3D bioprinting of thick vascularized tissues

David B. Kolesky*, Kimberly A. Homan*, Mark A. Skylar-Scott*, and Jennifer A. Lewis**

School of Engineering and Applied Sciences, Wyss Institute for Biologically Inspired Engineering Harvard University, Cambridge, MA 02138 (USA) *Authors contributed equally **Corresponding author e-mail: jalewis@seas.harvard.edu

SUPPORTING INFORMATION

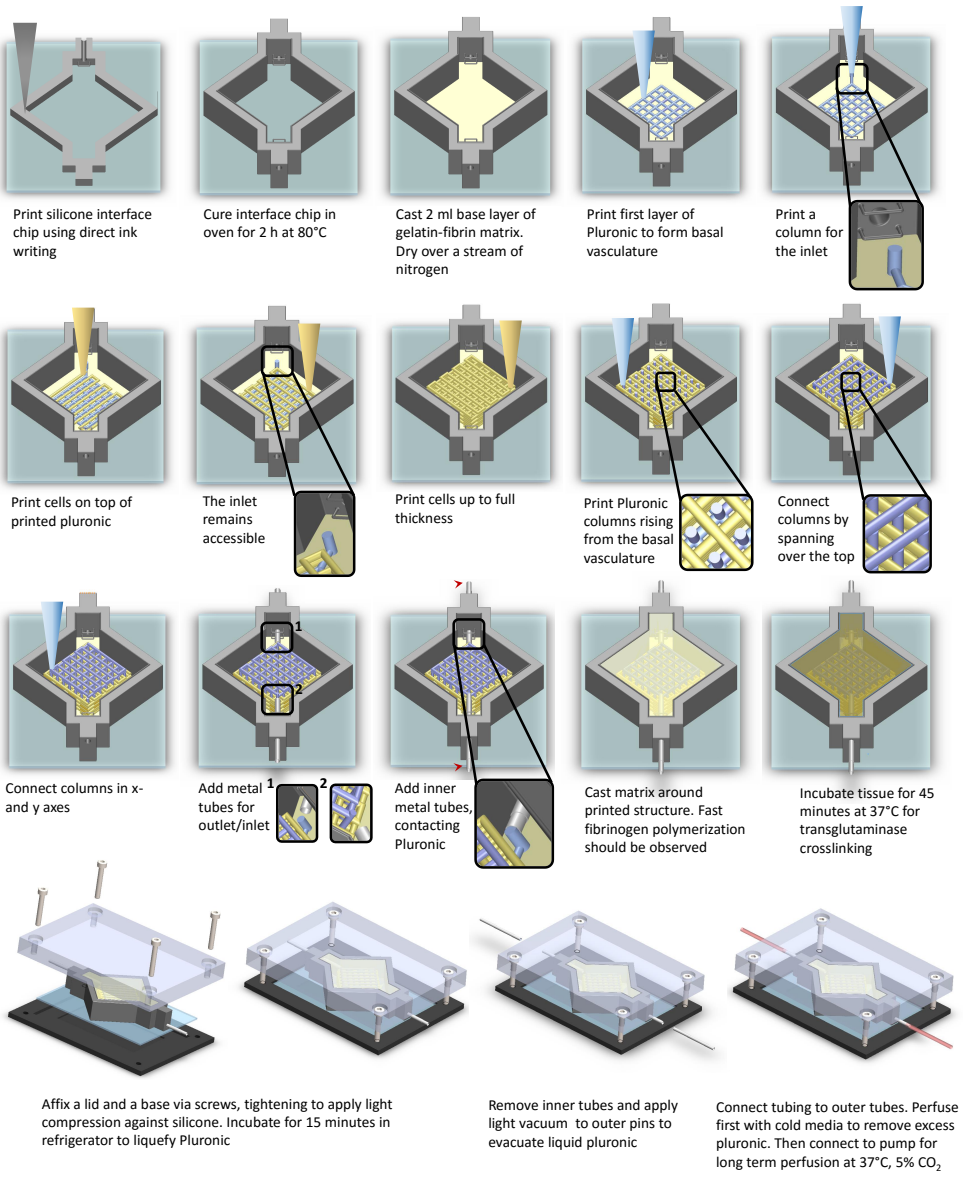


Figure S1: Schematic illustration of the construction and perfusion of 3D vascularized tissues on chip.

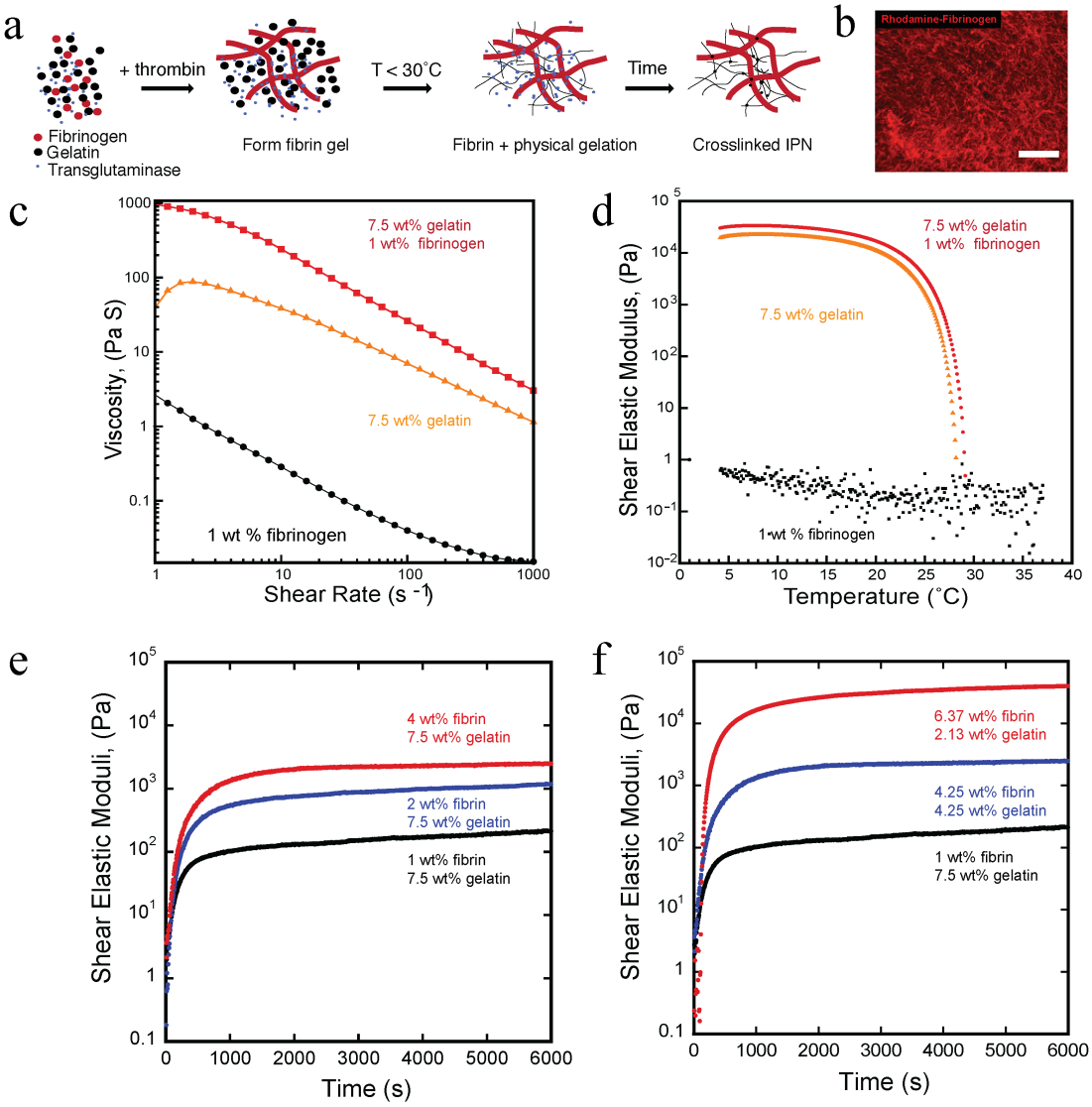


Figure S2: (a) Schematic illustration of the three-step formation of the gelatin-fibrin matrix. First, upon adding thrombin, the fibrinogen rapidly polymerizes into a fibrin gel. Second, gelatin undergoes a liquid-to-gel transition as the temperature decreases. Third, after an extended period of time (> 1h), the gelatin is cross-linked to fibrin via transglutaminase to form gelatin-fibrin matrix. (b) A fluorescent image of rhodamine-labeled fibrinogen within a gelatin-fibrin matrix demonstrates that a dense fibrillar network of fibrin is formed upon addition of thrombin.[Scale bar = 50μm] (c) At 37˚C, the presence of fibrinogen increases the solution viscosity of gelatin, yet only slightly enhances the shear plateau modulus (d) of matrix at room temperature. (d) Viscoelastic behavior of pure fibrin, gelatin and gelatin-fibrin inks. (e) Dynamic shear elastic modulus of gelatin-fibrin rapidly increases with time upon addition of thrombin, due to fibrin network formation, while the plateau elastic modulus increases with increasing fibrin content. (f) Within the gelatin-fibrin matrix, fibrin imparts more stiffness to the resulting gel.

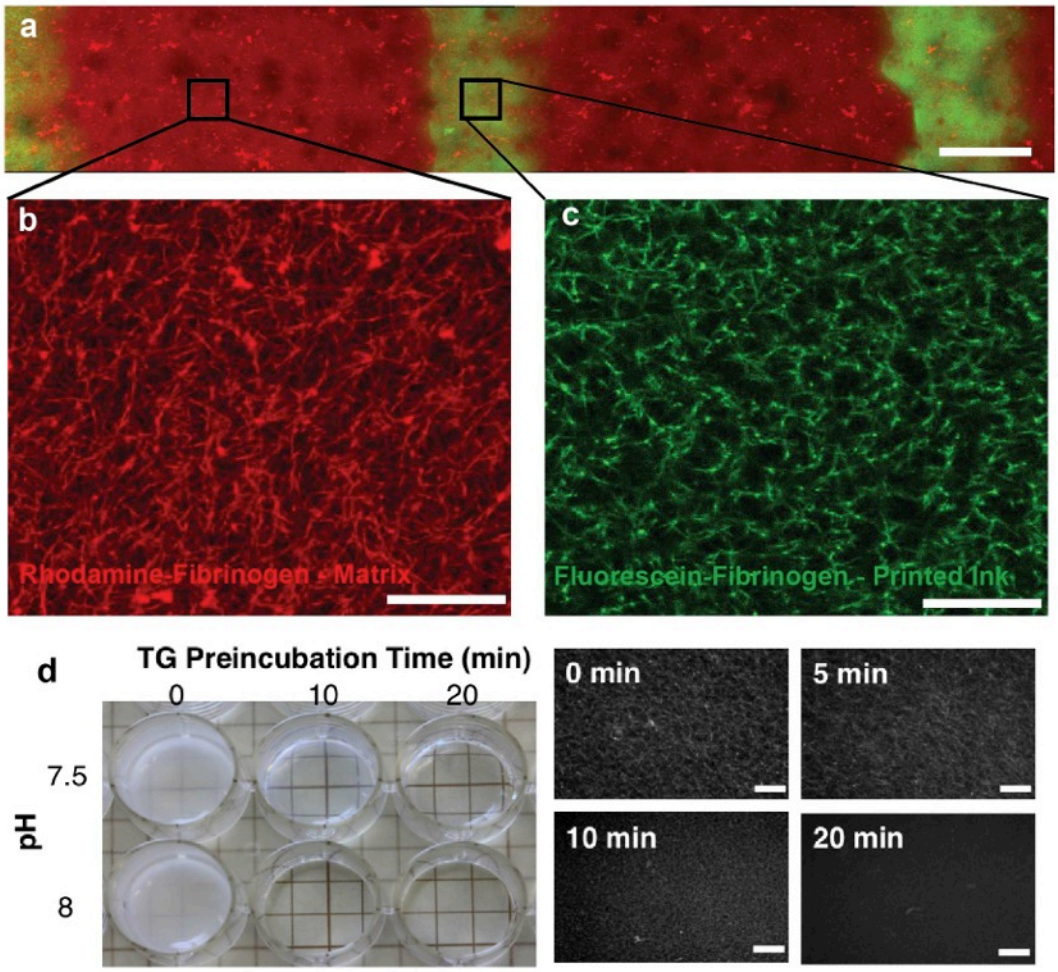


Figure S3: (a) Printed gelatin and fibrin-fluorescein filaments are encapsulated in a gelatin-fibrin-rhodamine matrix containing thrombin and transglutaminase. [Scale bar = 250μm] Upon gelation, the fibrin in the (b) matrix, and (c) printed filaments polymerizes into a continuous network. [Scale bar = 50μm] (d) Increasing the TG preincubation time before adding thrombin results in increasingly transparent matrices, and correspondingly, finer pore and filament sizes within the fibrin network. The TG concentration used in these gels is 0.2%wt. [Scale bar = 50μm]

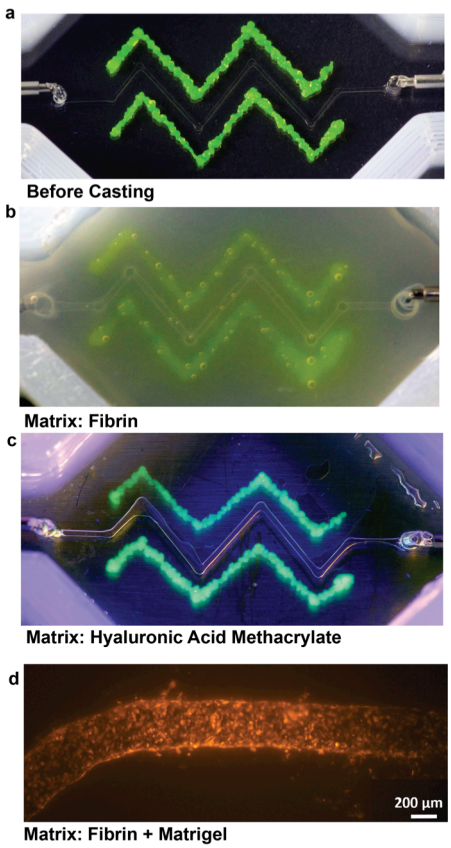
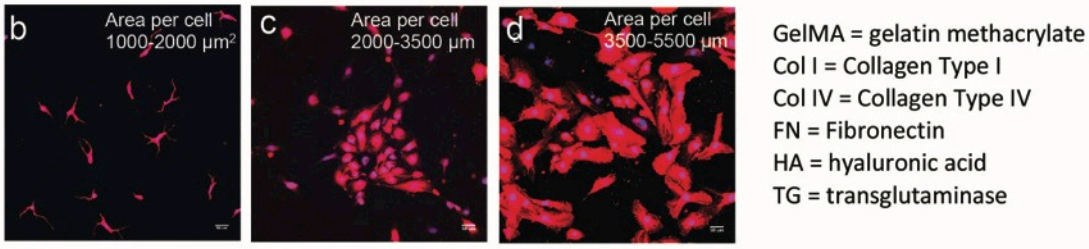


Figure S4: (a) Co-printed pattern of gelatin-fibrinogen (fluorescent green) and fugitive ink (clear) prior to casting. Image of evacuated channels surrounded by different matrices: (b) pure-fibrin gel, (c) hyaluronic acid methacrylate, and (d) fibrin-matrigel lined with RFP-HUVECs and perfused.

a

Matrix material	Low Area per cell (< 2000 μm^2)	Medium Area per cell (2000-3500 μm^2)	High Area per cell (> 3500 μm^2)
Polysyrene petri dish		X	
GelMA 10 wt%	X		
GelMA 10 wt% + 0.1 mg/ml Col I	X		
GelMA 10 wt% + 0.25 mg/ml Col I	X		
GelMA 10 wt% + 0.5 mg/ml Col I	X		
GelMA 10 wt% + 0.75 mg/ml Col I		X	
GelMA 10 wt% + 1 mg/ml Col I		X	
GelMA 10 wt% + 20 ug/ml Col IV	X		
GelMA 10 wt% + 50 ug/ml Col IV	X		
GelMA 10 wt% + 100 ug/ml Col IV	X		
GelMA 10 wt% + 150 ug/ml Col IV	X		
GelMA 10 wt% + 200 ug/ml Col IV	X		
GelMA 10 wt% + 250 ug/ml Col IV		X	
GelMA 10 wt% + 50 ug/ml FN	X		
GelMA 10 wt% + 200 ug/ml FN		X	
GelMA 10 wt% + 10 ug/ml Laminin	X		
GelMA 10 wt% + 125 ug/ml Laminin		X	
GelMA 10 wt% + 2.5 mg/ml 800 kDa HA	X		
GelMA 10 wt% + 2.5 mg/ml 200 kDa HA		X	
"Opaque" Fibrin 10 mg/ml, 2 U/ml thrombin		X	
"Clear" Fibrin (pH balanced to 7.5) 10 mg/ml, 2 U/ml thrombin		X	
"Clear" Fibrin (pH balanced to 7.5) 10 mg/ml + 1 mg/ml Col I, 2 U/ml thrombin			X
GelMA 7 wt%, 15 mg/ml Fibrin, 2.5 U/ml thrombin			X
GelMA 7 wt%, 15 mg/ml Fibrin, 1 U/ml thrombin			X
Gelatin 70C 12hr 5 wt%, 10mg/ml Fibrin, 1 U/ml thrombin, 0.2 wt% TG			X
Gelatin 70C 12hr 7.5 wt%, 10mg/ml Fibrin, 2 U/ml thrombin, 0.2 wt% TG			X
Gelatin 85C 12hr 7.5 wt%, 10mg/ml Fibrin, 2 U/ml thrombin, 0.2 wt% TG		X	
Gelatin 90C 12hr 12 wt%, 10mg/ml Fibrin, 2 U/ml thrombin, 0.2 wt% TG		X	
Gelatin 95C 12hr 12 wt%, 10mg/ml Fibrin, 2 U/ml thrombin, 0.2 wt% TG		X	



GelMA = gelatin methacrylate
Col I = Collagen Type I
Col IV = Collagen Type IV
FN = Fibronectin
HA = hyaluronic acid
TG = transglutaminase

Figure S5. (a) Table summarizing the adhesion behavior and relative cell spreading on various matrix formulations. Fluorescent images of (b) low, (c) medium, and (c) high levels of RFP-HUVECs spreading on different matrices. [Note: Fibrinogen is gelled at pH 7 to generate an opaque fibrin gel, and at pH 7.5 to generate a transparent or "clear" fibrin gel. pH is adjusted using 1M NaOH. [Scale bar = 50μm]]

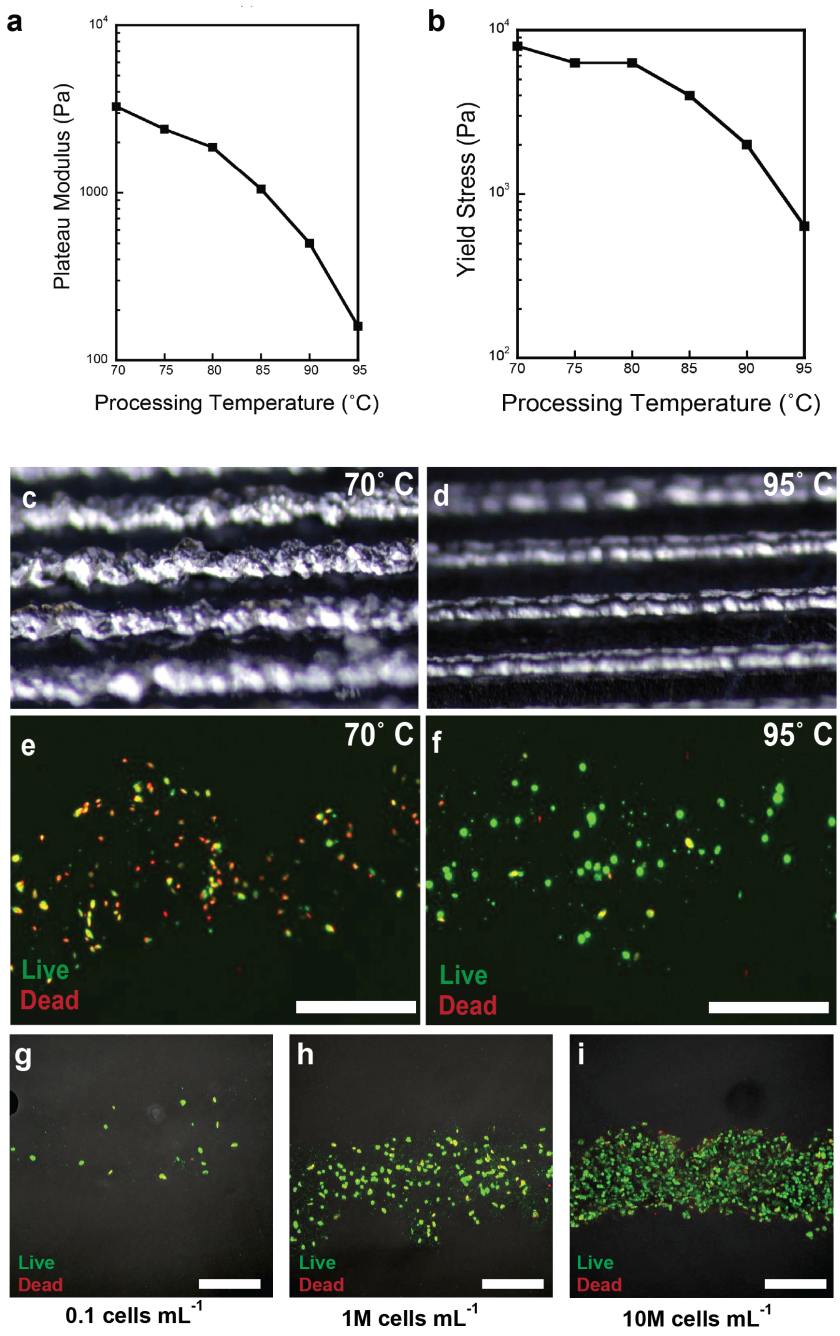


Figure S6. Gelatin processing temperature improves cell viability and printability. Dissolving gelatin at higher temperatures for 12 h results in gelatin-fibrinogen inks that exhibit lower (a) plateau shear elastic modulus and (b) shear yield stress. Printed cell-laden filaments are (c) less uniform when gelatin is solubilized at 70°C compared to (d) those produced by gelatin solubilized at 95°C. Correspondingly, live-dead assays demonstrate that printed fibroblast viability is (e) significantly lower in stiffer, 70°C solubilized gelatin than in (f) 95°C solubilized gelatin. hMSC-cell laden inks of varying cell densities: (g) 0.1M mL^{-1}, (h) 1M mL^{-1}, and (i) 10M mL^{-1} imaged immediately after printing, in which a live/dead assay is carried out using calcein (live) and ethidium homodimer (dead). [Scale bars = 250μm]

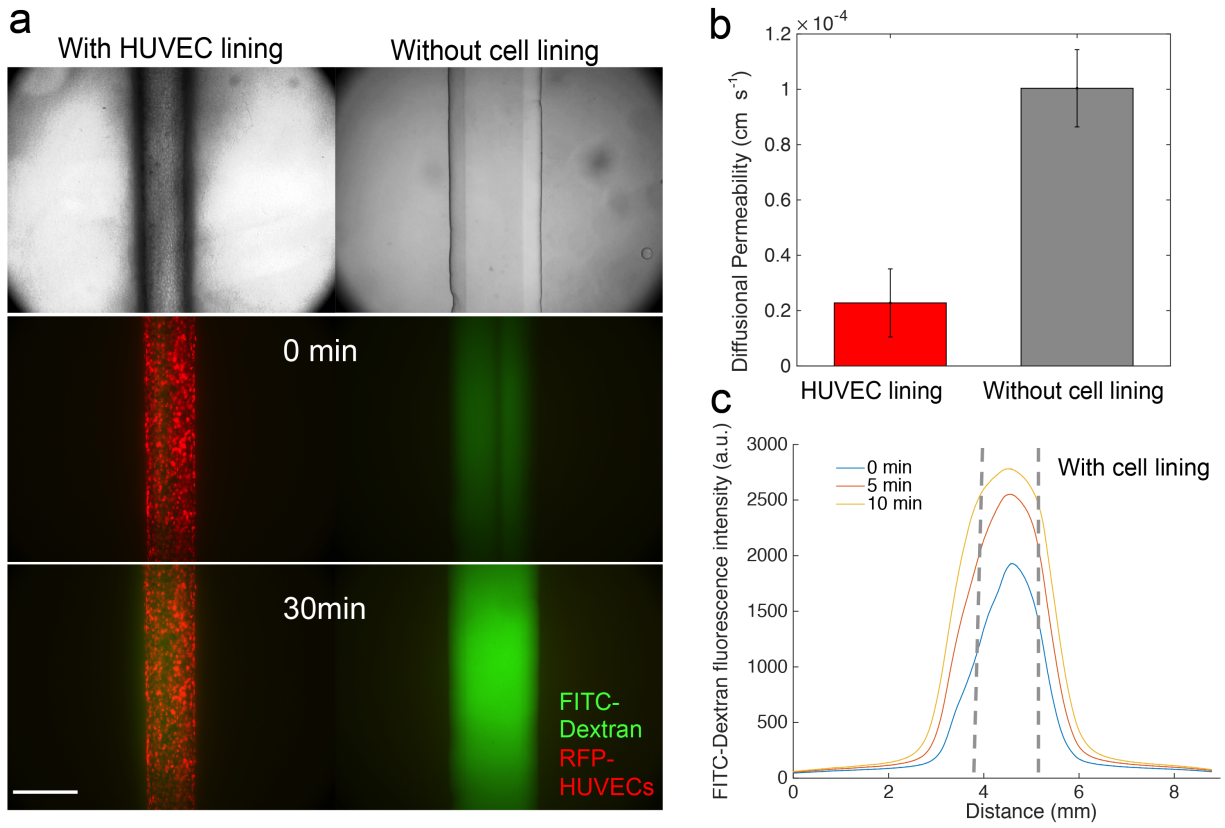


Figure S7. (a) FITC-labeled Dextran (70 kDa) is perfused through channels, with and without a lining of HUVECs, which are embedded in the extracellular gelatin-fibrin matrix. The fluorescence signal is recorded every 5 min up to 30 min to quantify their barrier function. (b) Calculated diffusional permeability with and without the HUVEC lining. (c) FITC-dextran fluorescence signatures within HUVEC-lined channels reveal a slow diffusive spreading over time. [Scale bar = 500 μm]

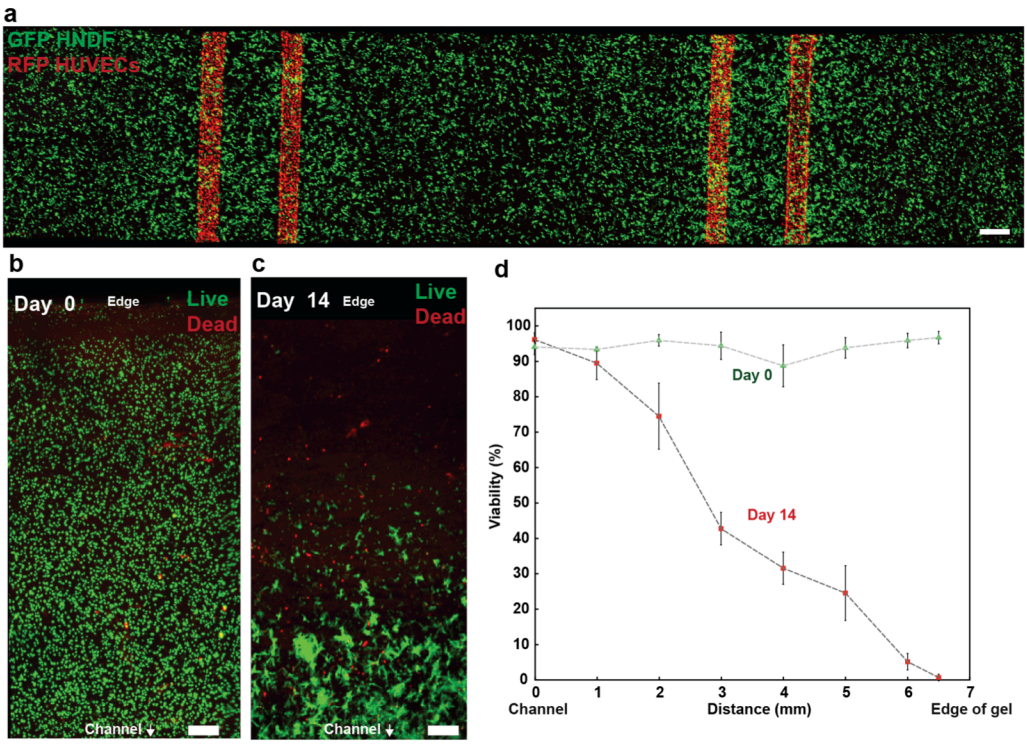


Figure S8. Fibroblast cell proliferation and viability within vascularized tissues. (a) A GFP-expressing fibroblast laden gelatin-fibrin hydrogel (50K cell mL^{-1}) is perfused through four embedded channels lined with RFP-expressing HUVECs. The cell density, as measured by GFP levels, is highest near the channels, and decreases in the central region far from the channels. [Scale bar = 500 μm] Viability, proliferation, and morphology of fibroblast-laden tissues (b-d) (500K cell mL^{-1}) are assessed during perfusion over a two-week period using calcein (live) and ethidium homodimer (dead). At Day 0 (b) cells are uniformly distributed and highly viable. At Day 14 (c) cells within 1 mm of vascular channel retain high viability forming a dense, interconnected network, while cells beyond this region exhibit a distant-dependent loss of viability and change in morphology. (d) Cell viability as a function of distance from the vascular channel for day 0 and day 14. [Scale bar = 500 μm]

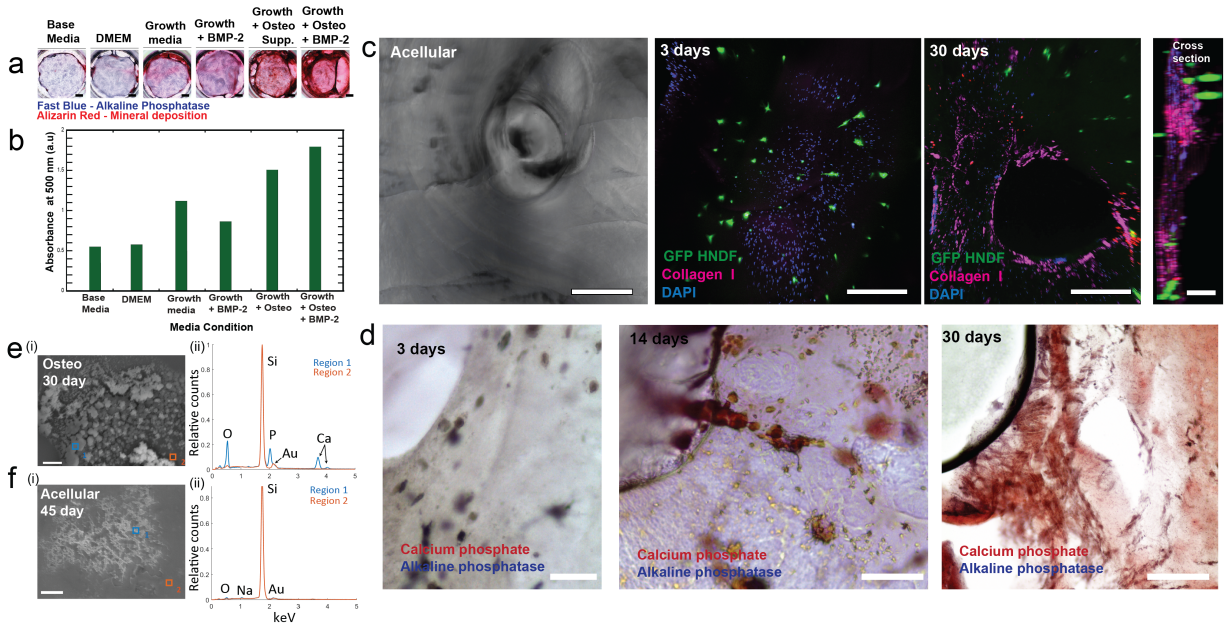
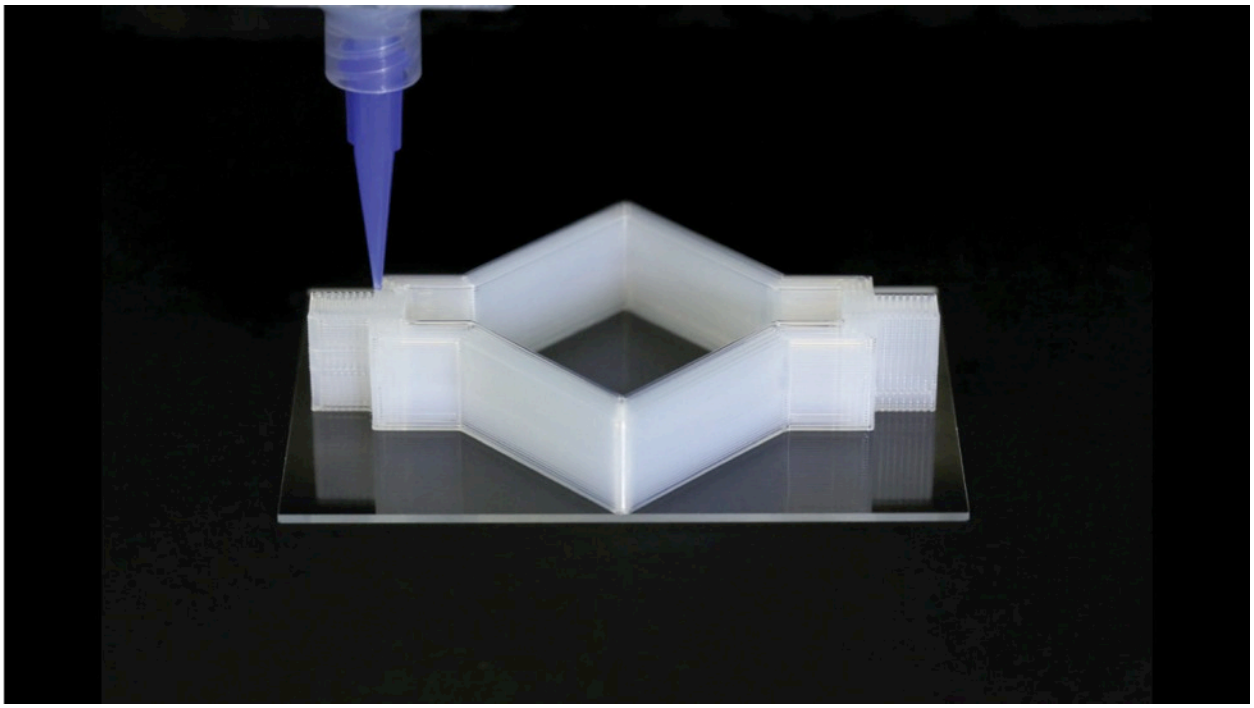


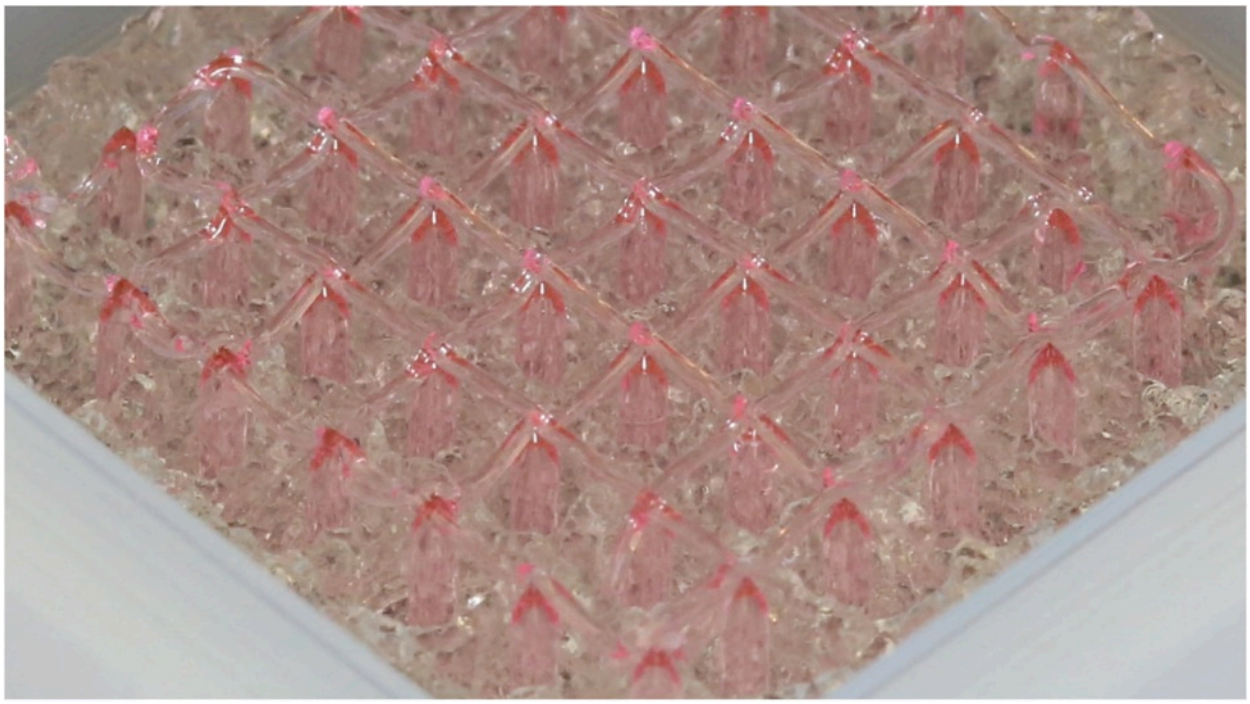
Figure S9. Characterization of osteogenic induction. (a) hMSC's are cultured in a polystyrene well in the presence of different media conditions. At Day 14, the cells are stained with fast blue and alizarin red to visualize osteocytes and deposited minerals, respectively. [Scale bar = 2 mm] (b) A plot of average absorbance at 500 nm at 9 different locations within each well. 500nm absorbance is a measure of the amount of alizarin red in the wells. Base media = Rooster Media. Growth media = Rooster media + GTX media booster. Osteo supplement = 10 mM beta-glycerophosphate and 50 µg mL¯1 L-ascorbic acid. BMP-2 concentration = 100 ng mL $^{-1}$. Thick vascularized tissue is analyzed at various time points to visualize maturation. (c) Collagen-I (pink) deposition is not observed in acellular scaffolds; by contrast, the thick vascularized tissues have very little collagen in regions near hMSCs and HNDFs after 3 days; however, by day 30, printed hMSCs have produced significant collagen in both filaments and circumferentially around the vascular channels. [Scale bars = 100 µm] (d) The delivery of an osteogenic cocktail through the vascular network leads first to the osteogenic lineage commitment of hMSCs, then to mineral deposition over the course of 30 days. This is evidenced by appearance of alkaline phosphatase expression, which is observable via fast blue, and subsequent calcium phosphate mineral deposition – observable via alizarin red stain. [Scale bars = 100 µm] (e-f) SEM/EDS of mineral deposits within printed thick vascularized tissues. (i) SEM image of collected mineral deposits (ii) elemental analysis in both a 30 day osteogenic sample (e) and (f) an acellular tissue perfused with osteogenic media. EDS of extracted particulates (e) possess distinct calcium and phosphorous peaks that are not present in (f). [Scale bars = 1 µm] [Note: The strong silicon peak arises from the silicon substrate on which the tissue sample is deposited.]

Supporting Table 1. Summary of staining protocols and reagents

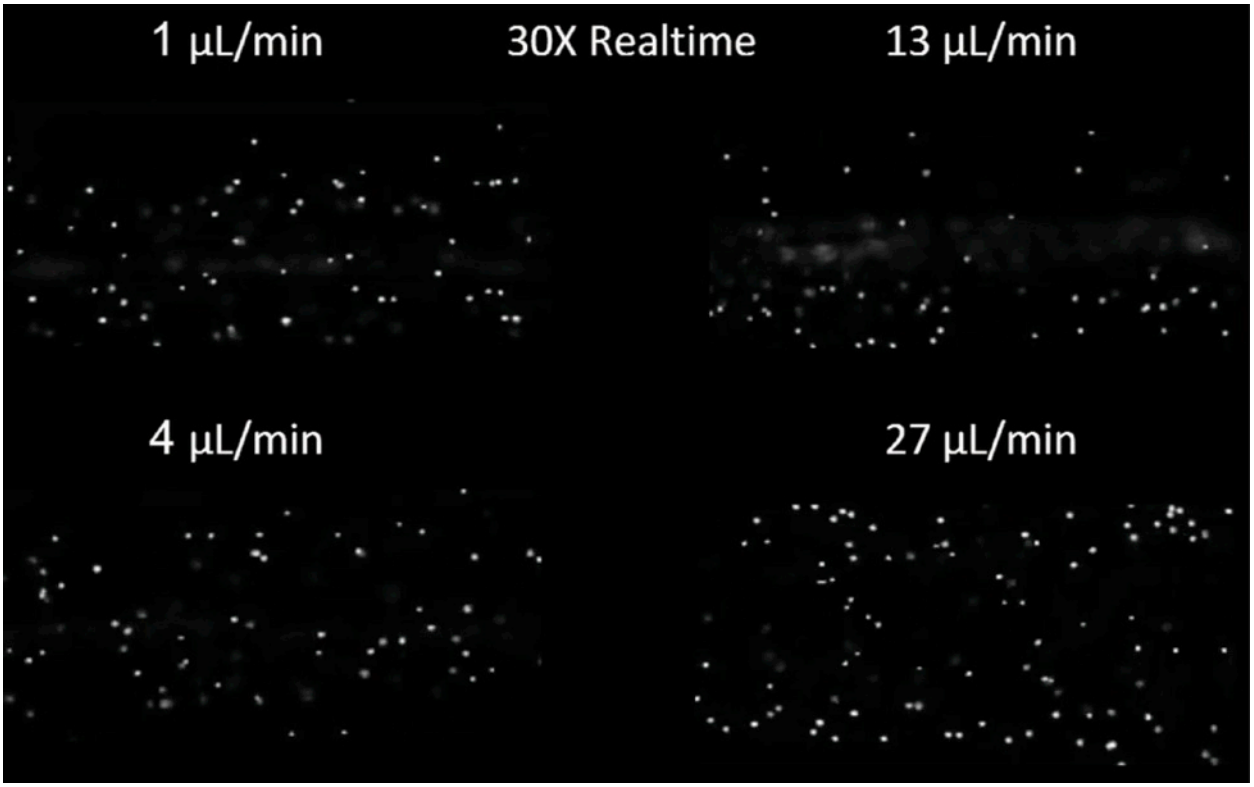
Antibody or stain	Source	Catalog #	Host Species & Reactivity	Concentration
CD31	abcam	ab24590	Mouse anti-human	1 : 200
VECadherin	Cell Signaling Technology	D87F2	Rabbit anti-human	1 : 250
von WIllebrand Factor	abcam	ab194405	Mouse anti-human	1 : 250
Alpha-smooth muscle actin	abcam	ab18147	Mouse anti-human	1 : 250
Osteocalcin	abcam	ab13418	Mouse anti-human	1 : 200
Collagen I	abcam	ab34710	Rabbit anti-human	1 : 400
ActinGreen	Life Technologies	R37110	N/A	2 drops per mL
NucBlue	Life Technologies	R37605	N/A	2 drops per mL
Fast blue	Sigma Aldrich	B5655	N/A	20 mg per mL
Alizarin Red	Sigma Aldrich	A5533	N/A	50 µg per mL



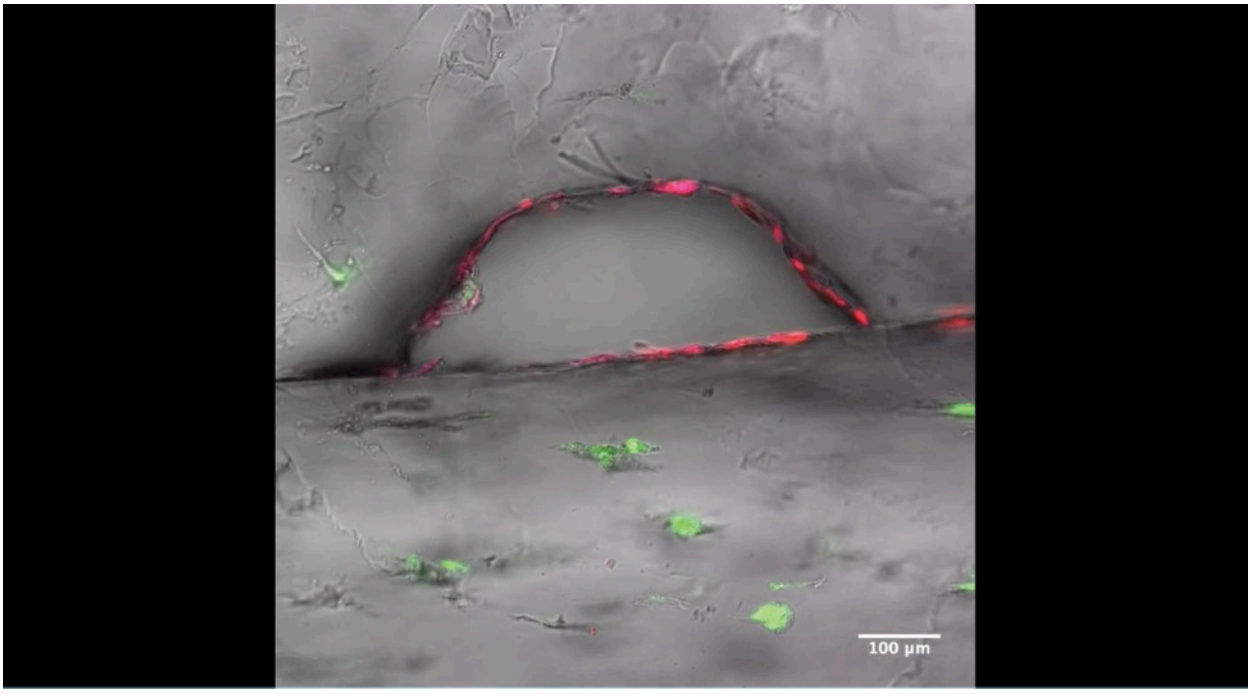
Movie S1. Time-lapse movie of printing the 3D perfusion chip using a silicone ink.



Movie S2. Video of 3D bioprinting of thick vascularized tissues.



Movie S3. Fluorescent microscopy video of different perfusion rates through the embedded vasculature within the printed 3D tissue microenvironments.



Movie S4. Confocal microscopy video of cross-section through vascularized tissue after 45 days of perfusion.