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BIOPRINTING PROTOCOL

CHITOINK

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 Chitoink using the INKREDIBLE, INKREDIBLE+, BIO X or BIO X6. It covers steps such as pre-print mixing with cells, 3D bioprinting and post-print processes such as ionic crosslinking. This protocol was optimized for Chitoink in both undiluted and diluted state and printed using the pneumatic printhead. Changing the parameters in the protocol might change the printing pressure or crosslinking time required.

Materials needed

- Chitoink*
- Cells* + cell culture medium*
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
- CELLMIXER* (optional)
- Clear cartridges, 3cc*
- Conical bioprinting nozzles, 22-25G recommended*
- BIO X*, BIO X6* or INKREDIBLE series* 3D bioprinter
- Well plate or Petri dish*
- TPP Crosslinking Agent (included with the bioink purchase)

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

Protocol

This protocol can be performed with printheads and print bed at room temperature (20-25°C).

1. Preparing the bioink

MATERIAL

Chitoink

DESCRIPTION

- Warm up Chitoink in a cartridge to room temperature.
- *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 Chitoink

Cell suspension

Cartridge, 3cc

CELLMIXER (optional)

DESCRIPTION

- Mix ten parts of bioink with one part of cell suspension without introducing air bubbles to the mixture. For detailed instructions see the *Mixing cells with bioink Protocol.*
- If preparing for quantities <2 mL of Chitoink, 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 the bioink 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 and transfer it 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 Chitoink before attaching it to the syringe with the cell suspension.

3. Preparation for printing

MATERIAL

Chitoink mixed with cells (if applicable) in cartridge

Conical bioprinting nozzles, 22-25G recommended

DESCRIPTION

- Cap the cartridge with a printing nozzle of choice and place the room tempered Chitoink in the printhead. Connect the cartridge to the air adapter.

Note: The recommended nozzle size is 22-25G. Decrease the nozzle diameter to achieve smaller filament diameter, however this also increases the pressure 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

- Bioprint structures onto a well plate or Petri dish with parameters suggested in Table 1. If printability is not as desired, adjust the pressure up/down by 1 kPa steps 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 $^{diluted}/_{undiluted}$. 'Diluted' assumes a mixture of one part of cell culture medium and 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	29 / 46	33 / 54	37 / 61	40 / 67
25	32 / 59	37 / 66	41 / 71	46 / 77
27	35 / 59	41 / 66	46 / 71	51 / 75

***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 a bioink temperature of 25°C and with a 10+1 bioink dilution with cell culture medium.*

5. Crosslinking

MATERIAL

TPP Crosslinking Agent

Cell culture medium

DESCRIPTION

- Chitoink is ionically crosslinked using the TPP Crosslinking Agent.
    - Submerge the cell-laden constructs in the TPP Crosslinking Agent for 1 to 5 minutes depending on construct size, infill density and desired construct stiffness. Remove crosslinking solution and rinse constructs with basal cell culture media once.

Note: 30 seconds is recommended for 10 µL droplets while 10 minutes might be required for dense 1 cm^3 blocks. In addition, optimize the crosslinking depending on the cell type.

Note: If constructs are large and solid, it is advised to lift them from the substrate while submerged in TPP Crosslinking Agent to ensure effective and stable crosslinking.

6. Incubation

MATERIAL

Cell culture medium

DESCRIPTION

- After crosslinking and washing, 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.