Ref No: BPR-IK-G106L3
Date: 25-NOV-2019
Author: EP. Version: 1

Bioprinting Protocol

GelMA FIBRIN

This is a suggested procedure, please adjust according to your experimental needs.

Protocol aim

The aim of this protocol is to provide instructions for bioprinting with GelMA FIBRIN bioink using the BIO X. This document covers pre-print mixing with cells, 3D bioprinting and post-print processes of crosslinking through photocuring and thrombin-fibrinogen interaction. This protocol was optimized for GelMA FIBRIN with LAP 0.25% in both undiluted and diluted (10+1 parts) states. The concentration of photoinitiator or bioink to cell suspension ratio effects the photocrosslinking time. Reference the *Photocrosslinking Crosslinking Optimization Protocol* to adjust and determine these numbers. This protocol was optimized using the Temperature-controlled printhead with the BIO X.

Material needed

- GelMA FIBRIN bioink*
- Thrombin (included when purchasing GelMA FIBRIN)
- 0.1% Bovine serum albumin (BSA) solution
- UV shielding cartridges, 3cc*
- Sterile conical bioprinting nozzles, 22-27G
- BIO X or INKREDIBLE series 3D Bioprinter*
- 365/405 UV modules for photocuring
- Petri dish* or well plate
- Cells + cell culture medium
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
- CELLMIXER*

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

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.

Boston, USA
75 Kneeland Street
Boston, MA 02111

Gothenburg, Sweden
Arvid Wallgrens Backe 20,
Gothenburg, 41346

Blacksburg, USA
2000 Kraft Dr, Suite 2125
Blacksburg, VA 24060

Kyoto, Japan
46-29 Yoshida-Shimo Adachi-cho, Sakyo-ku, Kyoto

www.cellink.com | +1 (833) CELLINK

Protocol

This protocol works best using the BIO X with the cooled print bed at 18°C and the Temperature-controlled printhead at 23°C with the thermal nozzle cover. GelMA FIBRIN can also be extruded using the BIO X pneumatic printheads or the INKREDIBLE series bioprinters, but with decreased shape fidelity if ambient temperature exceeds 23°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.

Step	Title	Material	Description
1	Prepare the bioink	- GelMA FIBRIN	- Heat up GelMA FIBRIN in a cartridge/syringe at 37°C for 5 min until the bioink becomes liquid. This can be tested by flipping the cartridge and observing if air bubbles move freely. Prolonged and repeated heating could negatively affect the photoinitiator stability and the homogeneity of the bioink. - Set the Temperature-controlled printhead to 23°C. Pre-cool the print bed to 18°C.
2	Mix GelMA FIBRIN with cells	- Cell suspension - CELLMIXER - Female/female Luer lock adaptor - 3 mL syringes with Luer lock connections - Prewarmed GelMA FIBRIN	If not printing with cells move directly to step 3. 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 Protocol GelMA Series*. - 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 other side of the Mixing unit. - Apply gentle pressure onto the Dispensing unit to mix the content of both syringes into the empty cartridge. Note: To avoid an air gap when mixing the bioink and the cell suspension, carefully pre-fill the Luer lock adaptor with GelMA FIBRIN before attaching the syringe with the cell suspension.

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			If preparing for quantities <2 mL of GelMA FIBRIN, it is recommended to connect two 3 mL Luer lock syringes and mix back and forth between the syringes until homogeneous.
3	Cool and load the cartridge	- UV shielding cartridges, 3cc loaded with GelMA FIBRIN (and cells) - Sterile conical bioprinting nozzles, 22-27G	- Cap the cartridge with a tip cap and place it in the Temperature-controlled printhead set to 23°C and wait for 5-10 min until the bioink reaches 23°C (air bubbles are no longer moving when flipping the cartridge). - Remove cartridge from the printhead, exchange the tip cap for a bioprinting nozzle of choice, and mount the cartridge back to the printhead.
4	Printing	- BIO X 3D bioprinter	Bioprint structures with parameters according to Table 1. If printability is not as desired, adjust the pressure up/down by 1 kPa to extrude more/less material. Note: If the bioink seems too liquid and the pressure needs to be decreased with >5 kPa, the printhead temperature might need to be shifted down to 22.5°C. If the bioink comes out chunky, heat it again at 37°C for 3 min, repeat step 3 and increase the printhead temperature to 23.5°C. 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. Note: GelMA FIBRIN becomes more solid over extended printing time (>20 min). If printing is paused and extrusion does not occur, heat the bioink again at 37°C for 3 min and repeat step 3 to 'reset' the bioink.

Table 1. Recommended minimal extrusion pressure** (±2 kPa) used for printing continuous filaments at 23°C $^{\text{diluted}}/_{\text{undiluted}}$. 'Diluted' assumes a mixture of one part of PBS to ten parts of bioink, which is the simulation of bioink and cell suspension mixing conditions. For smaller dilutions, the pressure needs to be increased towards the pressure used for undiluted bioink.

Printing speed (mm/sec) → Nozzle size (G) ↓	5	10	15	20
22	6 / 11	8 / 13	11 / 15	13 / 18
25	13 / 15	14 / 19	17 / 22	19 / 24
27	16 / 24	19 / 28	21 / 31	23 / 34

***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 printhead temperature at 23°C and with a 10:1 bioink dilution.*

Step	Title	Material	Description
5	Photocrosslinking	- 405/365 UV modules for photocuring	GelMA FIBRIN should first be photocrosslinked (using either the 405 or 365 nm photocuring module) and then immersed in Thrombin solution. - See Table 2 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 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. - Let the structure rest for 3-5 min to allow crosslinking after the light source is turned off. Note: It is recommended to use the 405 nm photocuring module instead of 365 if possible when photocuring GelMA FIBRIN. Overexposure at the 365 nm wavelength might damage the cells. Note: If crosslinking is unsure, add 37°C media to one printed construct to validate that it does not dissolve.
6	Thrombin crosslinking	- Thrombin 100 U - 0.1% BSA sterile - Cell culture media	- Thrombin-mediated generation of fibrin from fibrinogen is performed by addition of thrombin dissolved in media. - Prepare a 1 U/µL thrombin solution by addition of 100 µL 0.1% BSA solution to the vial containing 100 U freeze-dried thrombin. Remains active for 1 week if stored at -20°C. - Prepare a 10 U/mL thrombin solution in media by diluting the 1 U/µL solution 100 times, e.g. 50 µl 1 U/µL thrombin solution + 4.95 mL cell culture media. - Add enough 10 U/mL thrombin solution to the wells to cover the printed constructs. Incubate over night or until the next media

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Blacksburg, USA
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Kyoto, Japan
46-29 Yoshida-Shimo Adachi-cho, Sakyo-ku, Kyoto

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			change in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to your application.

Table 2. Recommended time required to photocrosslink the construct***. Distance from photocuring module to construct set at 5 cm using the BIO X 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 the exposure time at these conditions for more than 120 s when printing with cells.

	365 nm	405 nm
Construct depth (mm)/time (s)	1/10	1/20
	3/20	3/30

*****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.*

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