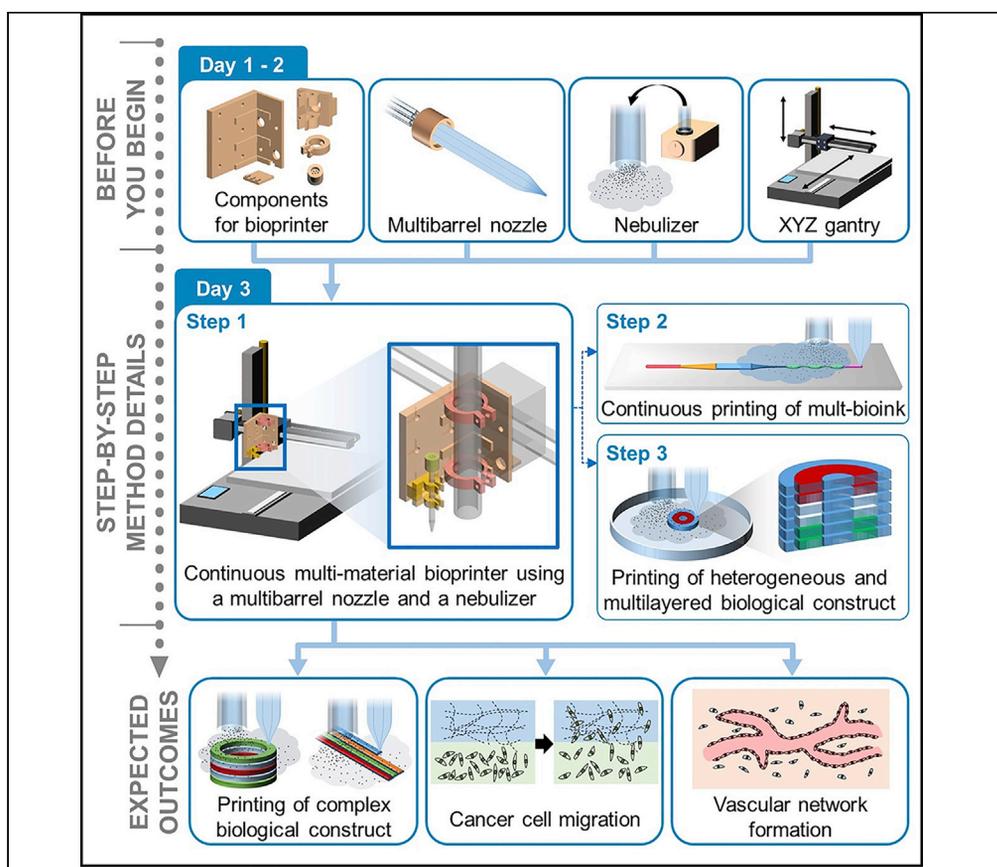# STAR Protocols

Protocol

Bioprinting of heterogeneous and multilayered cell-hydrogel constructs using continuous multi-material printing and aerosol-based crosslinking

Gihyun Lee, Soo Jee Kim, Je-Kyun Park

gihyunlee@kaist.ac.kr (G.L.)
jekyun@kaist.ac.kr (J.-K.P.)

Highlights

Set up a continuous multi-bioink printer to print a complex biological construct

Printing procedures of multilayered and heterogeneous constructs

Form a tumor microenvironment with four bioinks containing three cell types

Analyze cancer cell migration and vascular network formation

Bioprinting is a powerful biofabrication technique that mimics physiological environments and functions. Here, we describe a protocol to set up a continuous multiple-material bioprinting system that can replicate structurally complex and biologically functional microphysiological systems such as a tumor microenvironment. Although this bioprinting system uses a limited crosslinking agent, it is a versatile and advanced continuous multi-material printing technique.

Protocol

Bioprinting of heterogeneous and multilayered cell-hydrogel constructs using continuous multi-material printing and aerosol-based crosslinking

Gihyun Lee,[1,3,*] Soo Jee Kim,[1] and Je-Kyun Park[1,2,4,*]

[1]Department of Bio and Brain Engineering, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon 34141, Republic of Korea

[2]KAIST Institute for Health Science and Technology, 291 Daehak-ro, Yuseong-gu, Daejeon 34141, Republic of Korea

[3]Technical contact

[4]Lead contact

*Correspondence: gihyunlee@kaist.ac.kr (G.L.), jekyun@kaist.ac.kr (J.-K.P.)
https://doi.org/10.1016/j.xpro.2022.101303

SUMMARY

Bioprinting is a powerful biofabrication technique that mimics physiological environments and functions. Here, we describe a protocol to set up a continuous multiple-material bioprinting system that can replicate structurally complex and biologically functional microphysiological systems such as a tumor microenvironment. Although this bioprinting system uses a limited crosslinking agent, it is a versatile and advanced continuous multi-material printing technique.
For complete details on the use and execution of this protocol, please refer to Lee et al. (2021).

BEFORE YOU BEGIN

This protocol describes how to set up a continuous multi-material printing system and how to prepare the components and materials before printing a complex biological construct consisting of various biomaterials including different types of cells.

Preparation of bioprinting system components

⊙ Timing: 1 day

1. Order a commercial fused deposition modeling (FDM) 3D printer, a nebulizer, and multibarrel glass capillaries.
2. Prepare components of the printing head (Figure 1A).
   a. Download the component files (use STL files of supplemental information).
   b. Print the component files using a digital light processing (DLP) 3D printer to fabricate a printing head, a stopper, a nozzle adapter, a printing nozzle holder, and aerosol tube holders.
3. Prepare stainless steel tubes, Tygon tubes, and polyvinyl chloride (PVC) tubes for the supply of materials such as bioinks and aerosol.
4. To print biomaterials including cells, prepare a biological safety cabinet (BSC) and sterilize all components except for the multibarrel glass capillary.
   a. BSC: sterilize with 70% ethanol and UV treatment.
   b. Gantry, nebulizer, and polymer tubes: sterilize with 70% ethanol and UV treatment.
   c. Printed components and stainless steel tubes: sterilize with an autoclave at 121°C.
5. Dry the sterilized components in an oven at 80°C and put them inside the BSC.
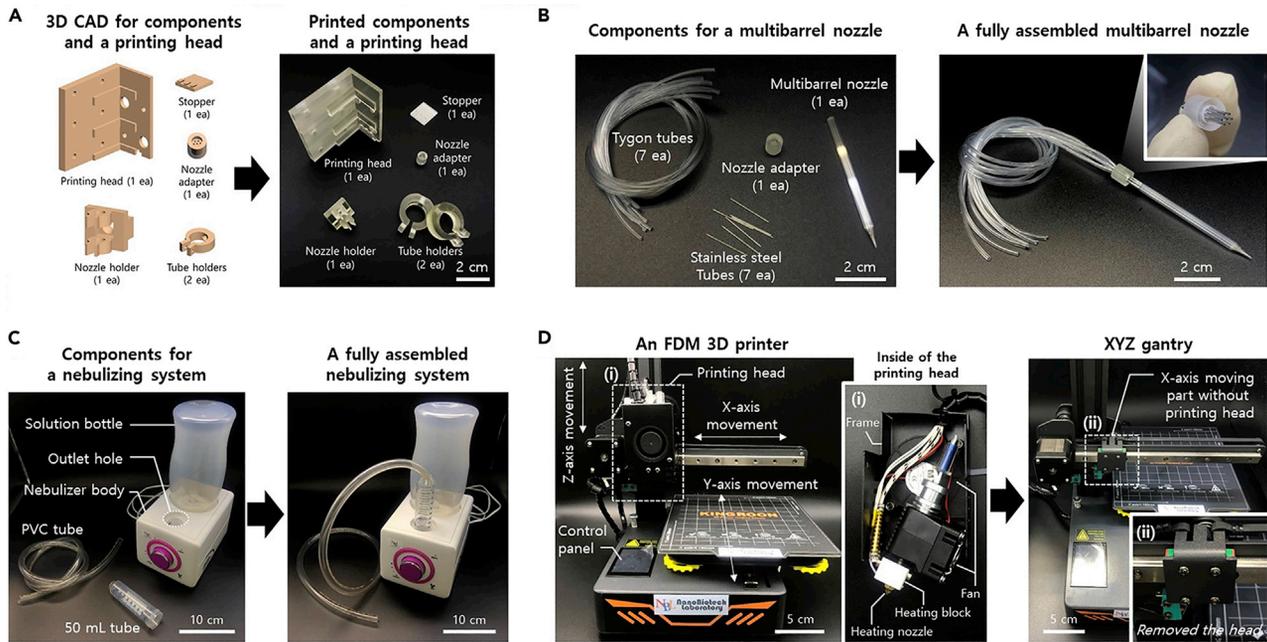
Figure 1. Preparation for continuous multi-material printing system setup
(A) Constructs required for a bioprinting system.
(B) A multibarrel nozzle.
(C) A nebulizer.
(D) A gantry.

Note: Here we used two different types of 3D printers. FDM was used as a gantry for a bio-printer and DLP was used to make the bioprinting components such as nozzle holder, tube holders, printing head, and stopper.

Multibarrel nozzle processing

⏱ Timing: 6 h

The goal of this step is to process a multibarrel glass capillary to create a printing nozzle that can continuously extrude multiple bioinks. To prepare the multibarrel nozzle of target diameter (here we targeted 400 μm), the glass capillary (inner diameter of 0.68 mm and outer diameter of 1.2 mm) was thermally and mechanically processed by a pulling machine and a microgrinder. The processed nozzle was sterilized for the printing of biological samples and assembled with the prepared printing head and adapter (Figures 1B and 2).

6. Pull a multibarrel glass capillary (Figure 2(ii)).
   a. Set the pulling conditions;
      i.   Heating level: 85.
      ii. Sub magnet level: 24.
      iii. Main magnet level: 70.
   b. Assemble and secure the multibarrel glass capillary on a holding chuck.
   c. Turn on the heat and initiate pulling.
   d. Uninstall and collect the thermally processed capillaries.

Optional: The shape of the nozzle tip affects the shear stress which occurs when extruding the bioinks. The end-users can adjust the resulting shape of the nozzle tip by controlling the magnet level and heating level of the pulling machine. The nozzle pulled at a low magnet level will
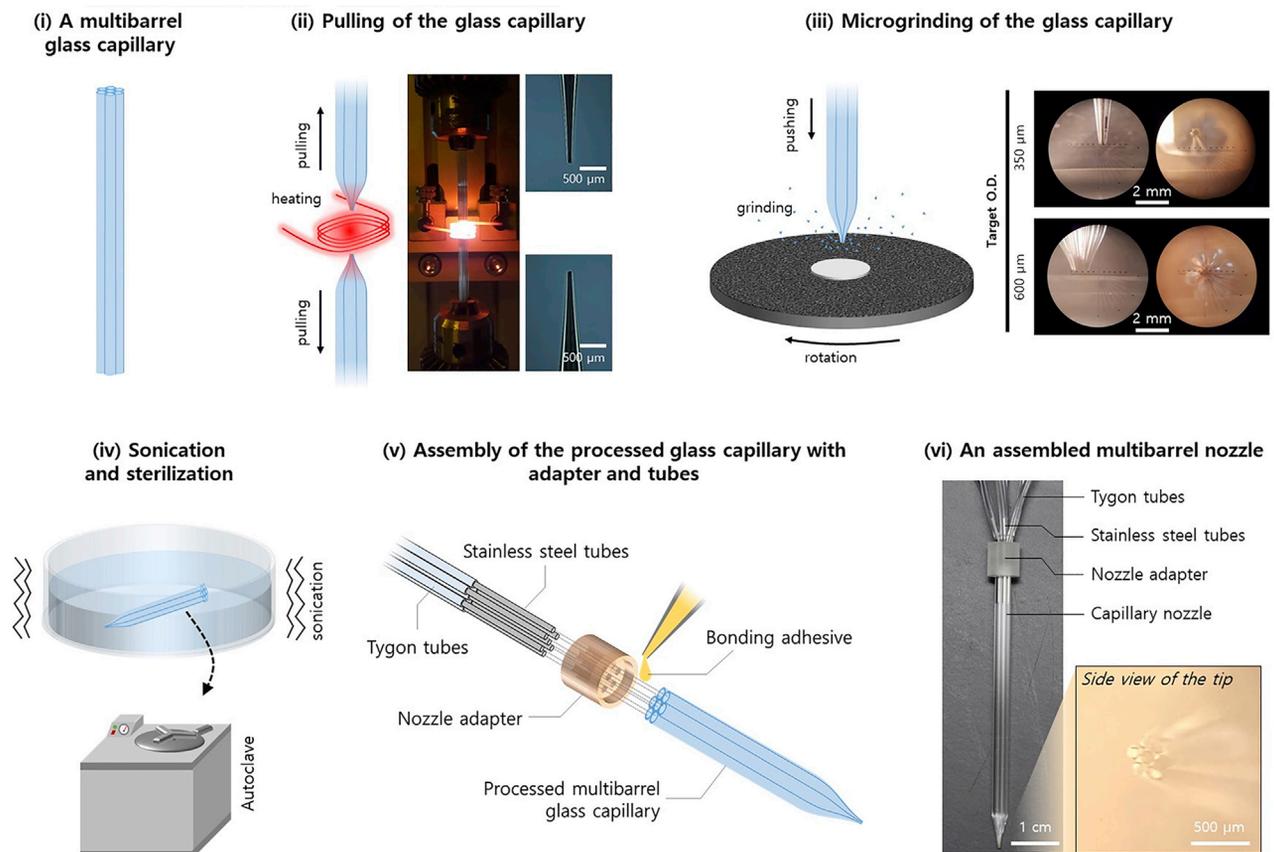
Figure 2. Procedures for preparing a multibarrel nozzle from thermal and mechanical processing to assembly

be a conical shape, while at a high magnet level it will be a sharp narrowed shape. The heating level affects the length of the nozzle tip.

Note: Wear gloves and goggles for protection from burns and visual damage caused by heat and light emissions during the capillary pulling process, as well as cuts and punctures by the sharp nozzle tip and glass fragments from the pulled glass capillary.

7. Microgrind the pulled glass capillary (Figure 2(iii)).
   a. Switch ON the microgrinder and set grinding conditions.
      i. Maximum speed (approximately 2,000 rpm).
      ii. Light ON.
   b. Set the pulled glass capillary on the XYZ controller and tighten the holder.
   c. Position the capillary tip vertically on the microgrinder and grind the tip by lowering the controller in the Z-axis.
   d. During the grinding of the nozzle, intermittently measure the nozzle tip using the scale bar in the microscope lens to obtain a nozzle tip with the desired dimensions.
   e. Uninstall and collect the mechanically processed capillary.

Optional: In this study, we prepared the multibarrel nozzles with a diameter of 400 μm. The end-users can adjust the resulting diameter of the nozzle by controlling its position in the Z-axis.

Note: Wear gloves and goggles to prevent cuts and punctures caused by the sharp nozzle tip and glass fragments from the ground glass capillary.

8. Clean the processed multibarrel capillary (Figure 2(iv)).
   a. Air blow the processed multibarrel capillary to remove glass particles and dust.
   b. Wash with 70% ethanol solution and rinse with distilled water (DW).
   c. Sonicate the nozzle while immersed in a 70% ethanol solution in a 15 mL Tube (30 min).
   d. Rinse the nozzle with DW.
   e. In the case of sterilization of the nozzle for the printing of biological samples, autoclave the nozzle at 121°C while immersed in DW in autoclavable labware (1 h).
   f. Dry in an oven at 80°C.
9. Assemble the sterilized multibarrel capillary (Figure 2(v) and Methods video S1).
   a. Bring the adapter inserted with seven stainless steel tubes and the multibarrel capillary to a BSC.
   b. Put a drop of a biocompatible adhesive on the side of the adapter that connects to the glass capillary (be careful not to clog the tube holes with the adhesive).
   c. Immediately, insert the seven stainless steel tubes of the adapter into each barrel of the multibarrel capillary.
   d. Let the adhesive cure.

   Note: As an adhesive, we recommend using sterilizable materials such as polydimethylsiloxane when assembling the nozzle adapter with the processed glass capillary. Beware of fracturing the glass capillary when inserting the stainless steel tubes. Wear gloves and goggles to prevent cuts and punctures caused by the sharp nozzle tip and glass fragments of the ground glass capillary.

Preparation of nebulizing system components

⏱ Timing: 3 h

The goal of this step is to generate an aerosol supply system for the printing bed by connecting a tube to a nebulizer. The aerosol flow rate can be controlled by changing the electrical resistor on the circuit and by adjusting the amount of solution inside the nebulizer (Figure 1C).

10. Order a commercial nebulizer.
11. Connect an aerosol tube from the nebulizer to the printing head to supply the crosslinking agent (Methods video S1).
    a. Cut the closed conical end of a 50 mL conical tube to insert a PVC tube (inner diameter of 9.5 mm and outer diameter of 12.7 mm).
    b. Plug the open end of the 50 mL conical tube into the aerosol supplying hole of the nebulizer.
    c. Insert the PVC tube into the hole cut in the 50 mL tube.

    Optional: To supply less aerosol, change a variable resistor on the nebulizer circuit to a smaller resistance element. Alternatively, keep the water level lower in the solution reservoir for generating less aerosol.

    Note: The nebulizer was sterilized with 70% ethanol and exposed to UV inside a BSC for 6 h. The crosslinking solution was filtered with a membrane of 0.22 μm pore. The sterilized components were handled inside BSC.

Preparation of 3D printer and modification of the printing head

⏱ Timing: 3 h

The goal of this step is to prepare a gantry machine for precisely controlling the movement of the printing head, and modify the printing head to assemble a multibarrel nozzle and an aerosol tube (Figure 1D).

12. Order a commercial FDM 3D printer.
13. Assemble the printer parts following a guideline provided by the company.
14. Turn on the printer and run a leveling in the menu on a touch screen for calibration.
15. After calibration, print a GCode sample file to test if the printer is well operated or not.
16. If the printer was operated well, remove all components and cables of extruder excepting an X-axis moving part connected with a timing belt (Figure 1D and Methods video S1).

> *Optional:* When choosing the 3D printer, check the specification of the printable maximum volume to print the desired model.

Maintenance of cancer cells, vascular endothelial cells, and fibroblasts

⏱ **Timing: 4–7 days**

The goal of this step is to maintain the three different types of cells to make the bioinks for printing a complex tumor microenvironment (TME) construct.

17. Order and thaw the cell stocks following the guideline provided by the company.
18. Culture the cells using a specific growth medium.
    a. Green fluorescent protein (GFP)-transfected breast cancer cells, GFP MDA-MB-231: Dulbecco's Modified Eagle Medium (DMEM), including 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin.
    b. Red fluorescent protein (RFP)-transfected primary human umbilical vein endothelial cells (HUVECs), RFP HUVECs: endothelial growth medium-2 (EGM-2).
    c. Normal human lung fibroblast (NHLF): fibroblast growth medium-2 (FGM-2).
19. Exchange the old medium to the new growth medium every 3–4 days of culture.
20. Subculture the cells when confluency is reached over 90%.
21. Match the passage of the cells and use it as a bioink.
    a. GFP MDA-MB-231: not significant.
    b. RFP HUVECs: passage 3–5.
    c. NHLF: passage 3–7.

> *Note:* The laboratory should be enrolled as an LMO facility for the use of the GFP and RFP transduced cells.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD31, diluted to 1/100 with buffer solution	Abcam	Cat#Ab215911
Chemicals, peptides, and recombinant proteins		
Dulbecco's Modified Eagle Medium (DMEM)	Corning	Cat#10-017-CV
Endothelial growth medium-2 (EGM-2)	LONZA	Cat#CC-3162
Fibroblast growth medium-2 (FGM-2)	LONZA	Cat#CC-3132
Penicillin–Streptomycin	Gibco	Cat#15140122

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fetal bovine serum (FBS)	Corning	Cat#35-015-CV
Phosphate-buffered saline (PBS)	Corning	Cat#21-040-CVC
Dulbecco's phosphate-buffered saline (DPBS)	LONZA	Cat#17-513F
Alginate	Alfa Aesar	Cat#B25266
Alginate RGD (NOVATACH MVG GRGDSP)	NOVAMATRIX	Cat#4270501
Collagen type I, Rat tail	Corning	Cat#354236
Fibrinogen, from bovine plasma	Sigma-Aldrich	Cat#F8630-1G
Thrombin, high purity grade, ≥1,500 NIH Units/mg protein, from bovine plasma	MPBio	Cat#154163
$CaCl_2$	Sigma-Aldrich	Cat#C7902-500G
Experimental models: Cell lines		
Human umbilical vein endothelial cell (HUVEC), used passage 3–5 for each experimental model	LONZA	Cat#C2519A
Normal human lung fibroblast (NHLF), used passage 3–7 for each experimental model	LONZA	Cat#CC-2512
MDA-MB-231	Korean Cell Line Bank	Cat#30026
Red fluorescent protein (RFP) HUVEC, used passage 3–5 for each experimental model	ANGIO-PROTEOMIE	Cat#cAP-0001RFP
Green fluorescent protein (GFP) MDA-MB-231	ANGIO-PROTEOMIE	Cat#cAP-0034GFP
Software and algorithms		
ImageJ	Schneider et al. (2012)	https://imagej.nih.gov/ij/
Fluigent All-in-One Software	Fluigent	A-I-O Software https://www.fluigent.com/product/microfluidic-components/fluigent-a-i-o-software/
Supplemental Data_GCODE files (GCode for continuous printing a line model with seven-different materials, GCode for continuous printing a heterogeneous and multilayered construct)	Mendeley data	https://doi.org/10.17632/6bycgdd9tp.1
Other		
Continuous multibioink printing system with aerosol-based crosslinking technique	Lee et al. (2021)	https://doi.org/10.1088/1758-5090/ac25ca
Pneumatic pumps	Fluigent	MFCS-EZ
Multibarrel glass capillary	WPI	Cat#7B120F-4
Capillary puller	Narishige	PE-22
Microgrinder	Narishige	EG-401
Fused deposition modeling (FDM) 3D printer	Kingroon	KP3S
Digital light processing (DLP) 3D printer	ASIGA	PICO2 HD
Supplemental Data_STL files (Printing head/ Nozzle adapter/ Nozzle holder/ Aerosol tube holder)	Mendeley data	https://doi.org/10.17632/6bycgdd9tp.1
Metal tubes (I.D./O.D. 0.3/0.51 mm)	EST	PN25G-2/1 http://www.i-est.co.kr/shop/item.php?it_id=1517469788
Tygon tub[es (I.D./O.D. 0.5/1.5 mm)	SAINT-GOBAIN PPL Corp.	Cat#AAD04103
PVC tubes (I.D./O.D. 9.5/12.7 mm)	SAINT-GOBAIN PPL Corp.	Cat#ACF00027

Alternatives: To print multiple bioinks, software that can precisely control multiple pneumatic pumps is essential. In this protocol, we controlled the Fluigent pneumatic pumps (MFCS-EZ) on the Fluigent All-in-One Software (A-I-O Software). If the end-user has already a pumping system in the laboratory, the established pumping system can be used for the experiments. However, if not, we recommend purchasing a pumping system from specialized companies such as Fluigent. In addition, if you are considering purchasing the A-I-O software we used in the experiments, we recommend the latest version, OxyGEN (https://www.fluigent.com/research/software-solutions/oxygen/), because the A-I-O software has been discontinued.

MATERIALS AND EQUIPMENT

Growth medium for breast cancer cell line MDA-MB-231		
Reagents	Final concentration	Amount
DMEM	N/A	500 mL
FBS	10%	50 mL
Penicillin–Streptomycin	1%	5 mL
Total	N/A	555 mL

Storage in refrigerator.

Bioink for cancer layer		
Resources	Final concentration	Amount
Alginate RGD	1%	0.5 mL
Collagen type I	1.5 mg/mL	0.5 mL
GFP MDA-MB-231	3×10^6 cells/mL	N/A
Total	N/A	1 mL

Use immediately to print after preparation.

Bioink for vascular layer		
Resources	Final concentration	Amount
Alginate RGD	0.5%	0.5 mL
Fibrinogen	2.5 mg/mL	0.5 mL
RFP HUVECs	5×10^6 cells/mL	N/A
NHLF	1×10^6 cells/mL	N/A
Total	N/A	555 mL

Use immediately to print after preparation.

Bioink for migration layer		
Resources	Final concentration	Amount
Alginate RGD	1%	0.5 mL
Collagen type I	1.5 mg/mL	0.5 mL
NHLF	2×10^6 cells/mL	N/A
Total	N/A	1 mL

Use immediately to print after preparation.

Bioink for supporting structure		
Resources	Final concentration	Amount
Alginate	8%	1 mL
NHLF	2×10^6 cells/mL	N/A
Total	N/A	1 mL

Use immediately to print after preparation.

Alternatives: The authors have optimized the bioink composition for printing a tumor micro-environment construct. The four bioinks for the cancer layer, vascular layer, migration layer, and supporting structure were listed up. If the end-user found an optimal composition of bioinks to mimic the target model, use the established bioinks instead of the above composition we suggested. In addition, when alternating the bioinks, the end-user also has to change the
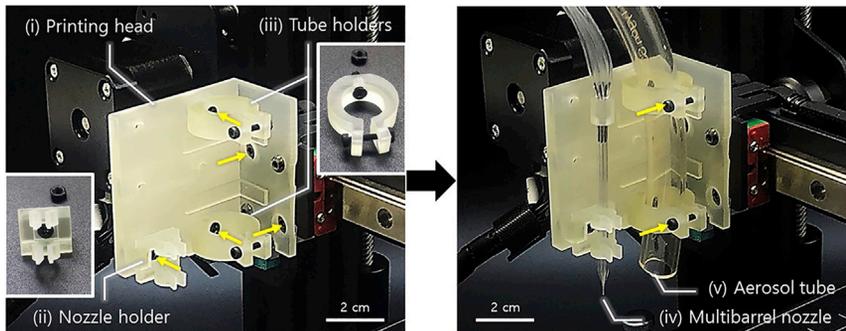
Figure 3. The continuous multi-material bioprinting system consisting of a multibarrel nozzle and a crosslinking aerosol tube
Yellow arrows indicate the tightening parts using screws.

crosslinking solution because the aerosol supplied from the bioprinting system in this paper was based on an ionic crosslinking mechanism.

STEP-BY-STEP METHOD DETAILS

Installation of the multibarrel nozzle and crosslinking aerosol tube on the printing head

🕐 Timing: 1 h

The role of this step is to assemble the multibarrel nozzle (Section of 'multibarrel nozzle processing' in Before you begin) and aerosol tube (Section of 'preparation of nebulizing system components' in Before you begin) using the 3D printed components (Section of 'preparation of bioprinting system components' in Before you begin) on the modified printing head (Section of 'preparation of 3D printer and modification of the printing head' in Before you begin) as shown in Figure 3 and Methods video S2.

1. Assemble the stopper into the groove on the back of the printing head.
2. Install the printing head in the gantry, and then assemble the prepared holders for the multibarrel nozzle holder and aerosol tube on the printing head (troubleshooting 1).
3. Connect the Tygon tubes from the pneumatic pump to the metal tubes of the nozzle adapter, and then insert the fully assembled nozzle into the holder of the printing head.
4. Connect the PVC tube with a nebulizer, and then insert the aerosol tube into the holder of the printing head.

   Optional: End-user can change the length of the nozzle-pump tube and aerosol tube. However, the range for movement of the printing head should be considered to avoid the tension of the tubes. All components were sterilized and handled inside BSC.

   ⏸️ **Pause point:** After the installation of the bioprinting system, the end-users can pause the protocol. Before printing with the biomaterials in the next steps, we recommend preparing the bioinks in advance and then using it to print immediately.

Continuous printing of multiple bioinks under crosslinking aerosol

🕐 Timing: 2 h

The role of this step is to print the multiple bioinks and confirm the continuous multibioink printing system. Here, the authors printed the seven-different colored alginate hydrogels under optimal printing conditions with crosslinking aerosol.

5. Prepare the seven-different colored 4% alginate inks by mixing food dye (pure alginate, red-, yellow-, green-, sky blue-, deep blue-, brown-colored alginates) and connect them to the pumping system via the Tygon reservoir and the tube.

6. Prepare the 2% and 10% $CaCl_2$ solution and pour into the Petri dish (for complete gelation of the alginate construct after printing) and the nebulizer (for retaining alginate structure by fast gelation during printing), respectively.

7. Fill the bioinks into the multibarrel nozzle by applying a pneumatic pressure of 2,000 mbar.

8. After checking the extruded bioinks through the nozzle tip, turn off the pump to stop extrusion.

9. Prepare the GCode for continuous printing and the pressure control program.

10. Before starting a printing process, turn on the nebulizer to supply 10% $CaCl_2$ aerosol (troubleshooting 2).

11. Start the pumping program and the GCode for continuous extrusion at the same time (see Figure 1B and supplemental data in our original report, Lee et al., 2021).

12. After the end of printing, immediately dip the printed sample into the crosslinking solution for 3 min for fully crosslinking of the printed hydrogels (troubleshooting 3).

13. After the crosslinking is complete, wash the printed sample with Dulbecco's phosphate-buffered saline (DPBS) three times.

14. Observe the sample on the microscope to optimize the printing conditions. Here we applied a pressure within the range from 200 mbar to 800 mbar and a speed of 100 mm/min as the optimal condition for printing seven-different colored alginate inks.

Printing of heterogeneous and multilayered cell-hydrogel construct

⊙ Timing: 30 min

The role of this step is to continuously print the complex biological construct such as the heterogeneous and multilayered cell-hydrogel construct under crosslinking aerosol. Here, to demonstrate a TME, we use the four bioinks, including cancer cells, vascular endothelial cells, and stromal cells. Each bioink consisted of the optimal composition of hydrogel blend and cells for observing cancer cell migration or vascular network formation. If the end-user wants to mimic a specific physiological microenvironment, we recommend firstly finding the optimal composition of bioinks and then using the bioink for printing (see Figure 6 in our original report, Lee et al., 2021).

15. Bring the four bioinks (cancer bioink, vascular bioink, fibroblast bioink, and supporting bioink) and connect them to the pumping system via the Tygon reservoir and the tube.

16. Fill the bioinks into the multibarrel nozzle by applying a pneumatic pressure of 2,000 mbar and a speed of 100 mm/min.

17. After checking the extruded bioinks through the nozzle tip, turn off the pump to stop extruding.

18. Before starting a printing process, turn on the nebulizer to supply 10% $CaCl_2$ aerosol (troubleshooting 2).

19. Start the pumping program and print the GCode for the TME construct at the same time.

20. After the end of printing, immediately harvest the printed sample and dip it into the crosslinking solution for 3 min for fully crosslinking of the printed TME construct (troubleshooting 3).

21. After the crosslinking is complete, wash the printed TME construct with DPBS three times.

22. Dip the TME construct into the EGM-2 culture medium.

23. Check the heterogeneous multilayered TME construct by observing the green fluorescent. cancer cells (GFP MDA-MB-231) in the bottom layer and red fluorescent vascular endothelial cells (RFP HUVECs) in the top layer on the microscope or confocal microscope over the culture time (The multilayered and heterogeneous TME construct should be compartmentalized with well-defined interfaces as shown in Figures 6B–6D in our original report, Lee et al., 2021).

24. For immunostaining of the tight junction CD-31 of HUVECs, see the protocol of Lee et al. (2021). After the fixation process of a TME construct, the CD-31 antibody which was diluted to 1/100 with phosphate-buffered saline (PBS) was treated with the fixed TME construct and incubated at 4°C for 24 h (troubleshooting 4).

25. Analyze the morphological characteristics of cancer cells and vascular endothelial cells tri-cultured in the printed TME (troubleshooting 5).

Note: If the end-user already has the verified protocol, we recommend following your protocol. All components were sterilized and handled inside BSC.

EXPECTED OUTCOMES

The established bioprinting system can continuously print the multiple bioinks via the multibarrel nozzle and immediately gelate by supplying the crosslinking aerosol during the printing process. The multibarrel nozzle can continuously extrude up to seven bioinks that have different rheological properties via one nozzle by individually controlling the applied pressure. The crosslinking aerosol can improve printability by gelating the biomaterials before the extruded bioinks spread on the substrate. Thus, the integration of the multiple-biomaterial printing technique and aerosol-based crosslinking technique satisfy as an effective and precise biofabrication approach for replicating a complex physiological microenvironment consisting of various biomaterials and different types of cells.

In this paper, we described the protocols from the setup of the bioprinting system to the printing of complex biological constructs. The expected outcomes from the established bioprinting system are versatile and effective printing performances that include continuous printing using seven different materials (Figure 1 in our original report, Lee et al., 2021) and improvement of printability (Figure 2 in our original report, Lee et al., 2021). Furthermore, the complex hydrogel construct such as the heterogeneous pattern and multilayer construct can be printed (Figures 3–5 in our original report, Lee et al., 2021). Using four bioinks, including different types of cells (breast cancer cells, vascular endothelial cells, and fibroblasts), a complex tumor microenvironment construct can be demonstrated. The morphological change and cellular behaviors of cancer cells and vascular cells cultured in the printed TME construct can be monitored and analyzed on the fluorescent microscope and confocal microscope (Figure 6 in our original report, Lee et al., 2021). In the printed TME construct, the breast cancer cells start to spread around the hydrogel and move to the vascular layer. In the case of the vascular endothelial cells, they start to aggregate each other and self-assemble to form the vascular endothelial networks. We expect that the established multiple-bioink printing system will have countless applications in printing the complex physiological microenvironments compartmentalized with various compositions, properties, and materials.

LIMITATIONS

A multi-material bioprinting system integrated with an aerosol-based crosslinking technique has significant advantages in replicating a complex physiological microenvironment, such as tumor (Lee et al., 2021), skin (Lee et al., 2009a), and neural tissues (Lee et al., 2009b). However, there is a limitation in fabricating a high-aspect-ratio construct using bioinks that require enough time to gelate. In our original report (Lee et al., 2021), we verified three different crosslinking mechanisms using ionic-, enzymatic-, and pH-crosslinkers to gelate the printed alginate, fibrinogen, and collagen type I by supplying the specific aerosol, respectively. While the alginate-based bioink could be crosslinked immediately by a divalent cation such as Ca^{2+}, the low viscosity collagen- or fibrinogen-based bioinks required a sufficient gelation time. The fast crosslinking mechanism was advantageous to retain the structure of the extruded bioink, however, the slow gelation caused the bioink spreading issue during the printing process and the relatively lower reproducibility for printing a high-aspect-ratio construct. Although the natural biopolymers with the slow crosslinking mechanism

were difficult to print at such a high-aspect-ratio construct, the crosslinking aerosol could compartmentalize the biopolymers. When printing an alginate/fibrin/collagen interfacing model, we confirmed that the printed construct showed clear interfaces between the alginate/fibrin and fibrin/collagen by the crosslinking aerosol (Figure 4 in our original report, Lee et al., 2021). In order to retain constructs using low viscosity bioinks, a supportive microstructured bioprinting substrate that can prevent the spreading of the extruded materials by a capillary pinning effect can be applied (Kim et al., 2021).

TROUBLESHOOTING
Problem 1
Problems related to the assembly of the 3D printed components (step 2).

Potential solution
During the assembly of the printing head and additional 3D printed components, the screws may not fit exactly into the screw holes. The final dimensions of the 3D printed components may vary due to the differences between the end-user's and our fabricating conditions.

To print the STL files provided in this protocol, we used a DLP 3D printer (PICO2 HD, ASIGA). If the screws still do not fit properly into the screw holes even when using a printer of the same model, the end-user should set the Z-axis unit at a higher resolution, or reduce the UV exposure time. When using other equipment, the optimal printer settings should be explored.

In our protocol, the printing head and its related components were installed on the gantry from which the original printing head was removed. If the end-user is using a gantry of a different model, the location of the screw holes should be altered to fit the model in use.

Problem 2
Problems related to the crosslinking drops accumulated inside the nebulizing tube (steps 10 and 18).

Potential solution
If the nebulizer is operated for a long time, crosslinking drops accumulated inside the nebulizing tube may grow and flow down to the printing bed. If the crosslinking drop falls on the resulting construct during printing, the printing process is difficult to continue because the printed hydrogel is immersed by the crosslinking drop and may deform its structure. To prevent this problem, we recommend the crosslinking drops accumulated inside the nebulizing tube should be removed by shaking off the tube before the printing operation.

Problem 3
Segmented construct when printing the continuous model (steps 12 and 20).

Potential solution
If the construct resulting from the continuous multi-material printing process is segmented, reduce the printing speed (the speed of the nozzle movement) or increase the applied pressure. When alternating between multiple materials, the extrusion of the bioink may stop as the change in pressure is not instantaneously applied to the bioink. Changing the pressure at a slightly earlier time to account for the time required to reach the set pressure can prevent the segmentation of the construct.

If the bioink does not extrude from the nozzle, check the nozzle and tubing for leakage. If the bioink is not leaking, the nozzle is most likely clogged. Nozzle clogging may occur from the drying of the bioink inside the nozzle from prolonged exposure to air. In general, this problem can be solved by immersing the nozzle in a buffer solution and applying high pressure to the clogged barrel. However, if the bioink has been exposed to the air for too long, the nozzle may no longer be usable.

In order to prevent nozzle clogging, we recommend immersing the nozzle in a buffer solution between printing.

Problem 4
Issues related to the antibody staining of the cells cultured in the printed construct (step 24).

Potential solution
The antibody staining cells within the printed construct may be of poor quality when using the conventional immunostaining protocol for 2D cell culture. In general, the incubation step should be modified. To achieve uniform staining throughout the volumetric 3D printed construct, sufficient time is required for the antibodies to fully diffuse into the hydrogel. We recommend incubation at 4°C on a shaking machine at a minimum speed for longer than 24 h. In addition, light can decrease fluorescence signals by photobleaching the fluorophore conjugated to the antibodies. The construct should be protected from light using an aluminum foil to minimize signal loss during the immunostaining procedure.

Problem 5
Issues related to the increase in the RFP HUVEC passage (step 25).

Potential solution
As a primary cell, HUVECs lose their characteristic morphology and show a reduced proliferation rate at high passage numbers. On top of the loss of properties specific to HUVECs, the distribution of RFP within the RFP HUVECs becomes uneven with an increase in the cell passage number. To observe a robust vascular network formation, an RFP HUVEC passage number of 5 or below is recommended.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Je-Kyun Park (jekyun@kaist.ac.kr).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The codes for 3D modeling and bioprinting in this study are provided in the supplemental information. Original data have been deposited to Mendeley Data: https://doi.org/10.17632/6bycgdd9tp.1.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101303.

AUTHOR CONTRIBUTIONS

Conceptualization, G.L. and J.-K.P.; methodology, G.L.; investigation, G.L. and S.K.; data curation, G.L.; formal analysis, G.L.; visualization, G.L.; writing, G.L., S.K., and J.-K.P.; supervision, J.-K.P.

DECLARATION OF INTERESTS

The authors have a patent related to this work.

REFERENCES

Kim, S.J., Lee, G., and Park, J.-K. (2021). Direct microextrusion printing of a low viscosity hydrogel on a supportive microstructured bioprinting substrate for the vasculogenesis of endothelial cells. Adv. Mater. Technol. 2101326. https://doi.org/10.1002/admt.202101326.

Lee, G., Kim, S.J., Chun, H., and Park, J.-K. (2021). Multilayered and heterogeneous hydrogel construct printing system with crosslinking aerosol.

Biofabrication 13, 045027. https://doi.org/10.1088/1758-5090/ac25ca.

Lee, W., Debasitis, J.C., Lee, V.K., Lee, J.H., Fischer, K., Edminster, K., Park, J.-K., and Yoo, S.-S. (2009a). Multi-layered culture of human skin fibroblasts and keratinocytes through three-dimensional freeform fabrication. Biomaterials 30, 1587–1595. https://doi.org/10.1016/j.biomaterials.2008.12.009.

Lee, W., Pinckney, J., Lee, V., Lee, J.H., Fischer, K., Polio, S., Park, J.-K., and Yoo, S.-S. (2009b). Three-dimensional bioprinting of rat embryonic neural cells. Neuroreport 20, 798–803. https://doi.org/10.1097/WNR.0b013e32832b8be4.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.