Ref No: BPR-IK-3X2102
Date: 18-DEC-2020
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Bioprinting Protocol

GelXA

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 GelXA bioink using the INKREDIBLE, INKREDIBLE+, BIO X or BIO X6, 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 GelXA with LAP 0.25% undiluted as well as a 10+1 cell suspension dilution. Changing the concentration of LAP or bioink to cell suspension ratio will change the photocrosslinking time. Reference the *Photocrosslinking Optimization Protocol* to adjust and determine these numbers. This protocol was optimized using the Temperature-controlled Printhead using the BIO X and BIO X6.

Materials needed

- GelXA bioink*
- Crosslinking Agent* (included with the bioink purchase)
- UV shielding cartridges, 3cc*
- Sterile conical bioprinting nozzles, 22-27G recommended*
- BIO X*, BIO X6* or INKREDIBLE series* 3D Bioprinter
- Well plate or Petri dish*
- 405 or 365 nm light modules for photocuring
- Cells + cell culture medium
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
  or
- CELLMIXER*

*The products 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 using the BIO X or BIO X6 with the cooled print bed at 15°C and the Temperature-controlled Printhead at 24°C. The GelXA can also be extruded using the pneumatic printheads or the INKREDIBLE series, but with decreased shape fidelity if ambient temperature exceeds 25°C and the printhead heats up. If using the INKREDIBLE series, the printing substrates such as Petri dishes or well plates 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	- GelXA - Female/female Luer lock adaptor - 3 mL syringes with Luer lock connections	If not printing with cells move directly to step 3. - Heat up GelXA in a cartridge/syringe at 37°C for 10 minutes. The heating of the GelXA can be performed in a pneumatic printhead, water bath or incubator. Prolonged and repeated heating could negatively affect the photoinitiator stability and the homogeneity of the bioink, requiring additional mixing before adding cells. If not using the entire 3 mL of the bioink in the cartridge, transfer the needed amount to a syringe using a female/female Luer lock adaptor and spare the rest of the bioink in the optimal storage conditions. Note: If there are bubbles in the bioink, make a quick centrifugation for 1.5 minutes at 1600 rpm.
2	Mix GelXA with cells	- Cell suspension - CELLMIXER - Female/female Luer lock adaptor - 3 mL syringes with Luer lock connections - Prewarmed GelXA	At this point, mix ten parts of 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 GelX Series.* - Transfer the cell suspension to the 1 mL cell syringe (PART 1) using a female/female Luer lock adaptor. - Transfer GelXA 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 from another 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 GelXA before attaching the syringe with the cell suspension. If preparing for quantities <2 mL of GelXA, it is recommended to connect two 3 mL Luer lock

			syringes and mix the bioink with the cell suspension back and forth between the syringes until it becomes homogeneous.
3	Cool and load the cartridge	- UV shielding cartridges, 3cc loaded with GelXA (and cells) - Sterile conical bioprinting nozzles, 22-27G	- Place cartridge on counter for 10-20 minutes to reach room temperature. If the bioink has been pre-heated, it can instead be placed in a fridge for 3-5 minutes, or in the Temperature-controlled Printhead at 24°C for 5 minutes for faster cooling. Note: Room temperature is within 20-25°C. - Place the room tempered GelXA in the printhead and cap with the printing nozzle. If using the BIO X or BIO X6, pre-cool the print bed to 15°C. Note: When printing with GelXA, the recommended printhead temperature for the highest printing fidelity is 20-25°C, though the bioink can be dispensed up to 32°C.
4	Printing	- Bioprinter (BIO X, BIO X6 or INKREDIBLE series) - Well plate or Petri dish	- 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 printheads heat up during a long printing session or ambient temperature exceeds 25°C, the bioink also heats up above 25°C and its viscosity decreases, observed as extrusion of very thick filaments even at low pressures. In this case, repeat step 3 to return to the temperature range for good printability. 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) for printing continuous filaments at 20-25°C using $^{\text{diluted}}/_{\text{undiluted}}$ 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 smaller dilutions, 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	19 / 21	21 / 25	25 / 31	29 / 34
25	25 / 34	30 / 40	34 / 46	38 / 51
27	30 / 41	25 / 50	40 / 59	45 / 68

***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 of 24°C and with a 10+1 bioink dilution with PBS.*

Step	Title	Material	Description
5	Crosslinking	- Crosslinking Agent AND/OR - 405 or 365 nm light modules for photocuring - Cell culture medium	GelXA can be photocrosslinked using the 405 or 365 nm light modules or ionically crosslinked using the $CaCl_2$-containing Crosslinking Agent. If using both, begin with photocrosslinking. Photocrosslinking only will generate a stiffer result than ionic crosslinking only. Photocrosslinking: see Table 2 below for recommended crosslinking times. Ensure that the bioprinted GelXA construct is thermally gelled after printing by cooling the print bed (if using the BIO X or BIO X6) or placing the printing substrates with the construct on ice for 10 seconds (if using the INKREDIBLE series). If photocrosslinking during bioprinting, set the crosslinking parameters appropriately in the G-code for the INKREDIBLE series or the printhead setup page for the BIO X or BIO X6. Note: It is recommended to use the 405 nm light module instead of 365 nm if possible. Overexposure might damage the cells. Note: To verify the crosslinking is sufficient, add 37°C media to one printed well and observe 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 time of the construct photocrosslinking***. Distance from each light module to construct was set to 5 cm using the BIO X or BIO X6 photocuring modules. If using the INKREDIBLE series photocuring modules, the time required can possibly be decreased. For crosslinking with other parameters, see *Photocrosslinking Optimization Protocol*. This table was generated using GelXA with mesenchymal stem cells. Don't exceed the exposure time to more than 120 seconds when printing with cells. To achieve the best structural integrity when printing thicker constructs, it is recommended to apply 3/5 seconds photocrosslinking with 365/405 nm light every fourth layer. If bioink is used above 25°C, the best results can be achieved when applying 3/5 seconds photocrosslinking with 365/405 nm light every second layer.

	365 nm, LAP 0.25%	405 nm, LAP 0.25%
Construct depth (mm)/time (s)	1/5	1/10
	3/15	3/30

****This is only a recommended reference of crosslinking times to start with. 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 crosslinking, add the desired medium to the constructs and place them in an incubator. - Incubate the constructs in cell culture medium in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to application.