Ref No: BPR-IKG5L300
Date: 16-JUL-2021
Author: PT, JB, EP Version: 3

Bioprinting Protocol

GelMA C

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 with the GelMA C bioink using the BIO X/BIO X6 and the Temperature-controlled Printhead or INKREDIBLE+ and the Pneumatic Printhead. This document covers pre-print mixing with cells, 3D bioprinting and post-print photocrosslinking. The protocol was optimized for GelMA C with LAP at 0.25% concentration, for printing both without cells and with a 10+1 cell suspension dilution. Changing the concentration of photoinitiator or bioink to cell suspension ratio will require a change of the photocrosslinking parameters. 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 system.

Materials needed

- GelMA C bioink*
- UV shielding cartridges, 3cc*
- Sterile Conical Bioprinting nozzles, 22-27G*
- BIO X*, BIO X6* or INKREDIBLE+* 3D Bioprinters
- 405 or 365 nm UV modules for photocuring
- 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.

Protocol

This protocol works best with the BIO X or BIO X6 with the cooled print bed as well as the Temperature-controlled Printhead. While the bioink can be used with the INKREDIBLE+ system due to its ability to heat the bioink, secondary steps are necessary to cool the printed structure to pre-gel it prior to crosslinking. Clogging may still occur due to lack of temperature control at the nozzle. Therefore, it is not recommended to use the bioink with the INKREDIBLE system since the bioink will not perform as expected and resulting filament characteristics may be inconsistent. If using the INKREDIBLE+ system, preheat a printhead to 26°C to achieve a similar temperature maintenance as in the Temperature-controlled Printhead. After deposition, the Petri dish or well plate being printed on should be placed on ice or another cooled surface to thermally gel the construct after printing prior to photocrosslinking.

Step	Title	Material	Description
1	Prepare bioink	- GelMA C	- Heat up GelMA C in a cartridge for 10 min at 37°C. The heating of the GelMA C can be performed in a printhead, water bath or incubator.
2	Mix GelMA C with cells	- Cell suspension - CELLMIXER - Female/female Luer lock adaptor - 3 mL syringes with Luer lock connections - Prewarmed GelMA C	If not printing with cells move directly to step 3. At this point, mix ten parts of the bioink with one part of cell suspension, taking care not to introduce 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 C 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 units other side. - 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 C before attaching the syringe with the cell suspension. If preparing for quantities < 2 mL of GelMA C, 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 C (and cells) - Sterile Conical Bioprinting nozzles, 22-27G	BIO X and Temperature-controlled Printhead: - Place the cartridge in the Temperature-controlled Printhead pre-set to 23°C and allow the bioink to equilibrate for 10 min. Pre-cool the print bed to 10°C. INKREDIBLE: - Place cartridge on counter for 15 min to reach room temperature (21-24°C). Pre-cool the Petri dish or well plate by placing on ice.
4	Printing	- Bioprinter (BIO X, BIO X6 or INKREDIBLE+)	- 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. If too high pressure is required to extrude filaments or the filaments look uneven, increase the print temperature with 0.5-2°C. If the filaments float out even at low pressures, decrease the temperature with 0.5-2°C. 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 23°C $^{\text{diluted}}/_{\text{undiluted}}$ bioink. '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 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	24 / 32	26 / 34	28 / 36	30 / 38
25	27 / 35	29 / 37	31 / 39	33 / 42
27	29 / 37	31 / 39	33 / 41	35 / 43

**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.*

Step	Title	Material	Description
5	Crosslinking	- 405 or 365 nm UV modules for photocuring	GelMA C with LAP can be crosslinked using either the 405 or 365 nm photocuring module. - Photocuring: see Table 2 below for recommended crosslinking times. Ensure that the bioprinted GelMA C construct is thermally gelled after printing by cooling the print bed. If you want

			to perform photocrosslinking during bioprinting, set the crosslinking parameters appropriately in the printhead setup page for the BIO X or BIO X6. Note: It is recommended to use the 405 nm photocuring module instead of 365 nm if possible, when photocuring GelMA C with LAP. Over exposure at the 365 nm wavelength might damage the cells. Note: If crosslinking is unsure add 37°C media to one printed well to validate that it does not dissolve.

Table 2. Recommended time to crosslink printed constructs**. Distance from photocuring module to construct set at 5 cm using the BIO X or BIO X6 photocuring modules. If using the INKREDIBLE+ photocuring modules, the time required might need to be decreased. For crosslinking with other parameters, see *Photocrosslinking Optimization Protocol*. This table was generated using GelMA C with mesenchymal stem cells. Do not exceed the exposure time to more than 120 seconds when printing with cells.

Construct depth (mm) /time (s)	365 nm LAP 0.25%	405 nm LAP 0.25%
1	10	20
3	20	25

***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.*

Step	Title	Material	Description
6	Incubation	- Cell culture medium	- After photocrosslinking, add the desired medium to the constructs and culture in standard conditions (37°C, 5% CO_2 and 95% relative humidity) or according to your application.