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Author: JB, MG Version: 2

Bioprinting Protocol

Chitoink

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

Material needed

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

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

Boston, USA
75 Kneeland Street
Boston, MA 02111

Gothenburg, Sweden
Arvid Wallgrens Backe 20,
Gothenburg, 41346

Blacksburg, USA
2000 Kraft Dr, Suite 2125
Blacksburg, VA 24060

Kyoto, Japan
46-29 Yoshida-Shimo Adachi-cho, Sakyo-ku, Kyoto

www.cellink.com | +1 (833) CELLINK

Protocol

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

Step	Title	Material	Description
1	Prepare bioink	- Chitoink	- Warm up Chitoink in a cartridge to room temperature.
2	Mix Chitoink with cells	- 3 ml syringes with Luer lock connections - Prewarmed Chitoink - Female/female Luer lock adaptor - Cell suspension in syringe	If not printing with cells move directly to step 3. 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 Protocol Chitosan Series*. - 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. Note: To avoid an air gap when mixing the bioink and the cell suspension, carefully pre-fill the Luer lock adaptor with Chitoink before attaching the two syringes. If preparing for quantities <2 mL of Chitoink, it is recommended to connect two 3 mL Luer lock syringes and slowly mix back and forth between the syringes until homogeneous consistency is reached.
3	Load the cartridge	- Clear cartridges, 3cc loaded with Chitoink (and cells) - Sterile conical bioprinting nozzles	- Place the Chitoink in a printhead and cap with a printing nozzle of choice. Note: The recommended nozzle size is 22-25G. Decrees the nozzle diameter to achieve smaller filament diameter, however this also increases the pressure needed.
4	Printing	- Bioprinter (BIO X or	- Bioprint structures onto a well plate or Petri dish with parameters suggested in Table 1. If printability

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		INKREDIBLE series) - Well plate or Petri dish	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 to 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/sec) → 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 printhead temperature of 25°C and with a 10:1 bioink dilution.*

Step	Title	Material	Description
5	Crosslinking	- TPP Crosslinking Agent - Cell culture medium	Chitoink can be ionically crosslinked using the TPP Crosslinking Agent. - Submerge the cell-laden constructs in the crosslinking solution for 1 to 5 min depending on construct size. Remove crosslinking solution and rinse constructs with basal cell culture media once. Note: if constructs are large and solid, it is advised to lift the them from the substrate while submerged in TPP Crosslinking Agent to ensure effective and stable crosslinking.
6	Incubation	- Cell culture medium	- 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 your application.

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