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BIOPRINTING PROTOCOL

GelMA Bioink

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 of GelMA Bioink using the BIO X and BIO X6, with and without cells. This document covers pre-print mixing with cells, 3D bioprinting and post-print processes such as crosslinking through photocuring. This protocol was optimized for GelMA 10% w/w concentration with LAP at concentration 0.25% undiluted as well as with a 10+1 cell suspension dilution. Changing the bioink to cell suspension ratio and thus also the concentration of photoinitiator, will change the photocrosslinking time. Refer to the *Photocrosslinking Crosslinking Optimization Protocol* to adjust and determine these numbers. This protocol was optimized using the Temperature-controlled printhead with the BIO X and BIO X6.

Materials needed

- GelMA Bioink*
- Cells* + cell culture medium*
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
- CELLMIXER* (Optional)
- UV shielding cartridges, 3cc*
- Conical bioprinting nozzles, 22-27G recommended*
- Temperature-controlled printhead
- BIO X* or BIO X6* 3D bioprinter
- Petri dish* or well plate

*The product can be purchased in the CELLINK shop at www.cellink.com/shop.

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

GelMA Bioink has been optimized for use with the BIO X and BIO X6 system and Temperature-controlled printhead with thermal nozzle cover and the use of a 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+ since the bioink will not perform as expected and resulting filament characteristics may be inconsistent. First time users of GelMA based bioinks are recommended to optimize the printing conditions without cells before proceeding to bioprint with cells. Perform the desired dilution using medium or PBS.

1. Preparing the bioink

MATERIAL

GelMA

Temperature-controlled printhead

DESCRIPTION

- Heat up GelMA in the cartridge at 37°C until the GelMA is liquid. The heating of the GelMA can be performed in a printhead or incubator and usually requires approximately 30 min if the bioink is taken directly from the fridge.
- Set the Temperature-controlled printhead to 25°C.
- *If not printing with cells move directly to Step 3.*

2. Mixing the bioink with cells

MATERIAL

3 mL syringes with Luer lock connections

Female/female Luer lock adaptor

Pre-warmed GelMA

Cell suspension

UV shielding cartridge, 3cc

CELLMIXER (optional)

DESCRIPTION

- 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 with bioink Protocol*.
- If preparing for quantities < 2 mL of GelMA, it is recommended to connect two 3 mL Luer lock syringes, one with the bioink and the other with the cell suspension and gently mix back and forth between the syringes until homogeneous. Transfer the mixture to an empty 3cc cartridge by connecting the syringe to the cartridge using the Luer lock adaptor. Cap the cartridge with a tip cap.
- If using larger quantities, the CELLMIXER can be used:
    - Transfer the cell suspension to the 1 mL cell syringe (PART 1) using a female/female Luer lock adaptor.
    - Transfer GelMA 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 unit's other side.
- Apply gentle pressure onto the Dispensing unit to mix the content of both syringes into the empty cartridge. Cap the cartridge with a tip cap.

Note: To avoid introducing air when connecting the syringes, carefully pre-fill the Luer lock adaptor with GelMA before attaching it to the syringe with the cell suspension.

Note: Keep the cartridge horizontal when tip and end cap are removed. This is to prevent air from entering the cartridge or bioink from dripping out when liquid.

3. Preparation for printing

MATERIAL

GelMA mixed with cells (if applicable) in UV shielding cartridge

Temperature-controlled printhead

Conical bioprinting nozzles, 22-27G

DESCRIPTION

- If the cartridge is just taken from the heat or if the cartridge still feels warm after mixing in the cells, place it in the pre-heated Temperature-controlled printhead at 25°C for 10 minutes. If not using the Temperature-controlled printhead, place the cartridge on counter for 5-10 minutes to reach approximately 25°C.
- If the cartridge has cooled down below 24°C, re-heat the cartridge at 37°C for 5 min to reset then restart equilibration in printhead.
- Cap the cartridge with a bioprinting nozzle and place the GelMA in the printhead. Connect the cartridge to the air pressure adapter. If using the BIO X or BIO X6, pre-cool the print bed to 15°C.

Note: When printing with GelMA, the recommended printhead temperature for the highest printing fidelity is 25°C, though the bioink can be dispensed up to 32°C. Below 24°C the bioink can become too viscous resulting in chunky filaments and too high extrusion pressures needed.

Note: Be careful not to touch the printhead with the nozzle tip and if using very liquid materials, make sure that the bioink does not drip through the nozzle especially when attaching the air adapter. Alternatively, the cartridge can be placed in the printhead with the tip cap on and when in place switched to a nozzle.

Note: Test the flow of the bioink after the calibration is performed and start with a low pressure and increase stepwise.

4. Printing

MATERIAL

BIO X or BIO X6 3D bioprinter

Well plate or Petri dish

DESCRIPTION

- Calibrate the nozzle to the well plate or Petri dish surface. Test the flow of the bioink first after calibration and start with a low pressure and increase stepwise. Bioprint structures with onto the well plate or Petri dish. If printability is not as desired, adjust the pressure up/down by 1 kPa to extrude more/less material.

Example: If printing continuous filaments with a Temperature-controlled printhead set to 25°C, a 25G nozzle, a printing speed of 5 mm/s and with 300 ms pre-flow delay, the suggested pressure range is between 23-33 kPa without cell suspension dilution and 18-28 kPa if diluted with cell suspension.

- If proper viscosity and printability is not achieved by extending temperature equilibration time or tuning pressure:
    - Too low viscosity (wide filaments despite using low pressure): decrease the printhead temperature 0.5-1°C to increase the viscosity and equilibrate an additional couple of minutes.
    - Too high viscosity (chunky filaments and high pressure required): increase the printhead temperature 0.5-1°C to decrease the viscosity and equilibrate an additional couple of minutes. If unsuccessful, the bioink might have over-gelled. In this case, re-heat the cartridge at 37°C for 5 min to reset and choose a 1°C higher printhead temperature than before. Let the bioink reach the new temperature before starting to print again.
- During print sessions longer than 20 min at 25°C the bioink can become too viscous due to continued gelling resulting in chunky filaments and too high extrusion pressures needed. To avoid this, a 0.5°C increase in printhead temperature after 20 min printing can extend the time the bioink remains at good printability.
- If the regular pneumatic printheads are used for a long period, they might heat up above the desired printing temperature. Then the bioink also heats up which decreases its viscosity, observed as extrusion of very thick filaments even at low pressures. Remove the cartridge from the printhead and allow to cool down to 25°C. In addition, remove the printheads from the BIO X/BIO X6 to let them cool down before continuing to print.

Note: If waiting too long between extrusions the bioink can dry in the nozzle causing it to clog. If this occurs, take a sterile tweezer and remove the dried GelMA part at the edge of the nozzle or replace with new nozzle.

5. Crosslinking

MATERIAL

405 or 365 nm LED modules for photocuring

DESCRIPTION

- GelMA with LAP can be crosslinked with photoinitiation using either the 405 or 365 nm photocuring module.

Note: It is recommended to use the 405 nm LED module instead of 365 nm if possible. Overexposure might damage the cells.

- See Table 1 below for recommended crosslinking times. Ensure that the bioprinted GelMA construct is thermally gelled after printing by cooling the constructs on the print bed on the BIO X or BIO X6 for 30 seconds.
- If photocrosslinking during bioprinting, set the crosslinking parameters appropriately in the printhead setup page for the BIO X or BIO X6.
- Let the structure sit for 3-5 minutes to allow crosslinking after the light source is turned off.

Note: To verify that the photocrosslinking is sufficient, add 37°C to one printed well and observe that it doesn't dissolve.

Table 1. Recommended seconds required to crosslink the construct**. Distance from photocuring module to construct set at 5 cm using the BIO X or BIO X6 modules. For crosslinking with other parameters, see *Photocrosslinking Optimization Protocol*. This table was generated using GelMA with mesenchymal stem cells. Don't exceed 120 seconds of exposure time when printing with cells. To achieve the best structural integrity when printing thicker constructs, it is recommended to apply 3 or 5 seconds photocrosslinking with 365 or 405 nm light respectively, every second or fourth layer.

	365 nm, LAP 0.25%	**405 nm, LAP 0.25%**
1 mm construct thickness	5 seconds	10 seconds
3 mm construct thickness	15 seconds	30 seconds

***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.*

6. Incubation

MATERIAL

Cell culture medium

DESCRIPTION

- After 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 application. Replace medium regularly.