

BIOPRINTING PROTOCOL

CELLINK FIBRIN

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 bioprinting of CELLINK FIBRIN using the INKREDIBLE, INKREDIBLE+, BIOX or BIO X6, and covers steps from pre-print mixing with cells, 3D bioprinting and post-print processes such as ionic crosslinking. This protocol was optimized for CELLINK FIBRIN, undiluted as well as using a 10+1 cell suspension dilution. Changing the parameters in the protocol might change the crosslinking time required. This protocol was optimized using the pneumatic printhead and the BIO X.

Materials needed

- CELLINK FIBRIN*
- Cells* + cell culture medium*
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
- CELLMIXER* (optional)
- Clear cartridges, 3cc*
- Conical bioprinting nozzles, 22-27G recommended*
- BIO X*, BIO X6* or INKREDIBLE-series* 3D bioprinter
- Well plate or Petri dish*
- Crosslinking Agent* (included with the bioink purchase)
- Vial with 100 U thrombin (included with the bioink purchase)

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

Protocol

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

1. Preparing the bioink

MATERIAL

CELLINK FIBRIN

3 mL syringe with Luer lock connections

Female/female Luer lock adaptor

Pipette tip or spatula

DESCRIPTION

- Mix the full 3 mL of CELLINK FIBRIN a few times to make sure it is homogenous. Connect the cartridge to a 3 mL syringe using a Luer lock connector and remove the end cap. Push 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. Gently mix the bioink back and forth between the cartridge and syringe to homogenize the bioink, taking care not to introduce air. If not using the entire 3 mL of the bioink in the cartridge, spare the rest of the bioink in the optimal storage conditions.
- Warm up CELLINK FIBRIN to room temperature. Only warm the needed volume.
- *If not printing with cells, move directly to step 3.*

2. Mixing the bioink with cells

MATERIAL

3 mL syringes with Luer lock connections

Female/female Luer lock adaptor

Pre-warmed CELLINK FIBRIN

Cell suspension

Cartridge, 3cc

CELLMIXER (optional)

DESCRIPTION

- At this point, mix ten parts bioink with one part cell suspension, taking care not to introduce air bubbles to the mixture. For detailed instructions see the *Mixing cells with bioink Protocol*.
- If preparing for quantities < 2 mL of CELLINK FIBRIN, it is recommended to connect two 3 mL Luer lock syringes, one with the bioink and the other with the cell suspension and gently mix back and forth between the syringes until homogeneous. Transfer the mixture to an empty 3cc cartridge by connecting the syringe to the cartridge using the Luer lock adaptor. Cap the cartridge with a tip cap.
- If using larger quantities, the CELLMIXER can be used:
 - Transfer the cell suspension to the 1 mL cell syringe (PART 1) using a female/female Luer lock adaptor.
 - Transfer the bioink to the 12 mL syringe (PART 2) using a female/female Luer lock adaptor.
 - Clip both syringes to the Dispensing unit (PART 3).
 - Connect the two syringes to the Mixing unit (PART 4), then connect the Empty cartridge (PART 5) to the Mixing unit's other side.
 - Apply gentle pressure onto the Dispensing unit to mix the content of both syringes into the empty cartridge. Cap the cartridge with a tip cap.

Note: To avoid introducing air when connecting the syringes, carefully pre-fill the Luer lock adaptor with CELLINK FIBRIN before attaching it to the syringe with the cell suspension.

3. Preparation for printing

MATERIAL

CELLINK FIBRIN mixed with cells (if applicable) in cartridge

Conical bioprinting nozzles, 22-27G recommended

DESCRIPTION

- Attach a nozzle to the cartridge and mount the room tempered CELLINK FIBRIN into the printhead. Connect the cartridge to the air adapter.

Note: The recommended nozzle size is 22G. Decrease the nozzle diameter to achieve smaller filament diameter, however this also increase the risk of the bioink clogging.

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.

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

4. Printing

MATERIAL

BIO X, BIO X6 or INKREDIBLE series bioprinter

Well plate or Petri dish

DESCRIPTION

- Bioprint structures with parameters according to Table 1 on to a well plate or Petri dish. 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.

Table 1. Recommended minimal extrusion pressure** (± 2 kPa) used for printing continuous filaments at 21-25°C with cells/without cells. Again, 'with cells' assumes a mixture of one part cell suspension and ten parts bioink. For highly concentrated cell suspensions, the pressure needs to be increased towards the pressure used for undiluted bioink.

Printing speed (mm/s) → Nozzle size (G) ↓	5	10	15	20
22	9 / 11	11 / 12	12 / 14	13 / 15
25	10 / 12	11 / 14	13 / 15	15 / 17
27	11 / 15	13 / 16	14 / 17	15 / 20

**Note this is only a recommended reference of starting pressures. The actual pressure needed will vary depending on the preparation procedures (amount of bioink and actual temperature of the bioink) as well as the fitting of the piston in the cartridge and the leveling of the print surface. This table was generated with bioink temperature at 23°C and with a 10+1 bioink dilution with cell suspension.

5. Crosslinking

MATERIAL

Crosslinking Agent
Thrombin
Cell culture medium

DESCRIPTION

- CELLINK FIBRIN is crosslinked with ions using the CaCl₂-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 ionic and thrombin 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 CaCl₂. 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% CO₂ and 95% relative humidity) or according to your application.
- **Alternative 2, sequential ionic and thrombin crosslinking:**
 - Ionic crosslinking: Submerge the cell-laden constructs in the Crosslinking Agent for 30 seconds to 5 minutes depending on construct size, infill density and desired construct stiffness. Remove Crosslinking Agent and rinse constructs with basal culture media once.
Note: 30 seconds is recommended for 10 µL droplets while 10 minutes might be required for dense 1 cm³ blocks. In addition, optimize the crosslinking depending on the cell type.
 - 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% CO₂ and 95% relative humidity) or according to your application.

6. 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. Replace medium regularly.