Ref No: BPR-IK-351102
Date: 06-JUN-2023
Author: EP,JH,VK. Version: 4

BIOPRINTING PROTOCOL

GelMA C

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 C bioink using the BIO X or BIO X6, with and without cells. This document covers pre-print mixing with cells, 3D bioprinting and post-print processes such as photocuring. This protocol was optimized for GelMA C with LAP at 0.25% concentration, undiluted as well as with a 10+1 parts 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 system.

Materials needed

- GelMA C 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*
- Temperature-controlled Printhead (optional)
- BIO X*, BIO X6* or INKREDIBLE+* 3D bioprinter

*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

This protocol works best with the BIO X or BIO X6 and the Temperature-controlled 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 pre-heat a printhead to 24°C to achieve the same temperature maintenance as the Temperature-controlled Printhead. After deposition, the Petri dish or well plate should be placed on ice or another cooled surface to thermally gel the construct after printing prior to photocrosslinking. 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 C

3 mL syringes with Luer lock connections

Female/female Luer lock adaptor

Pipette tip or spatula

DESCRIPTION

- Heat up the GelMA C cartridge at 37°C until it becomes a smooth viscous liquid. The heating of the GelMA C can be performed in a printhead or incubator and usually requires 30-60 min if the bioink is taken directly from the fridge.
- To make sure that the bioink is homogeneous after heating, connect the cartridge to a 3 mL syringe using a Luer lock connector and remove the end cap. Push approximately half of the bioink from the cartridge into the syringe by gently pushing the cartridge piston using a pipette tip or small spatula while simultaneously pulling the syringe plunger. To remove any air bubble derived from the dead volume in the syringe, separate the syringe and cartridge maintaining the Luer lock on the syringe. Hold the syringe with the tip facing upwards and gently tap the syringe to move air bubbles towards the tip. Carefully extrude air and pre-fill the Luer lock adaptor with GelMA C before re-attaching the cartridge. Gently mix the bioink back and forth between the cartridge and syringe to homogenize the bioink. If not using the entire 3 mL of the bioink in the cartridge, keep the rest of the bioink in the optimal storage conditions. Prolonged and repeated heating could negatively affect the photoinitiator stability.

Note: If there are bubbles in the bioink, make a quick centrifugation for 30 s at 600 x *g*.

- *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 C

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 C, 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 C 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 C before attaching it to the syringe with the cell suspension.

3. Preparation for printing

MATERIAL

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

Temperature-controlled Printhead (optional)

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 24°C for 10 minutes. If not using the Temperature-controlled Printhead, place the cartridge on counter for 5-10 minutes to reach approximately 24°C.
- If the cartridge has cooled down below 23°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 C 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 C, the recommended printhead temperature for the highest printing fidelity is 24°C, though the bioink can be dispensed up to 32°C. Below 23°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, BIO X6 or INKREDIBLE series 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 into 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 24°C, a 25G nozzle, a printing speed of 5 mm/s and with 300 ms pre-flow delay, the suggested pressure range is between 25-35 kPa without cells and 20-30 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.
- During print sessions longer than 20 min at 24°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 24°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, replace with new nozzle.

5. Crosslinking

MATERIAL

405/365 nm LED modules for photocuring

Cell culture medium

DESCRIPTION

- GelMA C can be photocrosslinked using the 405 or 365 nm LED modules. See Table 1 below for recommended crosslinking times. Ensure that the bioprinted GelMA C 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. Overexposure might damage the cells.

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 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 might need to be decreased. For crosslinking with other parameters, see *Photocrosslinking Optimization Protocol*. This table was generated using GelMA C 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 fourth layer. If the bioink is used above 25°C, the best results can be achieved when photocrosslinking every second layer.

	365 nm, LAP 0.25%	405 nm, LAP 0.25%
1 mm construct thickness	10 seconds	20 seconds
3 mm construct thickness	20 seconds	25 seconds

***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.*

6. Incubation

MATERIAL

Cell culture medium

DESCRIPTION

- After crosslinking, 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. Replace medium regularly.