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Bioprinting Protocol

GelXA BONE

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 the GelXA BONE bioink using the INKREDIBLE, INKREDIBLE+, or 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 GelXA BONE 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 pneumatic printhead using the BIO X.

Materials needed

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

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Protocol

This protocol works best using the BIO X with the cooled print bed. 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 BONE	If not printing with cells move directly to step 3. - Heat up GelXA BONE in a cartridge to 33-37°C. The heating of the GelXA BONE can be performed in a pneumatic printhead, water bath or incubator. Note: If there are bubbles in the bioink, make a quick centrifugation for 1.5 min at 1600 rpm.
2	Mix GelXA BONE with cells	- Cell suspension - CELLMIXER - Female/female Luer lock adaptor - 3 mL syringes with Luer lock connections - Prewarmed GelXA BONE	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 BONE 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 BONE before attaching the syringe with the cell suspension. If preparing for quantities <2 ml of GelXA BONE, it is recommended to connect two 3 mL Luer lock syringes and mix the bioink back and forth between the syringes until it becomes homogeneous.
3	Cool and load the cartridge	- UV shielding cartridges, 3cc loaded with GelXA BONE (and cells)	- Place cartridge on counter for 20 min to reach room temperature. Note: Room temperature is within 20-25°C.

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		- Sterile conical bioprinting nozzles, 22-27G	- Place the room tempered GelXA BONE in the printhead and cap with the printing nozzle. If using the BIO X, pre-cool the print bed to 15°C. Note: When printing with GelXA BONE, 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 or INKREDIBLE series recommended) - 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. 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 20-25°C $^{\text{with cells}}/_{\text{without cells}}$. 'With cells' assumes a mixture of one part of cell suspension to ten parts of 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	12 / 14	15 / 25	14 / 28	16 / 30
25	19 / 25	25 / 30	28 / 33	28 / 32
27	16 / 34	22 / 40	25 / 41	28 / 40

**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 of 24°C and with a bioink dilution with a low concentration of cells.*

Step	Title	Material	Description
5	Crosslinking	- Crosslinking Agent AND/OR - 405/365 nm light modules for photocuring - Cell culture medium	GelXA BONE 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: see Table 2 below for recommended crosslinking times. Ensure that the bioprinted GelXA BONE construct is thermally gelled after printing by cooling the print bed (if using the BIO X) or placing the printing substrates with the construct on ice for 10 s (if using the INKREDIBLE series). If photocrosslinking during bioprinting, set the crosslinking parameters

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			appropriately in the G-code for the INKREDIBLE series or the printhead setup page for the BIO X. Note: It is recommended to use the 405 nm light module instead of 365 nm one 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 s to 5 min 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 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 BONE with mesenchymal stem cells. Don't exceed the exposure time to more than 120 s when printing with cells.

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

***Note: 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 your application.