIN and if proper viscosity and printability is not achieved by extending temperature equilibration time or tuning pressure:
    - Too low viscosity (wide filaments despite using low pressure): decrease the printhead temperature 0.5-1°C to increase the viscosity and equilibrate an additional couple of minutes.
    - Too high viscosity (chunky filaments and high pressure required): increase the printhead temperature 0.5-1°C to decrease the viscosity and equilibrate an additional couple of minutes. If unsuccessful, the bioink might have over-gelled. In this case, re-heat the cartridge at 37°C for 5 min to reset and choose a 1°C higher printhead equilibration temperature.
- *If using the Skin Tissue Model Kit A, go to 10a. If using the Skin Tissue Model Kit B, go to 10b.*

10a. Crosslinking

MATERIAL

Crosslinking Agent

Thrombin

Cell culture medium

DESCRIPTION

- CELLINK SKIN is crosslinked with ions using the $CaCl_2$- containing Crosslinking Agent and thrombin.
    - Make a 100 U/mL thrombin stock: Add 1 mL of cell culture medium to the thrombin vial. If not using all the bioink, aliquots of the thrombin stock can be stored at -70°C for 2 months.
- The ionic and thrombin crosslinking can either be sequential or simultaneous.
- **Alternative 1, simultaneous crosslinking**:
    - Add 1 part of thrombin stock solution to a tube with 7 parts of cell culture medium and 2 parts Crosslinking Agent to receive a final concentration of 10 U/mL thrombin and 10 mM $CaCl_2$. Mix gently by pipetting up and down 2-3 times. Submerge the samples in the thrombin containing Crosslinking Agent and incubate overnight in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to your application.
- **Alternative 2, sequential crosslinking**:
    - 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: Add 1 part of thrombin stock solution to a tube with 9 parts of medium to receive a 10 U/mL final concentration of thrombin. 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.
- *Move to Step 11.*

10b. Crosslinking

MATERIAL

Crosslinking Agent

Thrombin

and/or

405/365 nm LED modules for photocuring

Cell culture medium

DESCRIPTION

- GelXA SKIN can be photocrosslinked using the 405 or 365 nm LED modules or ionically crosslinked using the $CaCl_2$-containing Crosslinking Agent with thrombin. If using both, begin with photocrosslinking. It is recommended to use both crosslinking methods which will generate the most robust constructs. Photocrosslinking only can be used for a softer construct, whilst it is not recommended to use only ionic crosslinking.

Note: It is recommended to use the 405 nm LED module instead of 365 nm one if possible. Overexposure might damage the cells.

- **Photocrosslinking:** see Table 1 below for recommended crosslinking times. Ensure that the bioprinted GelXA SKIN construct is thermally gelled after printing by cooling the print bed (if using the BIO X or BIO X6) or placing the printing substrates with the construct on ice for 10 seconds (if using the INKREDIBLE series). If photocrosslinking during bioprinting, set the

crosslinking parameters appropriately in the G-code for the INKREDIBLE series or the printhead setup page for the BIO X or BIO X6.

Note: To verify the crosslinking is sufficient, add 37°C media to one printed well and observe that it doesn't dissolve.

- Make a 100 U/mL thrombin stock: Add 1 mL of cell culture medium to the thrombin vial. If not using all the bioink, aliquots of the thrombin stock can be stored at -70°C for 2 months.
- The ionic and thrombin crosslinking can either be sequential or simultaneous.
- **Alternative 1, simultaneous crosslinking**:
    - Add 1 part of thrombin stock solution to a tube with 7 parts of cell culture medium and 2 parts Crosslinking Agent to receive a final concentration of 10 U/mL thrombin and 10 mM $CaCl_2$. Mix gently by pipetting up and down 2-3 times. Submerge the samples in the thrombin containing Crosslinking Agent and incubate overnight in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to your application.
- **Alternative 2, sequential crosslinking**:
    - 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: Add 1 part of thrombin stock solution to a tube with 9 parts of medium to receive a 10 U/mL final concentration of thrombin. 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.

Table 1. Recommended time of the construct photocrosslinking***. Distance from each light module to construct was set to 5 cm using the BIO X/BIO X6 photocuring modules. If using the INKREDIBLE series photocuring modules, the time required can possibly be decreased. For crosslinking with other parameters, see *Photocrosslinking Optimization Protocol*. This table was generated using GelXA SKIN with mesenchymal stem cells. Do not exceed the exposure time to more than 120 s when printing with cells. To achieve the best structural integrity when printing thicker constructs, it is recommended to apply 3 or 5 seconds photocrosslinking with 365 or 405 nm light respectively, every second or fourth layer.

	365 nm, LAP 0.25%	**405 nm, LAP 0.25%**
1 mm construct thickness	5 seconds	10 seconds
3 mm construct thickness	15 seconds	30 seconds

****Note: This is only a recommended reference of crosslinking times to start with. The actual time needed for crosslinking will vary depending on the size and temperature of the constructs as well as the intensity of the photocuring module and the distance to the construct.*

11. 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; 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 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.

12. Histological analysis

MATERIAL

Collagen Type 1 antibody

Keratin 10 antibody

Elastin antibody

DESCRIPTION

The antibodies provided with the Skin Tissue Model Kit A and B represents antibodies selective of key factors in a healthy skin tissue. Collagen type 1 is produced by the dermal fibroblasts and is, besides being the main component in the dermis, important for the functionality, structure and firmness of the skin. Keratin 10, also known as cytokeratin 10, is one of the main, early proliferation factors of keratinocytes and present in proliferative keratinocytes closest to the dermis. Elastin is the protein responsible for the crucial elastic properties of the skin, located in the dermis in close connection with the collagens.

- After culturing of the skin constructs for at least 14 days, fixate the constructs and prepare for the histological analysis. Follow the *Fixation Protocol*, *Embedding Protocol*, *Sectioning Protocol,* and *Immunofluorescence staining Protocol* found on CELLINKs webpage to prepare and to stain the tissue model with the antibodies.
- The dilutions for collagen type 1, keratin 10 and elastin antibodies are shown in Table 2.

Table 2. Recommended dilutions for the antibodies for use on sectioned slides.

Antibody	Dilution range	Recommended dilution
Collagen type 1*	1:50 – 1:200	1:50
Keratin 10*	1:200 – 1:800	1:200
Elastin*	1:50 – 1:200	1:50

*Host: Rabbit, Verified Species Reactivity: Human

- In-house immunofluorescence images of 3D bioprinted skin tissue models using Skin Tissue Model Kit A and/or B stained with included primary antibodies collagen type 1 (Figure 2), keratin 10 (Figure 3) and elastin (Figure 4).

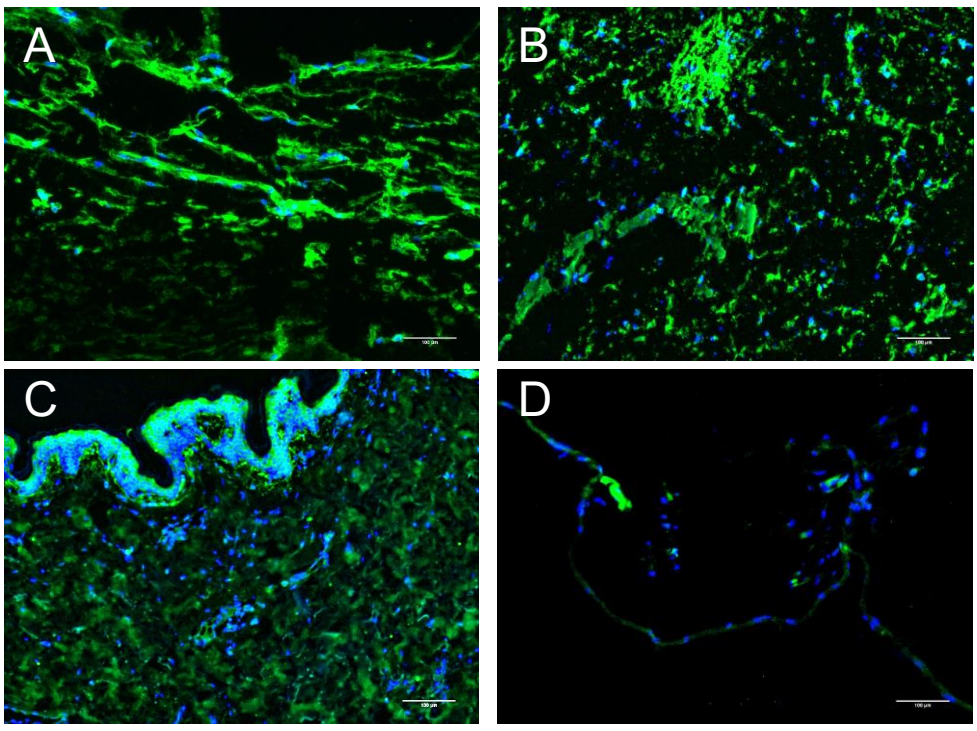

Figure 2. *Example of analysis of collagen type 1 expression in 3D bioprinted skin tissue models with GelXA SKIN (Skin Tissue Model Kit B). (A) Skin tissue model, (B) skin tissue model treated with compound and (C) native skin. (D) Control of unspecific antibody staining with only secondary antibody applied. Merged images of collagen type 1 expression (1:50 dilution), green, and nuclei stained with DAPI (1:50 dilution), blue. Secondary antibody: Alexa Fluor 488. Magnification: 10X, scale bar: 100 µm.*

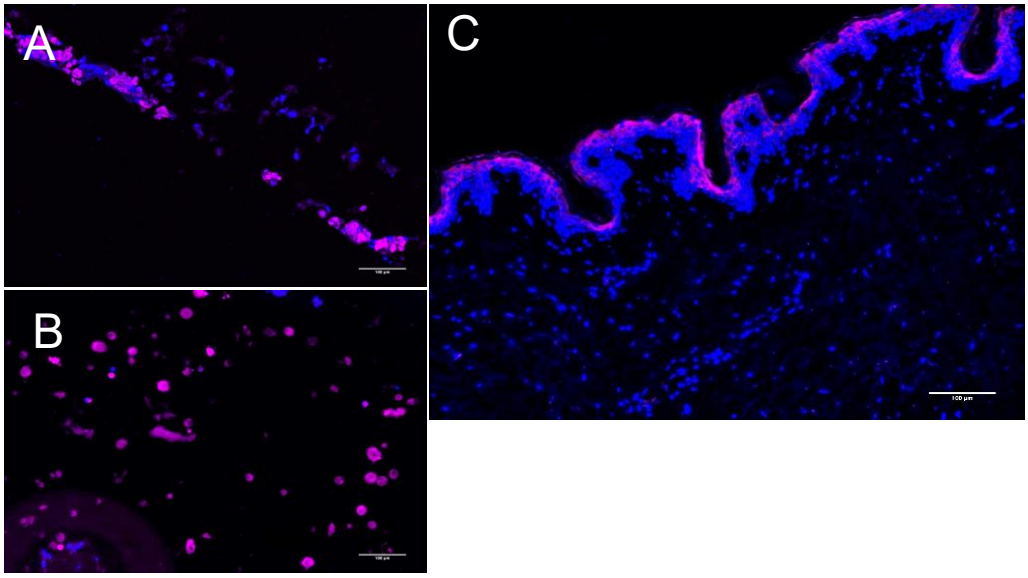

Figure 3. *Example of analysis of keratin 10 expression in 3D bioprinted skin tissue models with Skin Tissue Model Kit A and B. (A) Skin tissue model in CELLINK SKIN (Skin Tissue Model Kit A) with seeded keratinocytes. (B) Skin tissue model in GelXA SKIN (Skin Tissue Model Kit B) with embedded keratinocytes and (C) native skin. Merged images of keratin 10 expression (1:200 dilution), magenta, and nuclei stained with DAPI (1:50 dilution), blue. Secondary antibody: Alexa Fluor 488. Magnification: 10X, scale bar: 100 µm.*

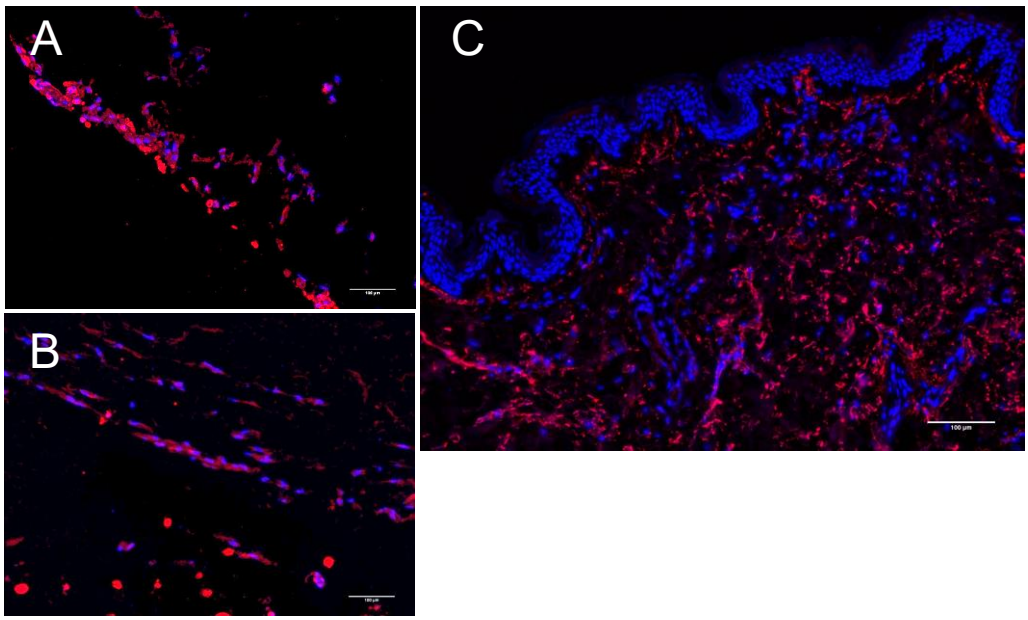

Figure 4. *Example of analysis of elastin expression in 3D bioprinted skin tissue models with Skin Tissue Model Kit A and B. (A) Skin tissue model in CELLINK SKIN (Skin Tissue Model Kit A) with seeded keratinocytes. (B) Skin tissue model in GelXA SKIN (Skin Tissue Model Kit B) with embedded keratinocytes and (C) native skin. Merged images of elastin expression (1:50 dilution), red, and nuclei stained with DAPI (1:50 dilution), blue. Secondary antibody: Alexa Fluor 488. Magnification: 10X, scale bar: 100 µm.*