Ref No: BPR-IK-G106L3
Date: 08-APR-2025
Author: EP, JH, VK. Version: 4

BIOPRINTING PROTOCOL

GelMA 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 GelMA FIBRIN bioink using the Temperature-controlled printhead on the BIO X/BIO X6 or using the INKREDIBLE+. This document covers pre-print mixing with cells, 3D bioprinting and post-print such as ionic crosslinking, photocuring and thrombin-fibrinogen interaction. This protocol was optimized for GelMA FIBRIN with LAP 0.25% both undiluted and diluted (10+1 parts) with cell suspension. Changing the bioink to cell suspension ratio and thus also the concentration of photoinitiator, will change the photocrosslinking time. Refer to the *Photocrosslinking Crosslinking Optimization Protocol* to adjust and determine these numbers. This protocol was optimized using the Temperature-controlled printhead with the BIO X.

Materials needed

- GelMA FIBRIN bioink*
- Cells* + cell culture medium*
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
- CELLMIXER* (optional)
- UV shielding cartridges, 3cc*
- Conical bioprinting nozzles, 22-27G
- Temperature-controlled printhead
- BIO X*, BIO X6* or INKREDIBLE+* 3D bioprinter
- Petri dish* or well plate
- Thrombin, vial with 100 U (included when purchasing GelMA FIBRIN)

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

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

Protocol

This protocol works best using the BIO X or BIO X6 with the cooled print bed at 15°C and the Temperature-controlled printhead at 23°C with the thermal nozzle cover. GelMA FIBRIN can also be extruded using the INKREDIBLE+, but with decreased shape fidelity if ambient temperature exceeds 25°C. If using the INKREDIBLE series, the printing substrates such as Petri dishes or well plates should be placed on ice or another cooled surface before and after printing to thermally gel bioprinted constructs prior to photocrosslinking. First time users of GelMA based bioinks are recommended to optimize the printing conditions without cells before proceeding to bioprint with cells. Perform the desired dilution using medium or PBS.

1. Preparing the bioink

MATERIAL

GelMA FIBRIN

Temperature-controlled printhead

DESCRIPTION

- Heat up GelMA FIBRIN in the cartridge at 37°C for 10-20 min until the bioink becomes liquid. Prolonged and repeated heating could negatively affect the photoinitiator stability and the homogeneity of the bioink.
- Set the Temperature-controlled printhead to 23°C.
- *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 GelMA FIBRIN

UV shielding cartridge, 3cc

CELLMIXER (optional)

DESCRIPTION

- Mix ten parts of bioink with one part of cell suspension without introducing air bubbles to the mixture. For detailed instructions see the *Mixing cells with bioink Protocol*.
- If preparing for quantities < 2 mL of GelMA 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 GelMA FIBRIN 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 GelMA FIBRIN before attaching it to the syringe with the cell suspension.

Note: 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 when liquid.

3. Preparation for printing

MATERIAL

GelMA FIBRIN mixed with cells (if applicable) in UV shielding cartridge

Temperature-controlled printhead

Conical bioprinting nozzles, 22-27G

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 23°C for 10 minutes. If not using the Temperature-controlled printhead, place the cartridge on counter for 5-10 minutes to reach approximately 23°C.
- If the cartridge has cooled down below 23°C, re-heat the cartridge at 37°C for 5 min to reset, then restart equilibration in printhead.
- Cap the cartridge with a bioprinting nozzle and place the GelMA Fibrin in the printhead. Connect the cartridge to the air pressure adapter. If using the BIO X or BIO X6, pre-cool the print bed to 15°C.

Note: When printing with GelMA Fibrin, the recommended printhead temperature for the highest printing fidelity is 23°C, though the bioink can be dispensed up to 32°C. Below 23°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.

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 or BIO X6 3D bioprinter

Well plate or Petri dish

DESCRIPTION

- Calibrate the nozzle to the well plate or Petri dish surface. Test the flow of the bioink first after calibration and start with a low pressure and increase stepwise. Bioprint structures into the well plate or Petri dish. If printability is not as desired, adjust the pressure up/down by 1 kPa to extrude more/less material.

Example: If printing continuous filaments with a Temperature-controlled printhead set to 23°C, a 25G nozzle, a printing speed of 5 mm/s and with 300 ms pre-flow delay, the suggested pressure range is between 22-32 kPa without cell suspension dilution and 18-28 kPa if diluted with cell suspension.

- 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 temperature than before. Let the bioink reach the new temperature before starting to print again.

- During print sessions longer than 20 min at 23°C the bioink can become too viscous due to continued gelling resulting in chunky filaments and too high extrusion pressures needed. To avoid this, a 0.5°C increase in printhead temperature after 20 min printing can extend the time the bioink remains at good printability.
- If the regular pneumatic printheads are used for a long period, they might heat up above the desired printing temperature. Then the bioink also heats up which decreases its viscosity, observed as extrusion of very thick filaments even at low pressures. Remove the cartridge from the printhead and allow to cool down to 23°C. In addition, remove the printheads from the BIO X/BIO X6 to let them cool down before continuing to print.

Note: If waiting too long between extrusions, the bioink can dry in the nozzle causing it to clog. If this occurs, take a sterile tweezer and remove the dried GelMA FIBRIN part at the edge of the nozzle or replace with new nozzle.

5. Photocrosslinking

MATERIAL

405/365 nm LED modules for photocuring

DESCRIPTION

- GelMA FIBRIN should first be photocrosslinked (using either the 405 or 365 nm LED module) and then immersed in thrombin solution.

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

- See Table 1 below for recommended photocrosslinking time. Ensure that the bioprinted GelMA FIBRIN construct is thermally gelled after printing by cooling the print bed on the BIO X/BIO X6 during printing or placing the well plate or Petri dish on ice for 10 s.
- If photocrosslinking is applied during bioprinting, set the crosslinking parameters appropriately in the printhead setup page for the BIO X/BIO X6.
- Let the structure rest for 3-5 min to allow crosslinking after the light source is turned off.

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

Table 1. Recommended time required to photocrosslink the construct**. Distance from photocuring module to construct set at 5 cm using the BIO X/BIO X6 modules. If using the INKREDIBLE+ photocuring modules, the time required can possibly be decreased. For crosslinking with other parameters, see *Photocrosslinking Optimization Protocol*. This table was generated using GelMA FIBRIN containing 0.25% LAP. Don't exceed 120 seconds of exposure time 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 fourth layer.

	365 nm, LAP 0.25%	**405 nm, LAP 0.25%**
1 mm construct thickness	10 seconds	20 seconds
3 mm construct thickness	20 seconds	30 seconds

***Note this is only a recommended reference of starting times. 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.*

6. Thrombin crosslinking

MATERIAL

Thrombin, vial with 100 U

Cell culture media

DESCRIPTION

- Thrombin-mediated generation of fibrin from fibrinogen is performed by addition of thrombin dissolved in media.
- 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.
- 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.
- Add enough 10 U/mL thrombin solution to the wells to cover the printed constructs. Incubate over night or until the next media change in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to your application.

7. 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.