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Author: JB, Version: 1

Bioprinting Protocol Example

CELLINK Bioink

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 CELLINK® bioink using the BIO X, and covers steps from pre-print mixing with cells, 3D bioprinting and post-print processes of ionic crosslinking. This protocol was optimized for CELLINK® bioink, undiluted as well as diluting the bioink with addition of 10% cell suspension. Changing the parameters in the protocol might change printing pressure or the crosslinking time required. This protocol was optimized using the pneumatic printhead.

Material needed

- 3 ml CELLINK® bioink*
- Clear cartridges, 3cc*
- Sterile conical bioprinting nozzles 22, 25 or 27G*
- BIO X* 3D Bioprinter
- 48-well plate
- Crosslinking agent (included with the bioink purchase)

- 15 million mesenchymal stem cells (MSCs) + 35 ml cell culture medium
- Female/female luer lock adaptor*
- CELLMIXER*
  or
- 3 ml syringes with luer lock connections

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

Protocol

This protocol can be performed with printheads and printbed at room temperature, where room temperature is between 20-25°C.

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Step	Title	Material	Description
1	Prepare Bioink	- CELLINK® bioink	If not printing with cells move directly to step 3. - Warm up CELLINK® bioink in a cartridge to room temperature.
2	Mix CELLINK® bioink with cells	- CELLMIXER or - 3 ml syringes with luer lock connections - Female/female luer lock adaptor - Prewarmed CELLINK® bioink - Cell suspension in syringe	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 CELLINK Series*. - Transfer the 15 million MSCs in 300 µl cell suspension to the 1 ml cell syringe (PART 1) using a Female/Female luer lock adaptor. - Transfer 3 ml of 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 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 CELLINK® bioink before attaching the syringe with the cell suspension. If preparing for quantities < 2 ml of CELLINK® bioink, 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	- Clear cartridges, 3cc loaded with CELLINK® bioink (and cells) - Sterile Conical Bioprinting nozzles 22, 25 or 27G.	- Place the CELLINK® bioink in the printhead and cap with a 22, 25 or 27G bioprinting nozzle. The recommended nozzle size is 22G. Decrees the nozzle diameter to achieve smaller filament diameter, however this also increase the risk of the bioink clogging.
4	Printing	- BIO X - 48-well plate	- Bioprint 5x5x1 mm rectilinear grids, see Figure 1, with parameters according to Table 1 into the 48-well plate. If printability is not as desired, adjust

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			the pressure up/down by 1 kPa to extrude more/less material. - Use the same layer height as the diameter of the 22, 25 or 27G nozzle. 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 $^{\text{with cells}}/_{\text{without cells}}$. 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.

Printing speed (mm/min) → Nozzle size (G) ↓	300	600	900	1200
22	6 / 11	8 / 13	9 / 14	11 / 17
25	8 / 12	10 / 15	11 / 17	12 / 17
27	10 / 13	14 / 15	15 / 17	16 / 20

Step	Title	Material	Description
5	Crosslinking	- Crosslinking solution - Cell culture medium	CELLINK® bioink can be crosslinked with ions using the $CaCl_2$ crosslinking solution. - Add 200 µl of crosslinking solution to the cell-laden constructs in each well and remove the entire volume after 45 seconds. Wash each well with 200 µl of cell culture medium to completely remove the crosslinking solution.
6	Incubation	- Cell culture medium	- After crosslinking and washing, add 500 µl of the desired cell culture 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. - Make a full medium change every Monday and Friday. Analyze the viability using Calcein AM and Propidium iodide (PI), see *Viability Protocol Calecin AM and PI* for full instructions. An example of the viability composite from Day 14 can be seen in Figure 2.

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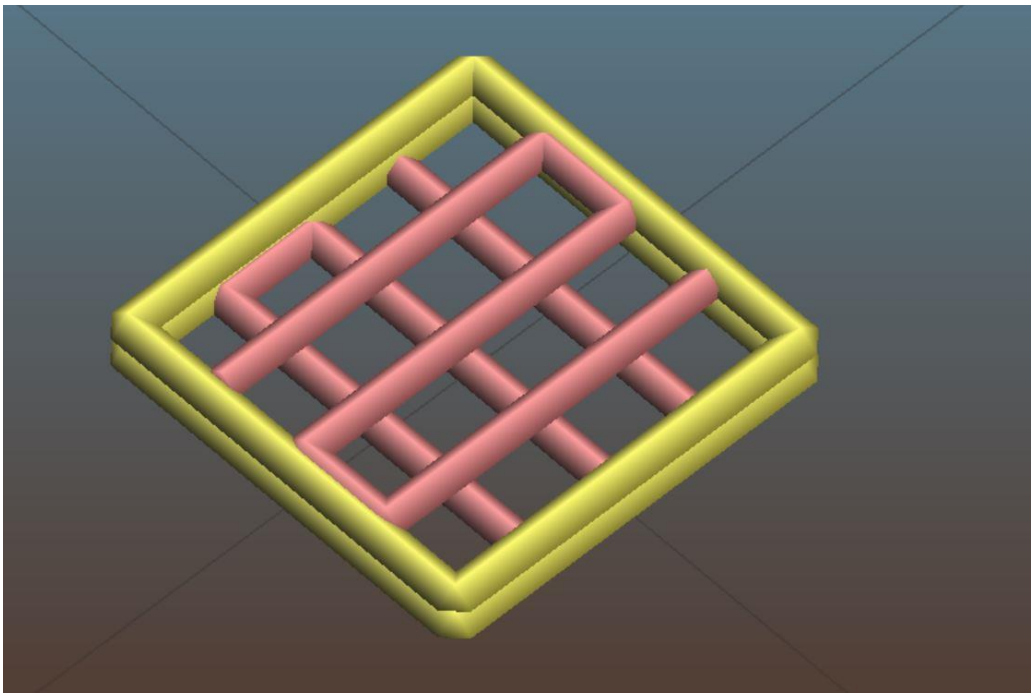

Figure 1. 5x5x1 mm rectilinear grid visualized in Slic3r.

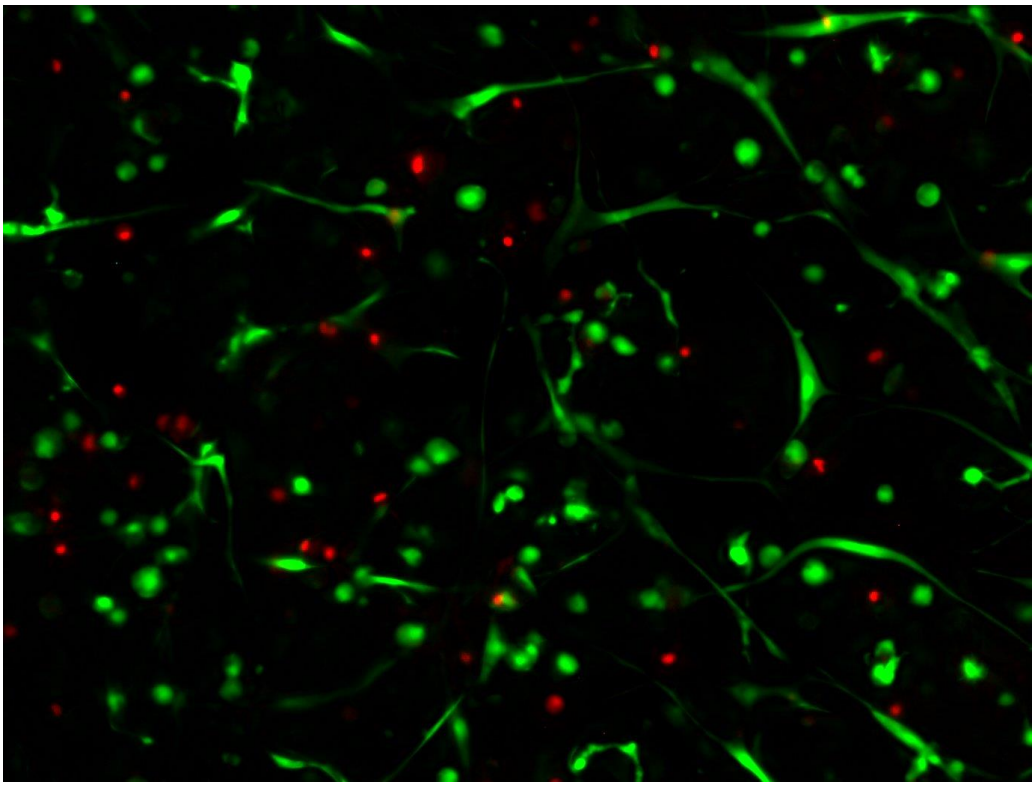

Figure 2. Viability composites where green represent the live MSCs and the red represents dead MSCs. The MSCS were bioprinted in CELLINK bioink and at Day 14 the viability was >80%.

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