Supporting Information

Digital Light Processing-based Bioprinting with Composable Gradients

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Materials and Methods

(Bio)printer setup: A DMD-based 3D (bio)printer was assembled in-house, featuring full control over the projection light, the projected patterns, and the movement of the build plate. As seen from **Figure S8**, a 450-nm light (power: 1 W; diameter: 15 mm, Ensfouy, China) was bounced onto the DMD panel after expanded by the beam-expander (magnification: 7×, Scorpii Nd:YAG Beam Expander, Edmund Optics, USA). The DMD device (DLP LightCrafter 6500 Evaluation Module, Texas Instruments, USA) contained an array of micromirrors (1920 × 1080 pixels) that could be switched between on and off states to reflect the projection patterns at the speed of 10 kHz. When the resulting image from the DMD was collected by a condenser (Nikon LWD 0.52, Japan) and concentrated as the clean pattern, two convex lenses (focal lengths: 10 cm and 7.5 cm, Edmund Optics) here served to narrow and focus the final pattern. A pinhole (diameter: 0.5 mm, Edmund Optics) was then placed between the two lenses to filter the projection from noise. Finally, an aluminum front-coated mirror (Edmund Optics) was used to reflect the projected image (0.5 mW cm^{-2} at 450 nm) onto the vat for the (bio)ink polymerization.

The (bio)printer was built on a combination of black poly(lactic acid) (Hatchbox, USA), black acrylic (McMaster-Carr, USA), and solid aluminum optical rods (Thorlabs, USA) to provide the firmness. The entire structure was assembled in an optical breadboard (Thorlabs). The developed (bio)printer used a commercially available Nema 11 (code: 11HS20-0674S) stepper motor to adjust the Z-axis position of the building platform. The rotational motion of the motor was turned into the linear motion using a 100-mm linear rail with a T6 × 1 lead screw (single start, 1 mm between peaks), which was controlled by a stepper motor-driver (Easydrive, USA) with a 1/8 micro-stepping configuration having a moving distance of 5 µm per step. The (bio)printer added a limit-switch to restrict the platform traveling and standardize the Z-axis starting point for the (bio)printing process. The building platform was manufactured using a

steel rod attached with a circular acrylic piece (diameter: 15 mm). A piece of circular cover glass (diameter: 15 mm, Carolina, USA) was placed on the surface of the build plate to achieve a strong material attachment, as a result of the hydrophilicity and smoothness of the glass. Different from the common DLP bioprinters, a custom-made (bio)ink vat integrated with the microfluidic chaotic mixer was specially designed to turn the (bio)printer into a composable-gradient DLP (bio)printing system, described in the next section.

A desktop application was developed using Python 3.6.5 to control the movement of the build plate and the images being projected. The software communicated via serial communication with an Arduino Mega board in a master-slave fashion, leaving the microcontroller the duty to manage the movement of the bioprinter parts. The projection images were obtained by slicing a 3D digital model with the open-source slicer (Form Labs, https://formlabs.com/blog/open-source-dlp-slicer/, USA). A programmable syringe pumping system (NE-1000, New Era Pump Systems Inc, USA) was implemented to control the flow rates of the infusing (bio)inks, which can also be fully automated as we previously reported.[23, 60]

Fabrication and assembly of the microfluidic mixing chip: The microchannel was designed in SolidWorks based on the chaotic mixers reported in the literature.[25, 28] It was fabricated with a commercial DLP 3D printer (Photon, Anycubic, China) using the UV-curable resin (Anycubic). To collect the mixed (bio)ink for (bio)printing, a cylindrical (bio)ink vat (diameter: 15 mm) was embedded at the end of the microchannel. The hydrophilic-treated FEP film (Anycubic) was placed at the base of the circular chamber to hold the mixed (bio)ink. By adding two pieces of properly cut polydimethylsiloxane (PDMS, Dow Chemical Company, USA) above and below the microchannel sheet, the chip was sealed between the top and bottom acrylic sheets using multiple screws distributed across the device. Acrylic sheets were manufactured with laser-engraving and cutting (VLS 2.30 Desktop Laser, Universal Laser

Systems, USA). Depending on the configuration, 2, 3, or 5 inlets can be made on a single chip connected to the infusing pump, plus 1 outlet after the (bio)printing vat.

Characterizations of the mixing efficiency: The characterizations of the mixing performance were carried out with the 2-, 3-, and 5-inlet mixers by simultaneously flowing two inks of 40 wt.% PEGDA (M_w = 575 Da, Sigma-Aldrich, USA) containing fountain pen inks with different colors (magenta, yellow, orange, and grey: J. Herbin, France; deep cerulean blue: Pilot, Japan). The inks were held in syringes mounted on programmable syringe pumps. A collective constant flow rate of 100 μL min^{-1}, 500 μL min^{-1}, 750 μL min^{-1}, or 1000 μL min^{-1} within the microchannel was tested on each device.

The photographs captured through the camera once the steady flows were obtained, were used for the RGB analyses and the quantification of the MI values. The RGB color profiles were obtained by analyzing the line colors at the inlet, before the microchannel, and after the microchannel using the ImageJ RGB-Profiler plugin (National Institutes of Health, USA). For the quantification of MI, which is defined as the ratio between the number of final mixed pixels to the number of total pixels, the photographs were segmented by colors and filtered to show only the completely mixed colors.

Printing methodology: 2D and 3D printing of acellular hydrogels were performed using inks consisted of 40 wt.% PEGDA (M_w = 575 Da) and 2-mM/20-mM of Ru/SPS (Advanced BioMatrix, USA).[61] The inks (1 mL) were colored by adding 20 to 50 μL of different dyes (fountain pen inks from J. Herbin or Pilot, and Easyou Marie's Chinese painting colors). By mixing these inks at different infusing speeds, variations in colors could be observed, making it possible for the gradients to be visualized under a camera. During the printing, the inks possessing different colors were filled into the inlets at the same time with different infusion speeds, keeping the collective flow rate within the microchannel constant at 1000 μL min^{-1}. The mixed ink in the vat was photocrosslinked layer-by-layer with the projected patterns. The

uncrosslinked ink was removed from the outlet by applying a negative pressure, leaving the chamber clean for the next iteration without any need for washing. 3D constructs were printed with a speed of 300 μm min^{-1} and a layer height of 300 μm. During printing, the build plate was lifted when the ink was being evacuated to avoid damaging the constructs.

GelMA synthesis: GelMA was synthesized by reacting gelatin (Sigma-Aldrich) with methacrylate anhydride (Sigma-Aldrich) at 50 °C for 1 h in phosphate-buffered saline (PBS, pH = 7.4, Thermo Fisher, USA), using our reported protocol.[48] The GelMA solid was achieved with freeze-drying after dialyzed against the dialysis membrane (molecular weight cut off: 12-14 kDa, Spectrum, USA), and was stored at -20 °C until use. Reconstituted GelMA solutions were sterilized with the filter (0.22 μm of pore size, Millipore, USA) before usage. Pore-forming GelMA bioink was formulated as we previously published.[51-53]

HepMA synthesis: HepMA was synthesized by adding methacrylate anhydride (10 wt.%) dropwise into the 2 wt.% solution of heparin (Sigma-Aldrich). The solution was stirred overnight at room temperature after adjusting the pH to 8.5 using 5-M NaOH (Sigma-Aldrich). Then, it was precipitated in 100% ethanol (Sigma-Aldrich) and dialyzed against the dialysis membrane (molecular weight cut off: 1 kDa, Spectrum) at room temperature for 7 days, followed by lyophilization and storage at -20 °C until use.

Cell viability measurement: The mixings were conducted under the flow rates of 800:200, 500:500, and 200:800 μL min^{-1} between 10 wt.% GelMA containing C2C12 cells and 10 wt.% GelMA without cells. The bioinks used for the control group were collected at the same pumping ratios but mixed by pipetting, which did not pass through the mixer. At 0, 3, and 7 days after bioprinting, the viabilities of the cells were evaluated with live/dead staining (Invitrogen, USA). In detail, the bioprinted structures were rinsed with PBS and incubated with 2 μM of calcein-AM and 4 μM of ethidium homodimer-1 for 30 min and then observed by the fluorescence microscope (Eclipse TE 2000U, Nikon, Japan). Live cells were quantified with

ImageJ. Additionally, the cellular metabolic activities were assessed using the MTS method with the CellTiter 96® AQueous Assay (Promega, USA) according to the manufacturer's instructions. Briefly, the bioprinted samples were incubated with the MTS assay solution for 4 h in the dark. The absorbance values were measured at 490 nm using a microplate reader (Tecan, Switzerland).

Cell-based bioprinting: The breast cancer cell line MDA-MB-231 (American Type Culture Collection [ATCC], USA) and mouse myoblast cell line C2C12 (ATCC) were cultured in the Dulbecco's modified Eagle medium (DMEM, Thermo Fisher) supplemented with 10% fetal bovine serum (Thermo Fisher). Cells were cultured in the incubator (Forma Scientific, USA) with 5% CO_2 at 37 °C and were passaged twice per week using 0.05% trypsin-EDTA (Thermo Fisher). GFP-HUVECs were cultured with the endothelial cell growth medium-2 (EGM-2, Lonza, Switzerland) and were passaged twice per week using 0.05% trypsin-EDTA.

To produce the cell-density gradients on-the-fly, two (bio)inks were separately loaded into the two inlets of the microfluidic mixer chip, consisting of 10 wt.% GelMA and 2-mM/20-mM Ru/SPS, with and without GFP-HUVECs (1×10^7 mL^{-1}), respectively. After arbitrarily adjusting the velocity ratios of these two (bio)inks, which were reversibly altered between 0.20 and 9.50, the mixed bioinks were bioprinted sequentially. The bioprinted structures were visualized using the fluorescence microscope. The intensities of green fluorescence of the captured images were analyzed using ImageJ.

For the bioprinted constructs with predictable cell-density gradients, MDA-MB-231 cells or HUVECs were stained with the CellTracker (Thermo Fisher) for 30 m at 37 °C before being mixed with the GelMA bioinks. The stained MDA-MB-231 cells in blue color (1×10^7 mL^{-1}) or HUVECs in green color (1×10^7 mL^{-1}) were suspended in the solution of 10 wt.% GelMA and 2-mM/20-mM Ru/SPS. Subsequently, they were mixed with the ink of 10 wt.% GelMA and 2-mM/20-mM Ru/SPS in the microfluidic mixer chip under the designed flow rates for

further bioprinting. The bioprinted scaffolds featuring cell-density gradients were visualized using the fluorescence microscope and analyzed using ImageJ. In addition, RFP-HUVECs (1×10^7 mL^{-1}) and GFP-HUVECs (1×10^7 mL^{-1}) suspended in 10 wt.% GelMA and 2-mM/20-mM Ru/SPS were mixed with four sets of flow ratios. The fabricated gradients of green fluorescence and red fluorescence in the two opposite directions were analyzed with ImageJ.

CAM assay: For the fabrication of VEGF gradient used for the CAM assay, HepMA (500 ng mL^{-1}) and VEGF (200 ng mL^{-1}, PeproTech, USA) were mixed for at least 4 h to ensure equilibration. Then, the samples were printed by properly mixing the ink containing 10 wt.% GelMA, 2-mM/20-mM Ru/SPS, 250-ng mL^{-1} HepMA, and 100-ng mL^{-1} VEGF with the ink containing 10 wt.% GelMA and 2-mM/20-mM Ru/SPS. The scaffolds were printed into four segments of increasing VEGF concentrations (0, 25, 50, and 100 ng mL^{-1}) via modulating the flow rates of the two inks.

The CAM assay was used as an ex ovo model to evaluate the angiogenesis efficiency of printed VEGF gradient hydrogel as previously reported.[62] Fertilized specific pathogen-free (SPF) chicken eggs were purchased from Charles River Laboratories (USA) and incubated at 37 °C at 60-65% humidity. On EDD 3, the eggs were opened into sterile weighting boats. After another 4 days of incubation, two of the printed VEGF-gradient scaffolds were gently placed on the outer regions of each CAM on EDD 7. On EDD 14, the printed hydrogels with the surrounding CAMs were observed under a stereomicroscope (Leica, Germany). The acquired digital photographs were utilized for the quantification of angiogenic responses using the NeuronJ plug-in (https://imagescience.org/meijering/software/neuronj/) of ImageJ. The total vessel lengths and the total numbers of blood vessels were assessed after tracing the vessels.

Fabrication of growth factor gradient and stiffness gradient: To investigate the HUVECs or MSCs attachment on the printed hydrogel possessing growth factor gradients, two bioinks of 10 wt.% GelMA and 2-mM/20-mM Ru/SPS with/without VEGF (100 ng mL^{-1}) or BMP-2

(6 μg mL^{-1}, PeproTech) were mixed in the mixer chip, followed by the printing of four segments. The GFP-HUVECs were seeded onto the VEGF gradient scaffolds with the cell density of 1×10^4 cm^{-2}. While the MSCs were seeded onto the BMP-2 gradient scaffolds with the cell density of 3×10^3 cm^{-2}. The fluorescence images of GFP-HUVECs were captured with the fluorescence microscope on day 7 after the cell seeding. The MSCs samples were collected on day 1 after the cell seeding and were stained with Alexa Fluor-488 Phalloidin (Thermo Fisher) for F-actin and 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, USA) for nuclei observation. The samples were then visualized using confocal laser scanning microscopy (LSM880, Zeiss, Germany).

The inks for the stiffness gradient fabrication were obtained by loading and mixing 10 wt.% GelMA and 2-mM/20-mM Ru/SPS with 30 wt.% GelMA and 2-mM/20-mM Ru/SPS, which were then printed into the constructs of GelMA concentration gradient. C2CL12 cells (1×10^3 cm^{-2}) were seeded onto the printed scaffolds and visualized with confocal laser scanning microscopy (LSM880, Zeiss, Germany) after the staining of F-actin and DAPI. The cell areas and aspect ratio were measured with ImageJ.

Osteogenesis for bone tissue engineering: MSCs (Lonza) were cultured with MSC growth medium (Lonza) and used at passages 4 and 5. For a 4-weeks in vitro osteogenesis process, the two bioinks loaded into the mixer chip were composed of 10 wt.% GelMA, 2-mM/20-mM Ru/SPS, 100-μg mL^{-1} HepMA with/without BMP-2 (6 μg mL^{-1}), and dextran (3.0 wt.%, M_w = 2,000,000 Da, Aladdin, China). The bioprinting of porosity- and BMP-2-gradient structures started by mixing these two bioinks under three sets of flow rates to achieve the gradients of 0, 3, and 6 μg mL^{-1} of BMP-2 and 0.5, 1.5, and 3.0 wt.% of dextran. The whole constructs were bioprinted with a layer height of 200 μm and 30 s for each layer. After 10 layers of bioprinting was completed of each bioink formulation (three bioinks in total), the 6-mm high, 10-mm long, and 8-mm wide 3D structures were obtained with dual gradients of porosity and BMP-2.

The bioprinted constructs were cultured in the osteogenic differentiation medium (Lonza), which was changed every 3 days. After 1, 2, and 3 weeks of culture, the differentiated scaffolds were harvested for further immunostaining and ARS staining examinations. The constructs were fixed by paraformaldehyde solution (Sigma-Aldrich) and treated with 0.3 v/v% Triton™ X-100 (Sigma-Aldrich) for 10 min at room temperature. After the blocking with 5 wt.% bovine serum albumin (BSA, Sigma-Aldrich) at room temperature for 1 h, they were incubated with Alex 488-phalloidin, or anti-RUNX2 (Abcam, UK) or anti-OCN (Abcam) antibody at 4 °C overnight, followed by the secondary antibody (Thermo Fisher) incubation for RUNX2/OCN staining. Fluorescence micrographs were captured using a confocal laser scanning microscopy. ImageJ was applied to analyze the IODs of fluorescence images. ARS staining was performed by staining the bioprinted constructs with 2 wt.% ARS solution (Sigma-Aldrich) for 20 min at room temperature. The stained scaffolds were washed with deionized water and then were observed by an inverted microscope (Leica DMi8, Germany).

The bioprinted constructs were collected at 4 weeks after differentiation for the evaluation of gene expressions. To isolate the total RNAs from the hydrogel samples, the 3D structures immersed in TRIzol (Thermo Fisher) were homogenized using the Precellys lysing kits (Precellys, France). The first-strand cDNA was synthesized using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the instructions of manufacturer. RT-PCR was conducted with the PowerTrack SYBR Green Master Mix (Thermo Fisher). Primers were obtained from Integrated DNA Technologies (USA) and their information is listed in **Table S1**. The RT-PCR was then run on the QuantStudio 5 Real-Time PCR instrument (Thermo Fisher) with duplicate copies and the results were normalized against the housekeeping gapdh gene.

Statistical analyses: The data are presented as means ± standard deviations (SDs). All statistical analyses were performed with one-way analysis of variance (ANOVA) followed by two-tailed student's t-test or Tukey's Honest Significant Difference test. $p < 0.05$ was

considered statistically significant.

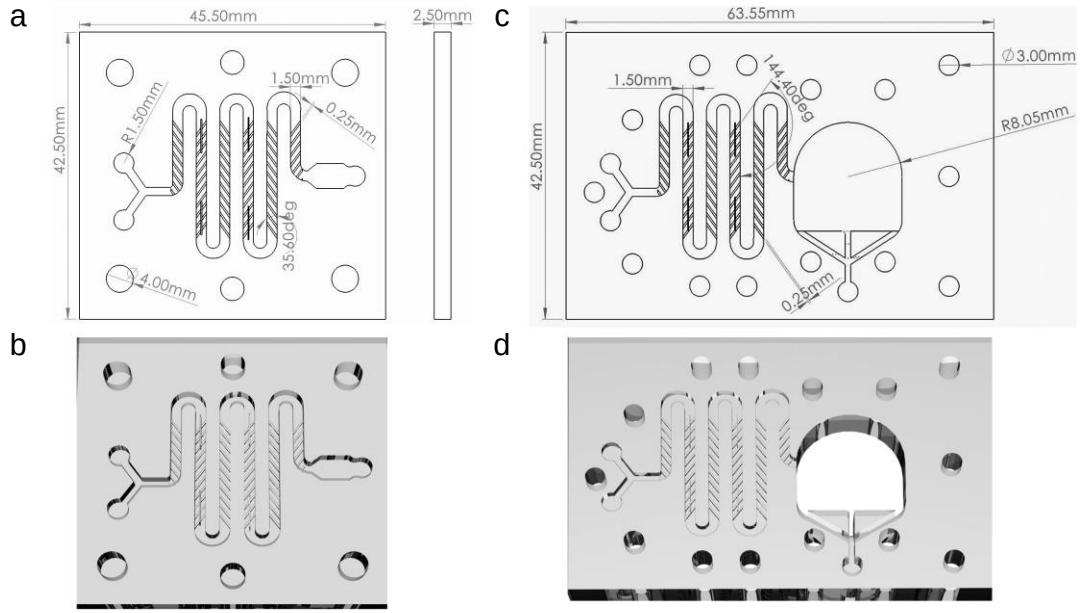

Figure S1. (a, b) Design and 3D rendering of the microfluidic chaotic mixer with 2 inlets. (c, d) Design and 3D rendering of the same microfluidic chaotic mixer with the integrated vat for (bio)printing.

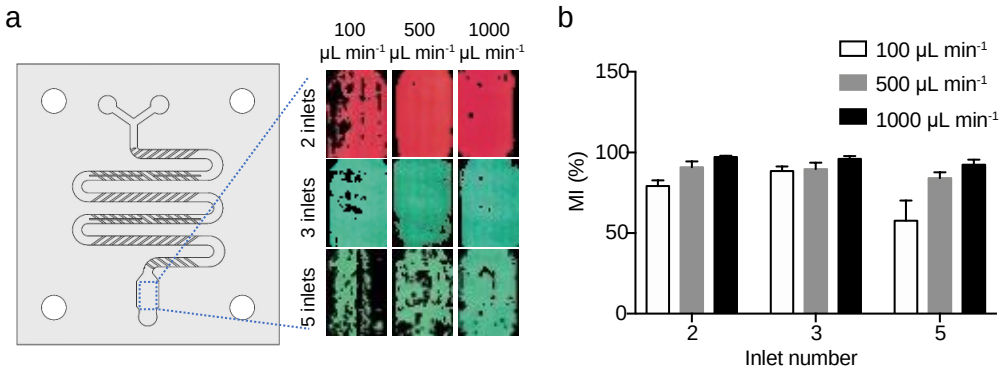

Figure S2. Analysis of mixing efficiency using MI. (a) Images showing the extracted final mixed colors at the end of the mixers when mixing 2, 3, and 5 inks at flow rates of 100 µL min^{-1}, 500 µL min^{-1}, and 1000 µL min^{-1}. (b) Quantification of percentage of MI values.

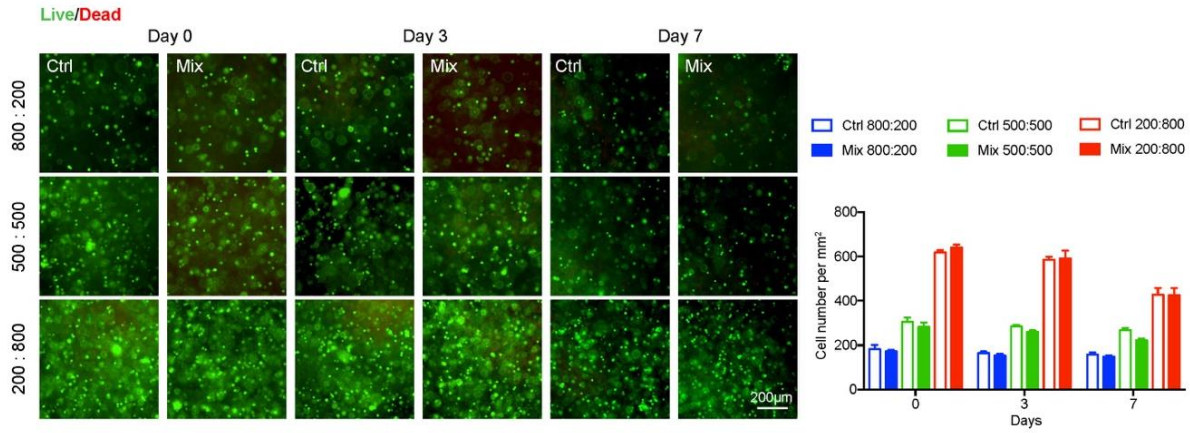

Figure S3. Micrographs showing the live/dead staining of NIH/3T3 cells bioprinted in GelMA constructs without passing through the mixer (i.e., mixed by pipetting; Ctrl) or mixed by the microfluidic mixing chip (Mix). The right panel is the corresponding quantitative results of the numbers of live cells at days 1, 3, and 7 after bioprinting.

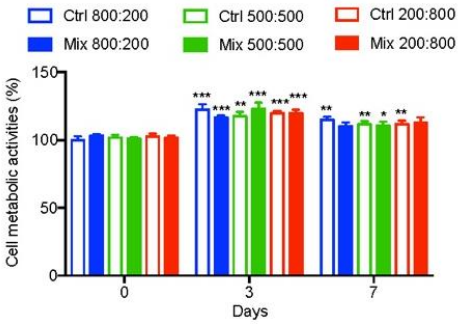

Figure S4. Quantification results of MTS assay of NIH/3T3 cells bioprinted in GelMA constructs without passing through the mixer (Ctrl) or mixed by the microfluidic mixing chip (Mix) at the days 1, 3, and 7 after bioprinting. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; one-way ANOVA (compared with the respective groups on day 0), mean ± s.d. (n = 5).

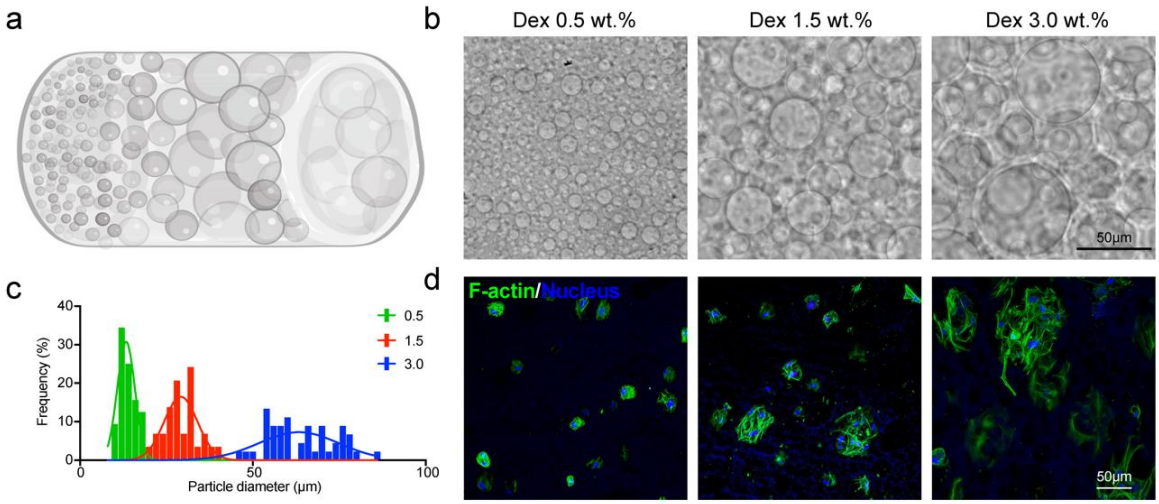

Figure S5. Bioprinting of GelMA hydrogel with a pore-size gradient based on the aqueous two-phase emulsion system. (a) Schematic of the porous hydrogel resulting from segmented 0.5 wt.%, 1.5 wt.%, and 3.0 wt.% porogen, enabled by the mixer. (b) Micrographs of pore morphologies within the porosity-gradient construct. (c) Quantification results showing the corresponding pore size distributions. (d) Fluorescence micrographs of MSCs stained for F-actin (green) and nuclei (blue) bioprinted in the pore-size gradient hydrogel showing the cell spreading at 3 days after culture.

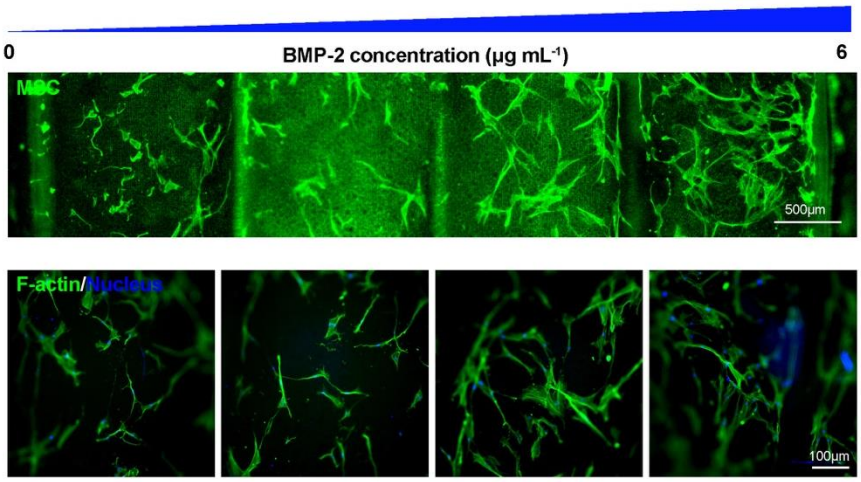

Figure S6. A construct with BMP-2 gradient produced by mixing two GelMA inks with or without BMP-2, followed by culture of MSCs on the matrix. Images of F-actin (green) staining were harvested after 1 day of culture.

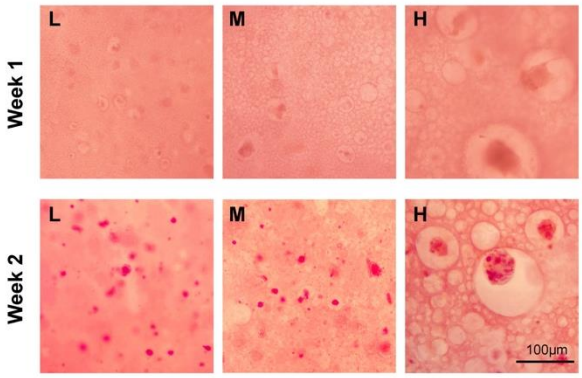

Figure S7. ARS staining of bioprinted dual-gradient hydrogel constructs at 1 and 2 weeks after osteogenic differentiation in the regions of low-gradient (L), medium-gradient (M), and high-gradient (H).

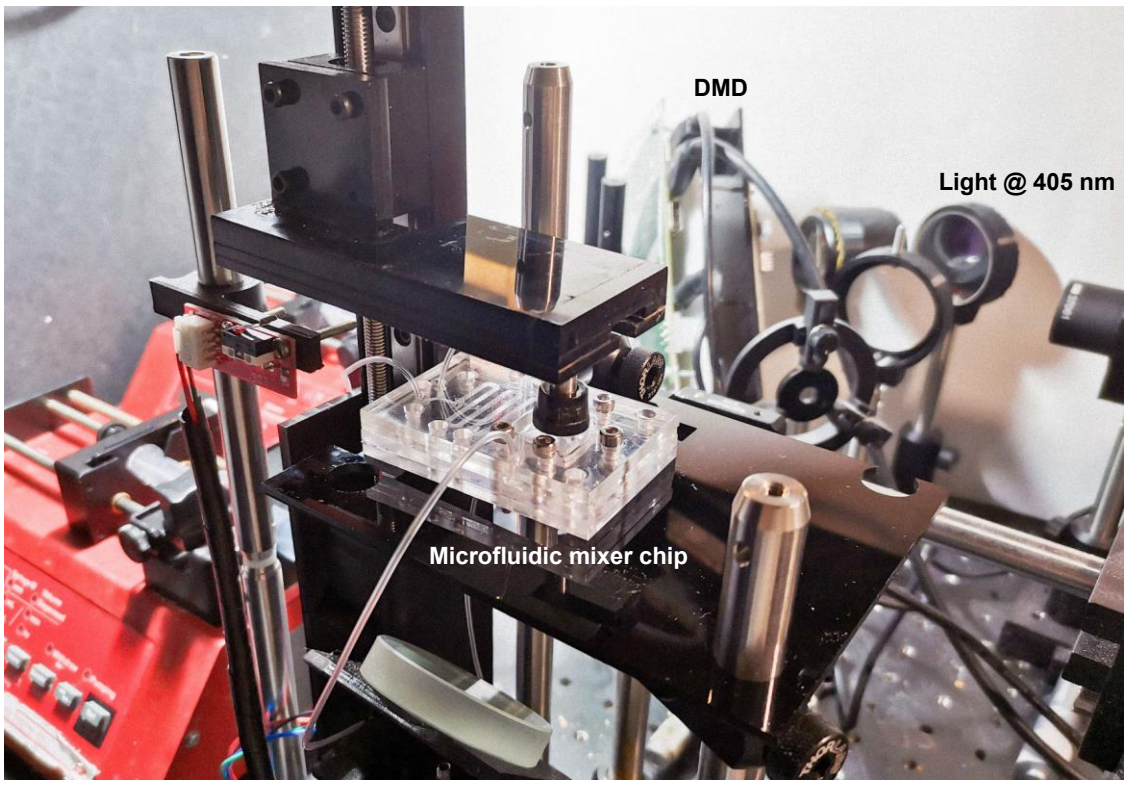

Figure S8. Photograph of the in-house-built composable-gradient DLP (bio)printing platform.

Table S1. Primers used for the RT-PCR of osteogenic gene evaluations.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
RUNX2	TGGTTACTGTCATGGCGG GTA	TCTCAGATCGTTGAACCTTGCTA
OSX	CTCCATCTGCCTGACTCCTT	GGGACTGGAGCCATAGTGAG
ALP	CCCTTGACCCCCACAATGT	GTTGTTCCTGTTGACCTCGTACTG
COL1A1	GGTGTAAGCGGTGGTGGTTAT	GCTGGGATGTTTTCAGGTTGG
BSP	GACAGTTCAGAAGAGGAGGAG	AGCCCAGTGTTGTAGCAGA
OCN	AGGAGGGCAGCGAGGTAG	GAAAGCCGATGTGGTCAGC

Video S1. Mixing of blue- and yellow-colored inks in the microfluidic mixing chip, followed by the ink filling in the vat and vacuum-enabled ink evacuation.