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We use this protocol and it's working

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Protocol for 3D bioprinting functional human brain tissues

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ABSTRACT

Detailed protocol for 3D bioprinting functional human brain tissues

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Reagents

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1. Thrombin (T7009, Sigma)
2. Fibrinogen (F3879, Sigma)
3. Aprotinin (A1153, Sigma)
4. CaCl2 (C7902, Sigma)
5. Deionized (DI) water
6. Dulbecco's phosphate buffered saline (DPBS) (14190144, ThermoFisher Scientific)
7. Transglutaminase (TG) from lyophilized Moo Glue powder (Modernist Pantry; ME, USA)
8. Hyaluronic acid (HA) (53747, Sigma)
9. Microneedle 30G (NZ5300505001, Cellink)
10. Micronozzle 27G (NZ3270005001, Cellink)
11. Sterile empty cartridges with end and tip caps, 3 mL (CSC010311101, Cellink)
12. DMEM/F12 (11330032, Thermo Fisher).
13. N-2 supplement 100X (17502048, Thermo Fisher).
14. MEM-NEAA (11140050, Gibco)
15. ROCK inhibitor Y27632 (72304, STEMCELL Tech.,).
16. 0.22µm Filter (SLGPR33RS, Millipore)
17. 30 µm CellTricsTM disposable filter (04-004-2326, Sysmex)

Equipment

2

1. CELLINK®, **INKREDIBLE+**TM (https://www.cellink.com/bioprinting/inkredible-3d-bioprinter/) and BIO XTM (https://www.cellink.com/bioprinting/bio-x-3d-bioprinter/)

2. SterilGARD® III Advance SG403 & SG603 biological safety cabinets (8038-30-1044, Baker)

Procedure Step 1: Preparation of bioink

4h

3 The bioink components were **prepared in advance** and stored for use as follows: 4h

1. 50 mg/mL Fibrinogen: Fibrinogen was dissolved in Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium for 04:00:00 at 37 °C. The solution was sterile-filtered with 0.22 µm filter and stored at -80 °C for use.

Tip: fibrinogen is very sensitive to temperature. Storage in -80 °C could keep it active for two months. Long-term preservation may cause clot formation.

2. 100 U Thrombin: was dissolved in DPBS and sterile-filtered with 0.22 µm filter. The solution was aliquoted and stored at -20 °C until use.

3. 10 mg/mL Aprotinin: aprotinin was dissolved in DPBS and stored at -20 °C until use.

4. 250 mM CaCl2: CaCl2 was dissolved in deionized (DI) water and filtered. The solution was stored at -4 °C until use.

5. 3% (w/v) Hyaluronic acid (HA): HA was prepared in DPBS at 80-90 °C until completely dissolved. The solution was kept at Room temperature.

Tip: Completely dissolving of HA is very difficult. It takes at least three hours.

6. 60 mg/mL Transglutaminase (TG): To prepare TG solution, lyophilized Moo Glue powder (Modernist Pantry; ME, USA) was dissolved in DPBS and stirring at 37 °C. The solution was filtered and stored at -20 °C until use.

7. **At the day of printing, prepare the crosslinking agent:**
The final working crosslinking solution contained 2.5 mM CaCl2, 1U thrombin and 0.2 % (w/v) of TG. The crosslinking was performed at 3-5 min at Room temperature. To avoid dehydration of gel, the crosslinking agent was added immediately after printing. The volume of the crosslinking solution was equal to the volume of bioinks.

Tip: the ideal working temperature is 37 °C. At Room temperature, it takes longer time for the gelation of fibrin gel.

8. **At the day of printing, prepare the gel mixed with fibrinogen and HA:**

a. Take fibrinogen from -80 °C and check whether clot formed. If clot formed, trash the fibrinogen and prepare fresh one.

b. Immediately add [M] 10 mg/mL aprotinin to avoid clot formation. The working concentration of aprotinin is [M] 0.5 mg/mL.

c. Dilute the fibrinogen solution with DMEM/F12 medium to [M] 15 mg/mL.

d. Then prepare the fibrinogen + HA solution with a volume ratio of 1: 2. The final concentrations of fibrinogen and thrombin in the fibrin gel constructs were [M] 2.5 mg/mL and 0.5 U.

Step 2: Preparation of cells

5m

4 9. When hPSC-NPCs grow to 3 weeks, start to prepare the cells for printing. 5m

Tip: The time is critical for printing. It is not better to use the cells older than 30 days. The best time range is 21-25 days. We tested the time of cells used for printing. The older cell used the less cell survived after printing.

10. Dissociate cells with accutase to make single cells of the NPCs.

11. The cells were then filtered with 30 μm filter to remove big cell clusters or aggregates.

Tip: Big cells cluster must be removed to avoid clogging of nozzle during printing.

12. Cells were centrifuged at 300 g for 00:05:00 to get cell pellet.

13. Cell pellet was mixed the above gel of fibrinogen + HA. The cell density for printing was 1 × 107/ mL of bioink.

Tip: Mix well cells with the gel to achieve even distribution of cells after printing. Avoid the generation of bubbles during mixing, because bubbles may reduce cell viability during printing. The volume of cells and gel mixture was dependent on the demanding of experiments. It suggests preparing at least 0.5 mL of the mixture in the cartridge, because of the existence of dead volume.

14. The mixture was transferred to 3 mL sterile cartridge for printing.

Tip: Make sure label clearly the cell types and which printhead will be chosen if using more than one printheads.

15. Cover the end and tip cap to avoid contamination.

Step 3: Printing preparation

5m

5

16. Before printing, autoclave any printing supplies, including nozzles, microneedles, cartridges, adaptors, pipettes, pipettes tips, waste beakers, etc. 5m

17. Place the Bio X printer in a sterile biosafety cabinet (BSC). Turn on the BSC.

18. Spray and wipe the printer and BSC with 70% ethanol to decontaminate.

Tip: Decontaminate inside and outside of the printer, including the each printheads and work station.

19. Plug in the power cord of the printer and turn on the machine.

20. Turn on the UV light to sterilize the printer chamber.
a. Select the “Clean chamber” in main menu on the touchscreen of Bio X.
b. Turn on “Clean chamber fan”
c. Click “Start” to perform UV sterilization.

Tip: The whole sterilization takes 00:05:00 .

21. After the UV sterilization is done, click the home button and return to the main menu.

Step 4: Printer Set-up

6

Loading printhead and cartridge

22. Insert the USB drive with the G.code file for the printing construct design

Tip: In the G.code file, the printheads would be chosen when writing the code. So, no need to choose printhead when setting the printer.

23. Remove the cap and end of the cartridge

24. Put the sterile 30G microneedle/nozzle on the end of the cartridge

Tip: Make sure the needle/nozzle tip is not bended or blocked.

25. Load the cartridge into the printhead
a. Connect the pressure hose on the top of cartridge
b. Insert the cartridge into the printhead

Tip: Make sure the cartridge is completely inserted. Don't push down the cartridge too hard, the printhead may get off. If the cartridge is loaded correctly, the blue light will be on behind the printhead. Otherwise, re-load the cartridge and printhead.

6.1 Printing parameters set-up

26. Select "Bioprint" in the main menu of the printer.

27. Choose the desired model from the "3D model" tab.

Tip: From pre-designed model, choose the desired G.code file on the left side of the screen. The G.code file from the USB drive shows in this tab. To preview the construct, click "Preview" on the right side of the screen. The printing construct will show.

28. Select the print surface.
a. In this study, choose well plate. There are other surfaces options like petri dish and glass slide.
b. Select the vendor to "Coning, COSTAR®", and set the number of wells to 24. And put the prepared 24 well plate on the work station of the printer.

Tip: The types of surface should be decided when design the printing model. We printed cells onto poly-ornithine coated coverslips in the well of the plate.

29. Set-up printhead parameters
In the "printer setup" tab, choose the printheads that will be used. In this study, we used printhead1 and printhead2. In the G.code file, the printheads were chosen. So the printheads was automatical on.
a. Click "Enable Printhead 1".
b. Select Bioink profile to "CELLINK START"
c. Set Printhead type to "Pneumatic 3mL" and make sure the Photo curable is off.
d. Set-up printing pressure, speed and temperature. In this study, we set printing pressure of "100 kPa", speed of "5 mm/s" and temperature of "25 °C".
e. Repeat steps a-d for printhead 2.

30. Set-up the layer properties in the "Layers" tab. In this study, the layer properties were set in G.code file.

31. Overview the printing parameters set-up.

Tip: In the "Overview" tab, the parameters could be previewed. Click "Test bioink flow" to test the printing.

6.2 Printing calibration

32. Click “Calibrate” at the bottom of the screen.

Tip: After clicking the button, the machine automatically calibrate to place the printhead 1 in the center of work station.

33. After the automatic calibration, manually calibrate each printhead.
a. Under “Printhead 1” menu, adjust the height and distance of the needle/nozzle from the printing surface using the left/right and up/down buttons.

Tip: This is a critical step. For 24-well plate, the printing starts from the first well at the very left-down corner. It is better to mark the center of the first starting well. There are three options for the adjustment 10, 1 and 0.1 mm. Start 10 mm first for large adjustment and choose 0.1 mm when the neezle/nozzle tip closes to the printing surface. It is suggested to calibrate the tip about 0.1 mm above the surface.

b. Press “Calibrate” button and start to calibrate the printhead 1.

34. Repeat the step 33 for calibration of printhead 2.

Step 5: Printing

7

35. Click “Print” to start printing.

36. When the printing of the first well is done, immediately add 50 µL crosslink reagents.

Tip: Carefully add the reagent and avoid destroying the printed construct. Make sure the liquid all cover the construct to prevent the dehydration of the construct.

37. Repeat step 36 for each well until the whole printing completes.

Tip: When the needle/nozzle is clogged, immediately pause the printing process. Replace the needle/nozzle to check whether the clogging issue is solved or not. If not, the problem may come from the bioink and cells.

Step 6: Post-printing culture

30m

8

38. After printing, place the plate in 37 °C incubator for 00:30:00 for gelation. 30m

39. **After 30 mi**n, add 1 mL fresh neural differentiation medium each well.

Tip: Carefully add the medium and avoid destroying the printed construct.

40. Re-place the plate in the incubator for long-term culture.

41. Chang the medium every 2-3 days.