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Bioprinting Protocol

GelMA A

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 A bioink using the BIO X, with and without cells. This document covers pre-print mixing with cells, 3D bioprinting and post-print processes of crosslinking ionically or through photocuring. This protocol was optimized for GelMA A with LAP at 0.25% concentration, undiluted as well as with a 10+1 parts cell suspension dilution. Changing the concentration of photoinitiator or bioink to cell suspension ratio, will change the photocrosslinking time. Reference the *Photocrosslinking Crosslinking Optimization Protocol* to adjust and determine these numbers. This protocol was optimized using the Temperature Control Printhead with the BIO X system.

Material needed

- GelMA A bioink*
- UV shielding cartridges, 3cc*
- Sterile Conical Bioprinting nozzles, 22-27G*
- BIO X*, BIO X6* or INKREDIBLE+* 3D Bioprinter
- Crosslinking agent (included with the bioink purchase)
- 405/365 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 and the Temperature Control Printhead as well as the cooled print bed. 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 the same temperature maintenance as the Temperature Control 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 A	- Heat up GelMA A in a cartridge to 35°C until the GelMA A is liquid, this can be tested by flipping the cartridge and observing if air bubbles move freely. The heating of the GelMA A can be performed in a pneumatic printhead, water bath or incubator.
2	Mix GelMA A with cells	- Cell suspension - CELLMIXER - Female/female Luer lock adaptor - 3 mL syringes with Luer lock connections - Prewarmed GelMA A	If not printing with cells move directly to step 3. 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 Protocol GelMA Series.* - Transfer the cell suspension to the 1 mL cell syringe (PART 1) using a female/female Luer lock adaptor. - Transfer GelMA A 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 A before attaching the syringe with the cell suspension.

			If preparing for quantities < 2 mL of GelMA A, 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 A (and cells) - Sterile Conical Bioprinting nozzles, 22-27G	- Place cartridge on counter for 20 min to reach room temperature. Observe the air bubble movement, once it begins to slow down, the GelMA A is almost ready to print. The viscosity needs to be like a thick syrup or honey. - Place the semi-gelled GelMA A in the Temperature Control Printhead preheated to 26°C and pre-cool the print bed to 10°C.
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. Note: Over time the GelMA A will become more solid (15-20 minutes). If printing is paused and this happens, replace the nozzle. If extrusion does not occur, repeat steps 1 and 3 to 'reset' the cartridge.

Table 1. Recommended minimal extrusion pressure** (±2 kPa) used for printing continuous filaments at 26°C $^{\text{with cells}}/_{\text{without cells}}$. Again, 'with cells' assumes a mixture of one part cell suspension to 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	3 / 2	4 / 4	4 / 5	4 / 6
25	3 / 4	5 / 6	5 / 8	7 / 10
27	5 / 6	8 / 9	8 / 12	9 / 15

***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 26°C and with a bioink dilution with a low concentration of cells.*

Step	Title	Material	Description
5	Crosslinking	- Crosslinking solution AND/OR	GelMA A can be crosslinking using the $CaCl_2$ crosslinking solution or with photoinitiation. GelMA A with LAP can be crosslinked with either the 405 or 365 nm photocuring module. If using both ionic and photocrosslinking, begin with photocrosslinking. - Photocuring: see Table 2 below for recommended crosslinking times. Ensure that

		- 405/365 nm UV modules for photocuring	the bioprinted GelMA A construct is thermally gelled after printing by cooling the print bed. If 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 if possible when photocuring GelMA A 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 doesn't dissolve. - Ionic crosslinking: Submerge the cell-laden constructs in the crosslinking solution for 30 seconds to 5 minutes depending on construct size. Remove crosslinking solution and rinse constructs with basal culture media once.

Table 2. Recommended seconds to crosslink the construct***. 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 can possibly be decreased. For crosslinking with other parameters, see *Photocrosslinking Optimization Protocol*. This table was generated using GelMA A with mesenchymal stem cells. Don't 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	10
3	15	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.*

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