Bioprinting Protocol
GelXA 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 the GelXA FIBRIN 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 FIBRIN 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 Crosslinking Optimization Protocol to adjust and determine these numbers. This protocol was optimized using the pneumatic printhead using the BIO X.

Material needed
- GelXA FIBRIN 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 UV modules for photocuring
- Crosslinking agent (included with the bioink purchase)
- Vial with 100 U thrombin (included with the bioink purchase)
- 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 using the cooled printbed. If using the INKREDIBLE-series system, 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.

<table>
<thead>
<tr>
<th>Step</th>
<th>Title</th>
<th>Material</th>
<th>Description</th>
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</table>
| 1    | Prepare Bioink | - GelXA FIBRIN | If not printing with cells move directly to step 3.  
- Heat up GelXA FIBRIN in a cartridge to 33-37°C. The heating of the GelXA FIBRIN 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 FIBRIN with cells | - Cell suspension  
- CELLMIXER  
- Female/female luer lock adaptor  
- 3 ml syringes with luer lock connections  
- Prewarmed GelXA FIBRIN | 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 GelX Series.  
- Transfer the cell suspension to the 1 ml cell syringe (PART 1) using a female/female luer lock adaptor.  
- Transfer GelXA 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 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 GelXA FIBRIN before attaching the syringe with the cell suspension.  
If preparing for quantities < 2 ml of GelXA 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 GelXA FIBRIN (and cells)
- Sterile Conical Bioprinting nozzles, 22-27G
- Place cartridge on counter for 20 min to reach room temperature.

Note: Room temperature is within 20-25°C.

4. Printing
- Bioprinter (BIO X or INKREDIBLE series recommended)
- Well plate or Petri dish
- Bioprint structures with parameters according to Table 1 or the Printing parameters in the Application note. 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 21-25°C with cells/without cells. Again, ‘with cells’ assumes a mixture of one part cell suspension to ten parts bioink. For information about filament diameter, see Printing parameters in the Application note. For highly concentrated cell suspensions, the pressure needs to be increased towards the pressure used for undiluted bioink.

<table>
<thead>
<tr>
<th>Printing speed (mm/sec) →</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
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</thead>
<tbody>
<tr>
<td>Nozzle size (G) ↓</td>
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<td>31</td>
<td>35</td>
<td>41</td>
<td>58</td>
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</table>

*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 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 UV modules for photocuring | GelXA FIBRIN can be crosslinked with photoinitiation using the 405 or 365 nm photocuring module or ionic crosslinking using the CaCl₂ containing crosslinking agent with thrombin. If using both, begin with photocrosslinking.
- Photocuring: see Table 2 below for recommended crosslinking times. Ensure that the bioprinted GelXA FIBRIN construct is...
thermally gelled after printing by cooling the printbed if using the BIO X or placing the well plates containing printed construct on ice for 10 sec if using the INKREDBILE-series. If photocrosslinking during bioprinting, set the crosslinking parameters appropriately in the G-code for the INKREDBILE-series or the printhead setup page for the BIO X.

Note: It is recommended to use the 405 nm photocuring module instead of 365 nm if possible. Over exposure 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: Prepare 10 ml 10 U/ml thrombin crosslinking solutions by adding 1 ml of crosslinking solution to the thrombin vial. Then transfer the 1 ml thrombin solution to 9 ml crosslinking solution. Mix gently by pipetting up and down 2-3 times.

- Submerge the cell-laden constructs in the crosslinking solution for 30 sec to 5 min depending on construct size. Remove crosslinking solution and rinse constructs with basal culture medium once.

Table 2. Recommended seconds to photocrosslink the construct**. Distance from photocuring module to construct set at 5 cm using the BIO X photocuring modules. If using the INKREDBILE-series photocuring modules, the time required can possibly be decreased. For crosslinking with other parameters, see Photocrosslinking Crosslinking Optimization Protocol. This table was generated using GelXA FIBRIN with mesenchymal stem cells. Don’t exceed the exposure time to more than 120 seconds when printing with cells.

<table>
<thead>
<tr>
<th>Construct depth (mm) / time (s)</th>
<th>365 nm LAP 0.25%</th>
<th>405 nm LAP 0.25%</th>
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<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>10</td>
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<td>3</td>
<td>15</td>
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**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% CO₂ and 95% relative humidity) or according to your application.