

Supporting Information

4D hydrogel for dynamic cell culture with orthogonal, wavelength-dependent mechanical and biochemical cues

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Methods

Synthesis of cyclo[RGD(DMNPB)fC].

Cyclic RGDfC peptide was synthesized according to a previous protocol.^[1] Briefly, a TCP -L-Gly-Fmoc resin (Intavis Peptides, Gly loading: 0.42 mmol/g) was deprotected using 20% piperidine in DMF, and then Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Cys(Trt)-OH, Fmoc-D-Phe-OH, and Fmoc-L-Asp(DMNPB)-OH were sequentially coupled in DMF, using HBTU/HOBt/DIPEA coupling mixture. After final Fmoc deprotection, the linear peptide was cleaved from the resin using AcOH:TFE:DCM (3:1:6) mixture and evaporated. Cyclization was performed in DMF using diphenylphosphoryl azide (DPPA) as coupling reagent and NaHCO₃ as base. Crude product was purified by precipitation in diisopropylether followed by preparative RP-HPLC (Reprosil 100 C18 column, gradient from 20%B to 100%B in 33 min; A: H₂O + 0.1% TFA; B: CH₃CN + 0.1% TFA+ 5 % H₂O), and freeze drying to obtain the cyclic deprotected peptide c[RGD(DMNPB)fC] as a yellow solid. ESI-MS+: 816.30 (M+H).

Synthesis of Dextran-methacrylate.

Dextran (MP Biomedicals, MW 86,000 kDa) was methacrylated by the reaction with glycidyl methacrylate, according to a previously reported procedure.^[2] In brief, dextran (20 g) and 4-dimethylaminopyridine (2 g) were dissolved in anhydrous dimethyl sulfoxide (100 mL) under vigorous stirring. Glycidyl methacrylate (24.6 mL) was added and the reaction mixture was heated to 45 °C for 24 h. The solution was cooled on ice for 20 min and precipitated into 1 L ice cold 2-isopropanol. The crude product was recovered by centrifugation, redissolved in milli-Q water and dialyzed against milli-Q water for 3 days. The final product was lyophilized and stored at -20 °C until use. The final product, named as DexMA, was characterized by ¹H-NMR (Fig. S1). The degree of functionalization was calculated as the ratio of the averaged methacrylate proton integral (6.22 ppm and 5.78 ppm in D₂O) and the anomeric proton of the glycopyranosyl ring (5.25 ppm and 4.99 ppm). Since the signal of the anomeric proton of α-1,3 linkages (5.25 ppm) partially overlaps with other protons, a pre-determined ratio of 4% α-1,3 linkages was assumed and the total anomeric proton integral was calculated solely based on the integral at 4.99 ppm. A methacrylate: dextran repeat unit ratio of 0.77 was determined.

Synthesis of methacrylated Alexa 647.

Alexa Fluor™ 647 NHS ester (succinimidyl ester) was methacrylated by reaction with 2-aminoethyl methacrylate hydrochloride. Alexa Fluor™ 647 NHS ester (succinimidyl ester) (1 mg) was dissolved

in anhydrous dimethyl sulfoxide (100 μL). 0.6 mg 2-Aminoethyl methacrylate hydrochloride was dissolved in 60 μL 0.2M sodium bicarbonate buffer (pH=8). 8 μL Alexa Fluor™ 647 NHS ester (succinimidyl ester) solution was added to the mixture and stirred at room temperature for 1 hour. The product was used without further purification.

Gel formation

DexMA (20 wt%) was dissolved in TEOA buffer (pH 8.0) inside sterile laminar flow. Solutions of cyclo(RGDfC) (3.45 mgmL^{-1} , 5 mM) or cyclo[RGD(DMNPB)fC] (4.2 mgmL^{-1} , 5 mM) were also prepared in sterile TEOA buffer (pH 8.0). These concentrations were kept constant during all experiments. DexMA (20 wt%, 10 μL) was mixed with cyclo(RGDfC) or cyclo[RGD(DMNPB)fC] (5 mM in TEOA buffer, 5 μL) and incubated for 30 min at 37 °C. The CD link (20 mmol/L thiol groups, 2.5 μL) and TEOA buffer (1.5 μL) were added to the solution and the gel was formed after incubated for 15 min at 37 °C.

Photolysis of cyclo[RGD(DMNPB)fC] in Dextran hydrogel

Photolysis of cyclo[RGD(DMNPB)fC] in dexMA hydrogel was monitored by binding cyclo[RGD(PMNB)fC] (1 mM) in dexMA hydrogels (prepared by the protocol described above) and irradiation at 360 nm (1.2 mWcm^{-2}) with LUMOS 43 (Atlas Photonics Inc.) for different time intervals, followed by washing with PBS to remove photolyzed by-product. After irradiation, photolyzed by-products were washed with water. Data were calculated from the absorbance values at $\lambda_{\text{max}} = 360$ nm by assuming 100% conversion at full photolysis.

Stiffness measurement of DexMA hydrogel

DexMA (20 wt%, 10 μL) was mixed with cyclo(RGDfC) (5 mM, 5 μL) and kept at 37 °C for 30 min. TEOA buffer (1.5 μL , pH 8.0), different amount (2.5 μL , 1.9 μL or 1.25 μL) of CD linker (20 mmol/L thiol groups) and Eosin Y (1 μL , 100 μM , pH 8.0) was added to this mixture and one drop of the mixture (10 μL) was loaded on the lower plate of a rheometer (8 mm parallel plate geometry). The upper plate of rheometer was adjusted above the sample and the area surrounding the drop was sealed with silicone oil to avoid evaporation. Time sweep measurements were performed at 25°C, with 0.5% applied strain at 0.5 N force and 0.1 rad/sec oscillation amplitude. Visible light exposure was applied through OmniCure® S2000 UV curing lamp system with a 400-500 nm filter (11.3 mWcm^{-2}).

Fluorescence patterns with methacrylated Alexa 647

Hydrogels were swollen in a solution containing 1 mg mL^{-1} methacrylated Alexa 647 and eosin Y (10 μM) for 5 mins. Gels were irradiated through a mask (200 μm glass with 200 μm chrome) by illumination at 470 nm (1.2 mWcm^{-2}) for 10 min with LED lamp (RoHS). Unreacted patterning agent and initiator were swollen into fresh media as the sample was gently agitated on an orbital shaker (2 hours), yielding the final patterned hydrogel. The sample was placed in Zeiss LSM 800 microscope and scanned with 488 nm laser with different scanning depth or time and predefined patterns were drawn by Zeiss LSM 800 ROI tool. In both the masked and scanned photopolymerization the photocoupling of the acrylate Alexa 647 was confined to volumes exposed to light within the material and was visualized by fluorescent confocal microscopy. The z-stack of obtained pattern was created by using confocal Zeiss LSM 800 microscope in Alexa 647 channel.

Fluorescence patterns with photoactivatable CMNB-caged fluorescein

DexMA (20 wt%, 10 μL) was mixed with NH_2 -PEG-SH (3.5 kDA, 1 mM in TEOA buffer, 1 μL) and incubated for 15 min at 37°C. CMNB-caged Fluorescein (Invitrogen, 10 mM in DMSO, 1 μL) was

added to the above solution and incubated further for 15 min at 37 °C. CD linker (20 mmol/L thiol groups, 2.5 µL) and TEOA buffer (5 µL) was added to the solution and the gel formed after incubated for 15 min at 37°C. Gels containing 1 mM photoactivatable fluorescein were irradiated through a chrome mask (200 µm glass with 200 µm chrome stripes) by illumination at 360 nm (1.2 mWcm⁻²) for 5 min with LED lamp (RoHS). Alternatively, the gels were placed in Zeiss LSM 800 fluorescence microscope and scanned with Rapp laser (500 mWcm⁻²) at 375 nm and predefined patterns were drawn by Rapp ROI tool. The z-stack of obtained pattern was created by using confocal Zeiss LSM 800 microscope in GFP channel.

Cell culture

Fibroblast L929 cell line was cultivated at 37 °C in 5 % CO₂ in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (Invitrogen) and 1 % P/S (Invitrogen). Cells were used between passages P4 and P16.

Live dead assay

Cell culture medium was removed and samples were incubated for 5 min with fluorescein diacetate (40 µgmL⁻¹, Sigma, F7378) and propidium iodide (30 µgmL⁻¹, Sigma, P4170) in PBS. Samples were washed twice with PBS and imaged with Zeiss LSM 800 confocal microscope.

Spheroid formation

20,000 cells were seeded in each well of a Corning® Costar® Ultra-Low attachment multiwell plate. Cell culture was incubated for 48 h. Cell medium was then removed carefully and the spheroids were washed once with PBS. Spheroids were collected into an Eppendorf and spin down for a short time at low speed (30 sec, 300 rpm). PBS was carefully removed. DexMA solution (20 wt%, 10µL) was mixed with cyclo(RGDfC) (5 mM, 5µL) and incubated at 37 °C for 30 min. The mixture was added to resuspend the spheroids. CD linker (20 mmol/L thiol groups, 2.5 µL) was added and the solution was placed in Ibidi® 15 µwell angiogenesis slide. After incubation for 15 mins at 37 °C, 50 µL cell medium was added on top of the hydrogel.

After 30 mins incubation in media, the media was replaced with PBS before UV light irradiation. UV light irradiation was performed at 360 nm (1.2 mWcm⁻²) with LED lamp (RoHS) for different time intervals, followed by washing with fresh media to remove photolyzed by-product.

For Vis light irradiation, the eosin Y (10 mM) was added during gelation. The gels were exposed to 420 nm (1.2 mWcm⁻²) with LED lamp (RoHS) for different time intervals. The eosin Y was removed by exchanging media three times after light exposure.

RGD or stiffness channels were patterned into the network with UV or visible light patterning ($\lambda = 375$ nm or 488 nm). Before stiffness patterning, the media was supplemented with eosin Y (10 mM), equilibrated for 30 mins, and selected regions within the gel were physically stiffened by $\lambda = 488$ nm. After light exposure, the gel was washed immediately 3 times with fresh medium. The samples were kept in cell culture incubator, observed after every 12 h and kept in culture for 3-5 days. Cell culture medium was substituted with fresh medium every day. The hydrogels were fixed in 4% PFA for 1h, followed by cell permeabilization with 0.5% Triton® X-100 (Fisher) in PBS for 2 h. The samples were blocked with 1% bovine serum albumin (BSA, Sigma) in PBS for 1 h and rinsed with PBS. F-actin was visualized using Alexa Fluor® 546 Phalloidin Conjugate (5 U/mL, Invitrogen), while nuclei were stained with DAPI (500 nM, Invitrogen), each for 1 h. The samples were washed with PBS prior to confocal visualization.

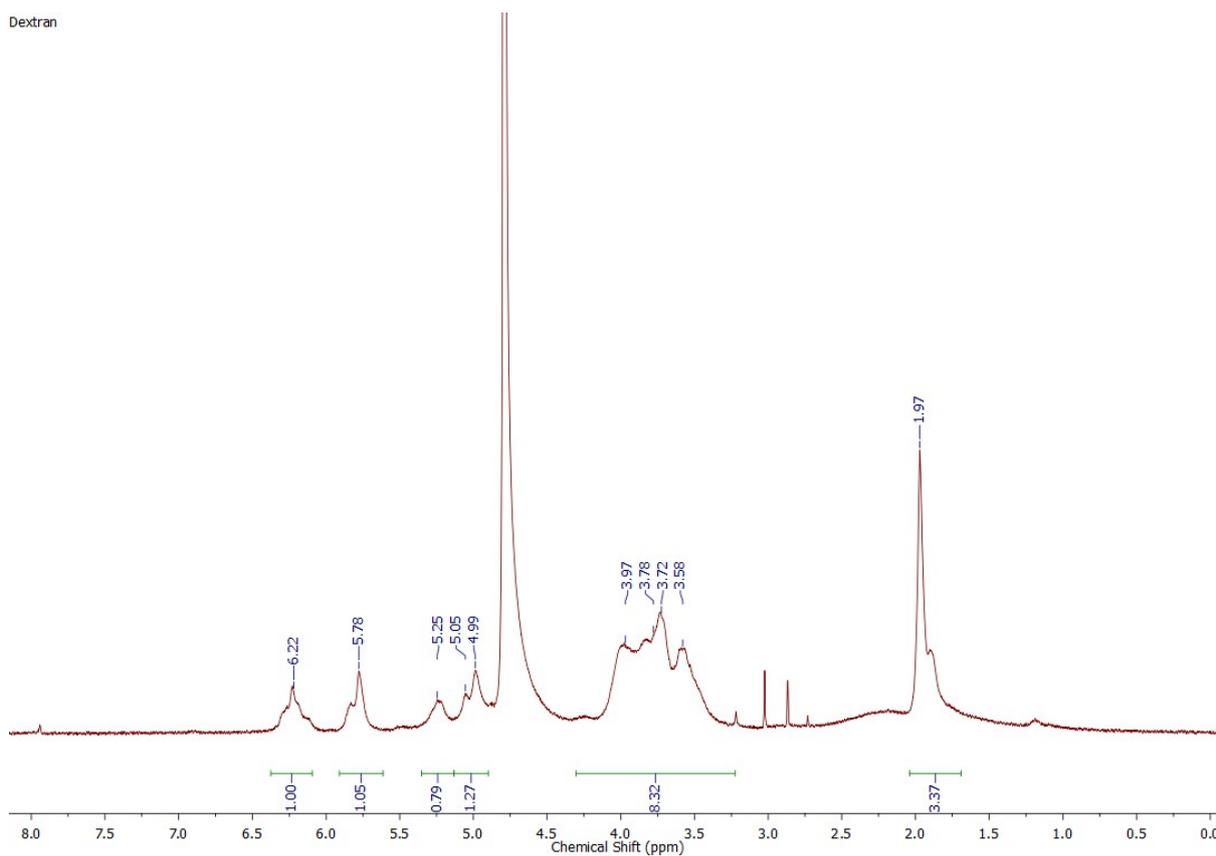


Figure S1. ^1H NMR spectrum of methacrylated dextran (DexMA) in D_2O . The degree of functionalization was calculated as the ratio of the averaged methacrylate proton integral (6.22 ppm and 5.78 ppm in D_2O) and the anomeric proton of the glycopyranosyl ring (5.25 ppm and 4.99 ppm). Since the signal of the anomeric proton of α 1,3 linkages (5.25 ppm) partially overlaps with other protons, a pre-determined ratio of 4% α -1,3 linkages⁴³ was assumed and the total anomeric proton integral was calculated solely based on the integral at 4.99 ppm. A methacrylate:dextran repeat unit ratio of 0.77 was determined.

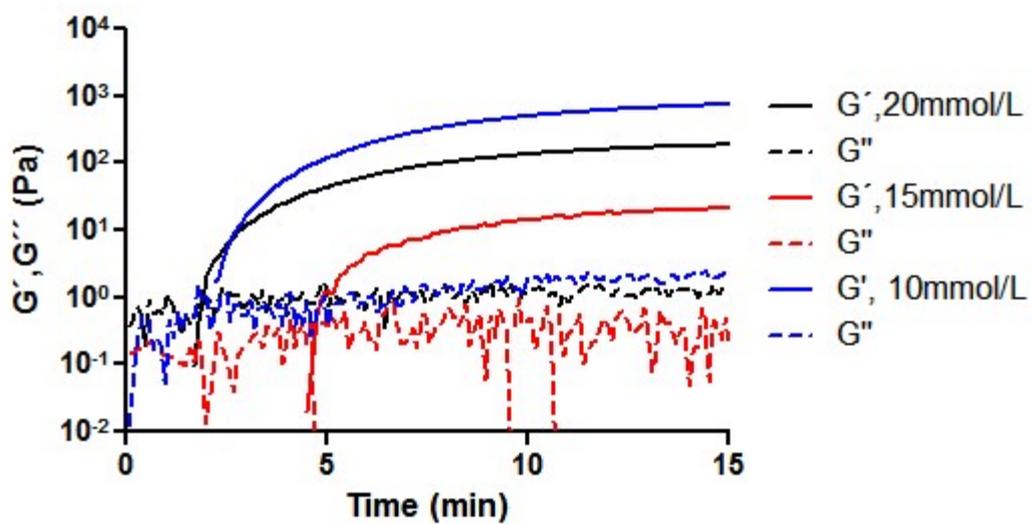


Figure S2. Storage modulus (G' , solid lines) and loss modulus (G'' , dash lined) as a function of time obtained in a rheology measurement during gelation of dextran-MA (20 wt%) in TEOA buffer (pH 8) using different amount of MMP degradable link.

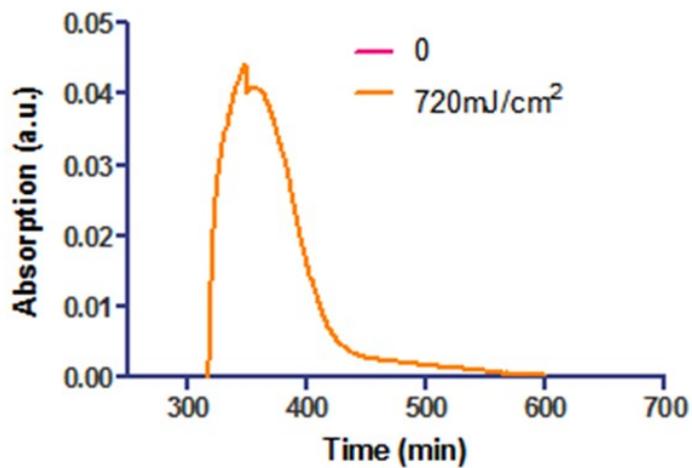


Figure S3. UV-Vis spectra of a Dextran hydrogel film functionalized with cyclo[RGD(DMNPB)FC] (1 mM) with 10 min of light irradiation (450 nm, 1.2 mW/cm²).

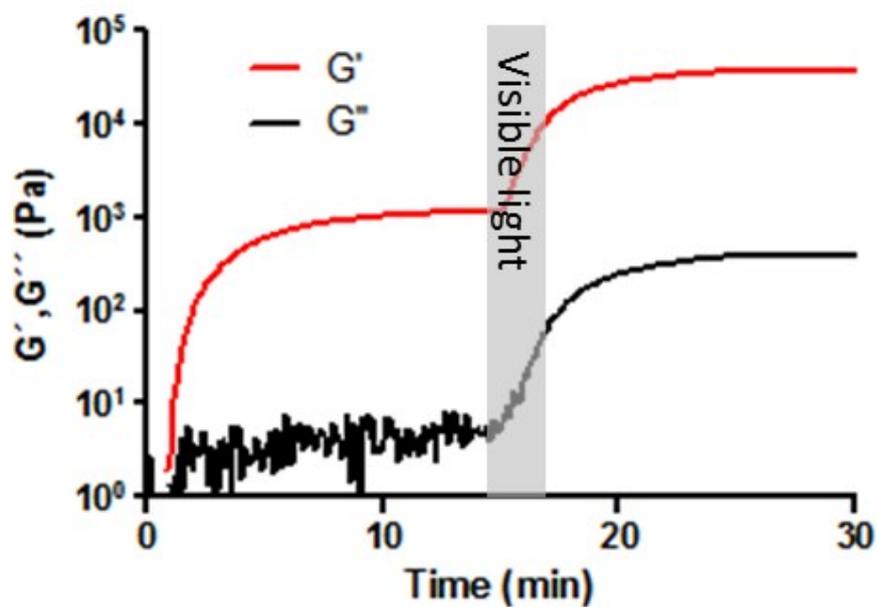


Figure S4. Rheology profiles showing hydrogel formation via addition reaction only (0-15 min) and hydrogel stiffening by radical polymerization (soft to stiff) after visible light irradiation (400-500 nm) for 2 min.

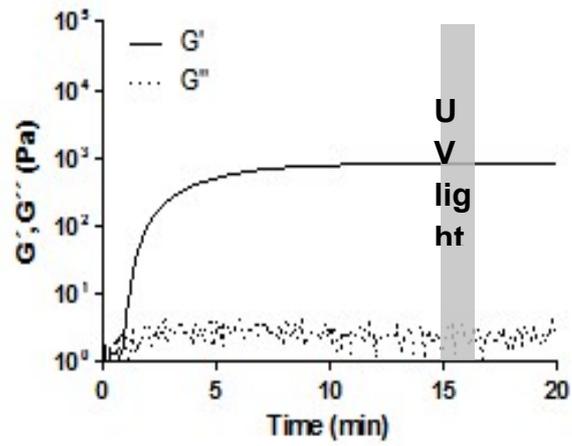


Figure S5. Rheology profiles showing hydrogel formation via addition reaction only (0-15 min) and no stiffness change in the absence of photo initiator after UV irradiation (365 nm) for 2 min.

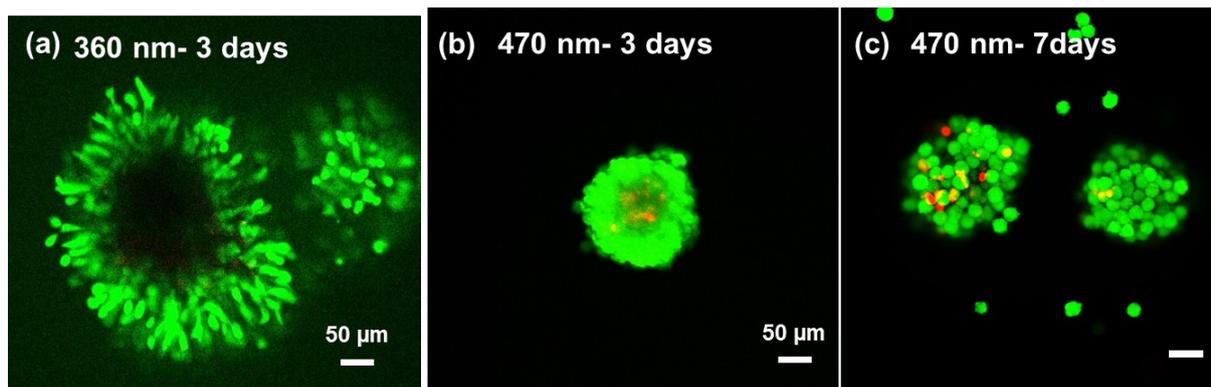


Figure S6. Life and dead assay of fibroblasts spheroids performed on Day 3 and Day 7 after exposure to (a) 360 nm, 1.2 mW/cm² for 5 mins and (b) 470 nm, 1.2 mW/cm² for 10 mins.

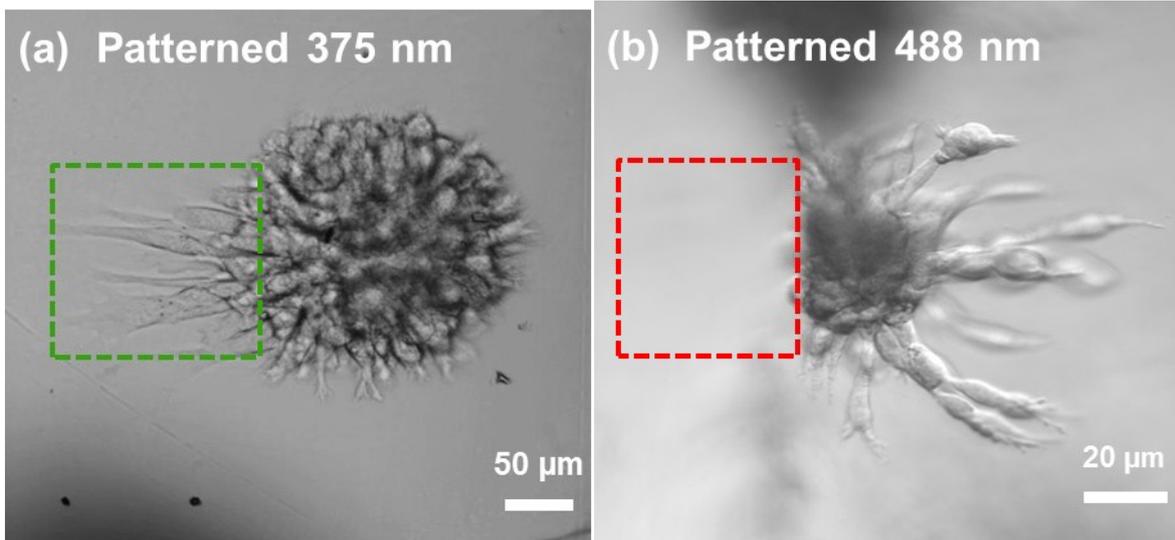


Figure S7. (a) Patterned adhesive cyclo[RGD(DMNPB)fC] functionalized (500 mW/cm^2 , 1s) after 5 days. (b) Patterned stiffening RGDfC functionalized (488 nm, speed 1) hydrogels after 5 days.

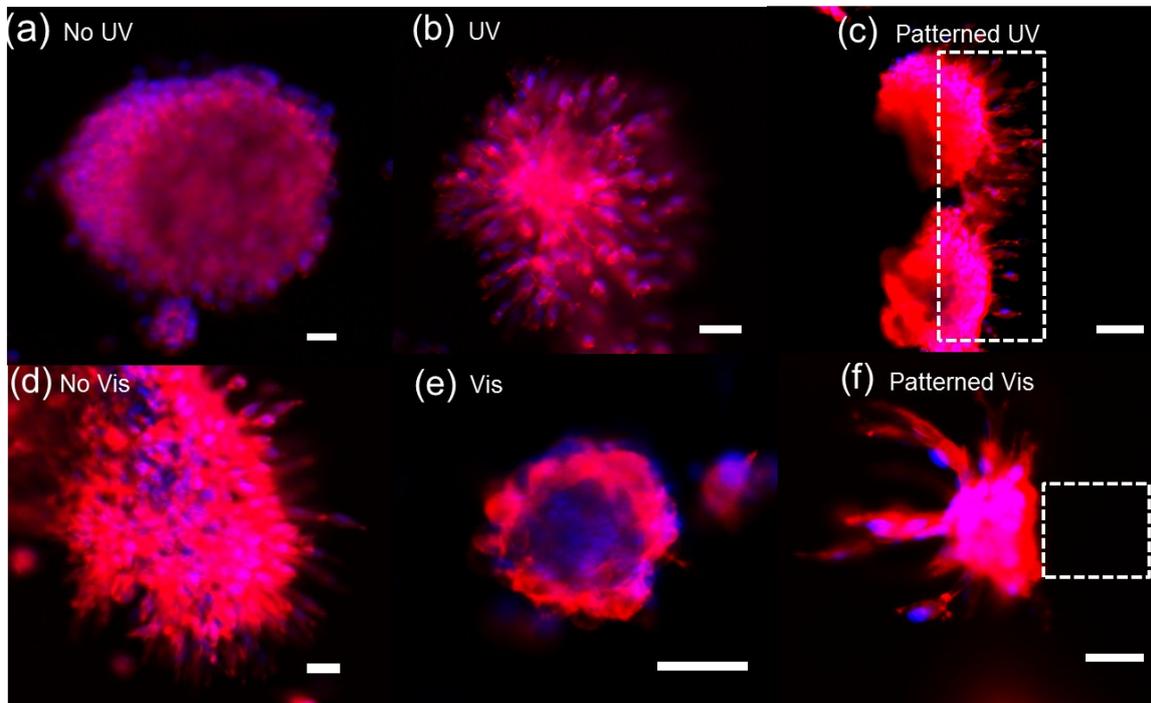


Figure S8. Immunofluorescence images of fibroblasts spheroids within (a) nonadhesive, (b) fully adhesive (360 nm, 1.2 mW/cm², 5 mins) or (c) patterned adhesive cyclo[RGD(DMNPB)fC] functionalized (500 mW/cm², 1s) after 5 days. Fibroblasts spheroids within (d) nonstiffening, (e) fully stiffening (470 nm, 1.2 mW/cm², 10 mins) or (f) patterned stiffening RGDfC functionalized (488 nm, speed 1) hydrogels after 5 days. The regions of light irradiation areas are depicted by the dashed square in (c) and (f). Scale bars 20 μ m.

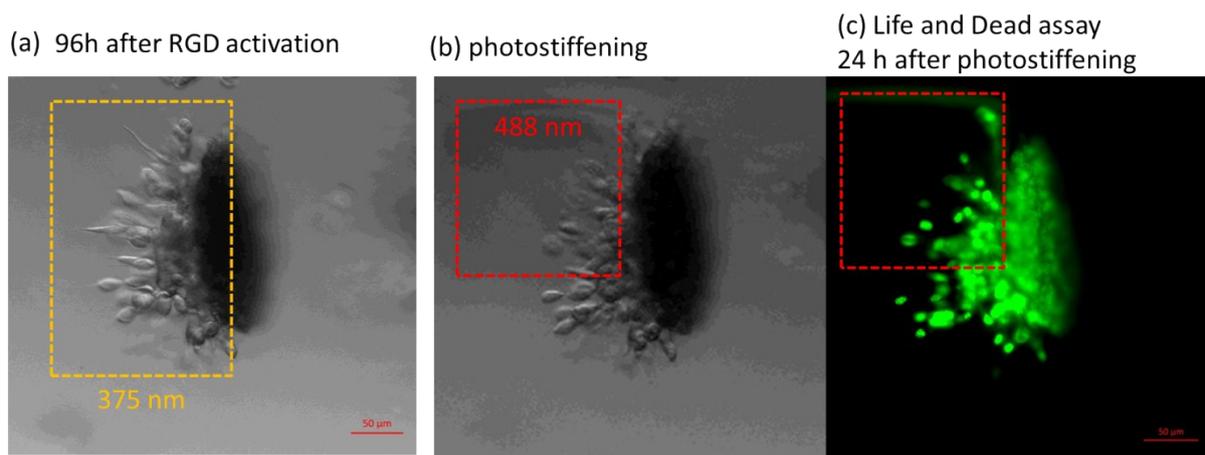


Figure S9. (a) fibroblast spheroids 96 h after RGD activation. (b) Photostiffening and (c) Life and Dead assay 24 h after photostiffening.

References:

[1] T. T. Lee, J. R. García, J. I. Paez, A. Singh, E. A. Phelps, S. Weis, Z. Shafiq, A. Shekaran, A. del Campo, A. J. García, *Nat. Mater.* 2015, 14, 352.

[2] B. M. Baker, B. Trappmann, W. Y. Wang, M. S. Sakar, I. L. Kim, V. B. Shenoy, J. A. Burdick, C. S. Chen, *Nat. Mater.* 2015, 14, 1262.