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Author: SJ, JH, VK. Version: 1

CELLINK
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BIOPRINTING PROTOCOL

CELLINK Vivoink

This is a suggested procedure, please adjust it according to your experimental needs. Work under aseptic conditions.

Protocol aim

The aim of this protocol is to provide instructions for bioprinting of CELLINK Vivoink using the BIO X or BIO X6. It covers steps of pre-print mixing with cells, 3D bioprinting, and post-print processes such as ionic crosslinking. This protocol was optimized for CELLINK Vivoink, undiluted as well as using a 9+1 cell suspension dilution. Changing the dilution parameters in the protocol might change the recommended bioprinting parameters and crosslinking time required. This protocol was optimized for the Pneumatic Printhead and Syringe Pump Printhead on the BIO X6.

Materials

- CELLINK Vivoink bioink*
- $CaCl_2$ crosslinking agent* (included in the bioink purchase)
- Cells* + cell culture medium*
- 3 mL and/or 5 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
- Clear cartridges, 3cc* (if printing with Pneumatic Printhead)
- Conical bioprinting nozzles, 22-25G recommended*
- BIO X* or BIO X6* 3D bioprinter
- Well plate or Petri dish*

*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. CELLINK Vivoink can be printed between 10°C and 37°C with little to no alterations to the printability.

1. Bioink preparation

MATERIAL

CELLINK Vivoink, 3 mL in 5 mL syringe

5 mL syringe with Luer lock connections

Female/female Luer lock adaptor

DESCRIPTION

- If not using the full 3 mL of CELLINK Vivoink, mix it a few times to make sure it is homogenous before taking an aliquot. For mixing, connect the syringe with the bioink to a 5 mL empty syringe using a Luer lock connector. Gently mix the bioink back and forth between the syringes to homogenize the bioink, taking care not to introduce air. Spare the rest of the bioink in optimal storage conditions.
- Warm up the needed volume of CELLINK Vivoink to room temperature.

2. Mixing the bioink with cells

MATERIAL

Pre-warmed CELLINK Vivoink

Cell suspension

3 mL or 5 mL syringes with Luer lock connections

Female/female Luer lock adaptor

Cartridge, 3cc (if printing with the Pneumatic Printhead)

DESCRIPTION

- Prepare a cell suspension with the desired number of cells in a volume which is 10% of the final total volume (cells suspension + bioink), for example dissolve 1 million cells in 100 µL cell culture medium to be mixed with 900 µL CELLINK Vivoink.
- At this point, mix nine parts bioink with one part cell suspension, taking care not to introduce air bubbles to the mixture. For mixing of CELLINK Vivoink, it is recommended to connect the 5 mL syringe of CELLINK Vivoink with an empty 3 mL or 5 mL Luer lock syringe and push half of the bioink to the empty syringe. Disconnect the two syringes and pipette the cell suspension into one of the syringes very gently while pulling on the plunger to create room for the cell suspension in the syringe. Remove any air introduced into the syringe and connect the two syringes again. Gently mix back and forth between the syringes until the mixture is homogeneous. If detecting any air bubbles during mixing, disconnect the syringes and evacuate the air. Mix until homogeneous. The number of mixing cycles will depend on the cell type and bioink volume, optimize depending on application.
- If printing using the Pneumatic Printhead, transfer the mixture to an empty 3cc cartridge by connecting the syringe to the cartridge using the Luer lock adaptor.
- If printing using the Syringe Pump Printhead, make sure that the mixture is in a 3 mL syringe compatible with the Syringe Pump Printhead.

Notes:

- To avoid introducing air when connecting the syringes, carefully pre-fill approximately half of the Luer lock adaptor with CELLINK Vivoink before attaching it to a second syringe.

- The number of mixing cycles required for a homogeneous mixture need to be optimized according to application but as a starting point if mixing 1 mL bioink; 15 mixing cycles are often required to get a homogeneous distribution throughout the full volume while more than 30 cycles can start decreasing the cell viability.
- The maximum filling level of the 3 mL syringes which fits in the Syringe Pump Printhead is 2.7 mL, while the cartridge for printing using the Pneumatic Printhead can be slightly overfilled to 3.4 mL to fit the full volume of 3 mL bioink + 0.33 mL cell suspension.

3. Preparation for bioprinting

MATERIAL

CELLINK Vivoink mixed with cells in a cartridge or syringe.

Conical bioprinting nozzles, 22-25G recommended.

DESCRIPTION

- Cap the cartridge or syringe with a printing nozzle of choice and place in the printhead. Connect the cartridge to the air adapter or connect the syringe plunger to the Syringe Pump Printhead.
- Bioprinting can be performed using both Pneumatic and Syringe Pump Printheads. However, for a higher resolution and reproducibility, it is recommended to use Syringe Pump Printhead. *Go to 4a for starting parameters using the Pneumatic Printhead or go to 4b is using the Syringe Pump Printhead.*

Note:

- It is not recommended to use a nozzle with a smaller diameter than 25G (such as 27G) due to the risk of clogging.

4a. Bioprinting using the Pneumatic Printhead

MATERIAL

BIO X or BIO X6

Well plate or Petri dish

DESCRIPTION

- Perform autobed leveling and the calibration of the nozzle to the substrate before testing the flow of the bioink to avoid drying of the bioink at the nozzle tip during these procedures.
- Before starting the print, test the flow of the bioink using the Test Flow bottom with the recommended starting parameters in Table 1 or even lower pressure to avoid losing material during the nozzle priming procedure. Increase the pressure stepwise until a good flow is detected.

Notes:

- If waiting too long (approximately 8-10 min) between extrusions, the bioink can dry in the nozzle causing it to clog. However, this time could vary depending on environmental conditions such as humidity and temperature. If this occurs, replace the clogged nozzle with a new nozzle.
- If printing more than three layers, optimization of the construct design (infill density, infill pattern, size and layer height) is required.
- If printability is not as desired, adjust the pressure ±1 kPa to extrude more/less material or adjust the speed ±1-2 mm/sec.
- Additionally, pre- and post-flow delay functions can be used to adjust the printability.
    a) For instance, if you observe an accumulation of bioink at the starting point of each new filament, it could be because too much material is being pushed out before printing begins. To solve this problem, use the pre-flow delay function.

b) The post-flow delay function could be used when the filament is not complete due to the start of the next movement before the bioink is extruded.
c) Nevertheless, print speed, and bioink viscosity, can also influence extrusion consistency. So, it may be necessary to fine-tune multiple parameters to achieve the best results when bioprinting.

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 and nine parts bioink. For highly concentrated cell suspensions, the pressure needs to be increased towards the pressure used for undiluted bioink.

Printing speed (mm/s) → **Nozzle size (G) ↓**	**10**	**15**	**25**
22	7 / 9	9 / 12	13 / 14
25	11 / 12	13 / 14	15 / 17

** *This is only a recommended reference for 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 23°C and with/without a 9+1 bioink dilution with cell suspension.*

4b. Bioprinting using the Syringe Pump Printhead

MATERIAL

BIO X or BIO X6

Well plate or Petri dish

DESCRIPTION

- Perform autobed leveling and the calibration of the nozzle to the substrate before testing the flow of the bioink to avoid drying of the bioink at the nozzle tip during these procedures.
- Before starting the print, test the flow of the bioink using the Test Flow bottom with the recommended starting parameters in Table 2 or even lower extrusion rates to avoid losing material during the nozzle priming procedure. Increase the extrusion rate stepwise until a good flow is detected.

Notes:

- If waiting too long (approximately 8-10 min) between extrusions, the bioink can dry in the nozzle causing it to clog. However, this time could vary depending on environmental conditions such as humidity and temperature. If this occurs, replace the clogged nozzle with a new nozzle.
- If printing more than three layers, optimization of the construct design (infill density, infill pattern, size and layer height) is required.
- If printability is not as desired, adjust the extrusion rate by 0.1-0.2 µL/sec to extrude more/less material. Alternatively, change the speed by ±1 mm/sec.
- It is not recommended to use an extrusion rate of less than 1.3 µL/sec for printing of continuous filaments.

Table 2. Recommended minimal extrusion rate*** (±0.1 µL/sec) 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 and nine parts bioink. For highly concentrated cell suspensions, the extrusion rate needs to be increased towards the extrusion rate used for undiluted bioink.

Nozzle size (G)	**Extrusion rate (µL/sec)**	**Retraction volume (µL)**	**Printing speed (mm/sec)**
22	1.4 / 1.5	5 / 5	18 / 18
25	1.4 / 1.5	5 / 5	14 / 14

*** *This is only a recommended reference for starting extrusion rate, retraction volume and printing speed. This table was generated with a bioink temperature of 23°C and with/without a 9+1 bioink dilution with cell suspension.*

5. Crosslinking

MATERIAL

$CaCl_2$ crosslinking agent

Cell culture medium

DESCRIPTION

CELLINK Vivoink is crosslinked using the $CaCl_2$ crosslinking agent.

- Submerge the cell-laden constructs in the $CaCl_2$ crosslinking agent for 30 seconds to 5 minutes depending on construct size, infill density, and desired construct stiffness.
- Remove the $CaCl_2$ crosslinking agent and rinse constructs with complete or basal culture media once.

Notes:

- 30 seconds crosslinking time is recommended for 10 µL droplets, while 5-10 minutes might be required for dense 1 cm^3 blocks. In addition, optimize the crosslinking time depending on the cell type.
- Longer crosslinking times will generate a stiffer construct, but this initial stiffness will decrease with time as calcium levels in the construct equilibrate with the surrounding media, hence the final stiffness will depend on the calcium content in the medium. Culture medium should contain at least 1 mM of calcium for construct stability.
- After crosslinking the construct might detach from the substrate. Be mindful when removing liquid to not accidently aspirate or puncture the construct with the pipette tip.

6. Incubation

MATERIAL

Cell culture medium (with calcium supplement)

DESCRIPTION

- After crosslinking and washing, add the desired medium to the constructs and place them in an incubator.
- Incubate the constructs in a cell culture medium in standard culture conditions (37°C, 5% CO_2, and 95% relative humidity) or according to application. Replace the medium regularly.

Note:

- Culture medium as well as solutions for downstream analysis, washing or incubations should contain at least 1 mM of calcium for construct stability. Therefore, do not let the constructs stay in PBS, instead use a solution containing calcium, for example HBSS++ with calcium.