Supporting Information

User-controlled 4D biomaterial degradation with substrate-selective sortase transpeptidases for single-cell biology

*Ross C. Bretherton, Amanda J. Haack, Irina Kopyeva, Fariha Rahman, Jonah D. Kern, Darrian Bugg, Ashleigh B. Theberge, Jennifer Davis, and Cole A. DeForest**

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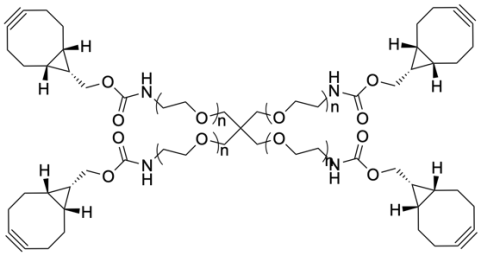
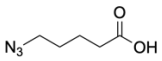
Experimental Procedures

Synthesis of previously reported compounds

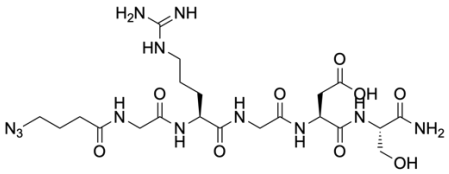
PEG-tetraBCN



N_3-COOH



RGD-azide



Poly(ethylene glycol) tetra-bicyclononyne (PEG-tetraBCN, M_n = 20 kDa), 4-azidobutanoic acid (N_3-COOH), and N_3-GRGDS-NH_2 (RGD-azide) were synthesized and characterized as previously described.[1,2]

Method S1 – Isolation and sortase treatment of primary cardiac fibroblasts for RNAseq

All animal procedures were conducted under a protocol approved by the University of Washington Institutional Animal Care and Use Committee. Fibroblasts were isolated from the hearts of wild type FVB/NJ mice at 2 mos of age by retrograde Langendorff perfusion of collagenase (2 mg mL^{-1}; Worthington Biochemical; Lakewood, NJ) and Liberase TH (0.4 mg mL^{-1}; Roche; Mannheim, DE) in a modified Krebs-Henselheit Buffer (113 mM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 12 μM $NaHCO_3$, 10 μM $KHCO_3$, 10.8 μM HEPES) as previously described.[3,4] Following 15 mins of perfusion, hearts were minced and digested further for 30 mins on a rocking platform in a tissue culture incubator, with intermittent trituration. Digestion was halted in Dulbecco's Modified Eagle Medium (DMEM) with high glucose containing 20% fetal bovine serum (FBS), and cells were collected via centrifugation. Cells were expanded over two passages in DMEM with high glucose and 20% FBS prior to use.

Sortase A pentamutant (5M) in pET29, eSrtA(2A-9) (2A9) in pET29b, and eSrtA(4S-9) (4S9) in pET29b were gifts from David Liu (Addgene Plasmids #75144, #75145, #75146). Electrically competent BL21 cells were individual transformed with expression plasmid and selected on kanamycin-containing LB agar plates. 5 mL Luria Broth (LB) with kanamycin was inoculated with plasmid-expressing colony and expanded overnight at 37 °C under agitation (200 rpm). The following morning, 500 mL of LB with kanamycin was inoculated with 5 mL overnight culture and incubated at 37 °C, 200 rpm until OD600 reached 0.6. Isopropyl β-d-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mM to induce protein expression, which was carried out at 18 °C, 200 rpm overnight. Cells were centrifugally pelleted, resuspended in ice-cold lysis buffer (20 mM Tris, 50 mM NaCl, 5 mM imidazole; pH 7.52) containing Pierce protease inhibitor (Thermo Scientific; Waltham, MA), and sonicated 6x on a 3 min cycle with 30% amplitude (Fisher Scientific; Waltham, MA). The lysate was clarified by centrifugation (5000 x g, 5mins) and sterile filtered through a 0.45 μm syringe filter (Sartorius; Göttingen, DE). Sortase protein was then purified from the clarified

lysate using an ÄKTA Pure 25 L FPLC (Cytiva; Marlborough, MA) equipped with a 5 mL HisTrap HP column at a flow rate of 5 mL min^{-1}. The column was first equilibrated with 5 column volumes of lysis buffer, after which the sample was loaded and washed with 8 column volumes of endotoxin removal buffer (20 mM Tris, 50 mM NaCl, 20 mM imidazole; 0.1% Triton-X 114; pH 7.5) and 8 volumes of wash buffer (20 mM Tris, 50 mM NaCl, 20 mM imidazole; pH 7.5) while monitoring Triton-X 114 removal by ultraviolet (UV) absorbance (λ = 280 nm). The sample was then eluted over an 8-column volume gradient from 5-250 mM imidazole into a 96-well plate. Purified protein-containing fractions were pooled and dialyzed into reaction buffer (20 mM tris, 50 mM NaCl; pH 7.56) for dye-release studies or phosphate-buffered saline (PBS) for cell culture experiments. For long-term storage, purified sortases were concentrated using an Amicon Ultra 15 centrifugation filter (10 kDa cutoff), diluted in buffer containing 20% glycerol to 2x working concentration (100 μM), and frozen in working aliquots to avoid repeated freeze-thaw cycles. Sortase purity was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sortase identity was confirmed by electrospray ionization mass spectrometry on a Sciex 5600 mass spectrometer (AB Sciex; Framingham, MA).

Fibroblasts at 80% confluency on a six-well plate were treated with either a sortase reaction solution (50 μM sortase, 18 mM triglycine (GGG; Sigma Aldrich; St. Louis, MO) in DMEM with 20% glycerol) or control containing GGG and glycerol but no sortase enzyme. After 45 mins of treatment at 37 °C, cells were briefly rinsed with 1X PBS and then lysed *in situ* with 200 μL of TRIZol reagent (Thermo Fisher Scientific; Waltham, MA). RNA was isolated from lysate using a Direct-Zol RNA microprep kit (Zymo Research; Irvine, CA) according to manufacturer's instructions. RNA integrity was verified using an Agilent Tapestation 4200 and RNA Screentape (Agilent Technologies; Santa Clara, CA). After flash freezing on liquid nitrogen, samples were transferred to BGI Genomics for strand-specific paired-end RNA library prep and sequencing on the DNBseq platform (20 million clean reads per sample).

Cleaned reads from RNASeq were aligned to the *mus musculus* mm10 genome using RNA STAR on the Galaxy Web Server.[5,6] Reads were then counted using featurecounts, and differential expression was evaluated using EdgeR.[7,8] Differentially expressed genes were input into G:Profiler for pathway enrichment analysis against the Reactome and Wikipathways databases.[9–11]

For the experiments shown in Figure S2, fibroblasts were treated sequentially with the sortase variants under the same conditions as above, with a brief PBS rinse between each treatment, then lysed for RNA. Isolated RNA was reverse transcribed using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific; Waltham, MA), and amplified by real-time PCR on a CFX96 Touch Real Time PCR Detection System (Bio-Rad; Hercules, CA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad; Hercules, CA) and the primers listed below.

Gene Name	**Forward Primer**	**Reverse Primer**
C3	5'-CCAGCTCCCCATTAGCTCTG-3'	5'-GCACTTGCCTCTTTAGGAAGTC-3'
Clu	5'-AGCAGGAGGTCTCTGACAATG-3'	5'-GGCTTCCTCTAAACTGTTGAGC-3'
Col1a2	5'-TTGTGGATACGCGGACTCTG-3'	5'-CTGAGCAGCAAAGTTCCCAG-3'

18s	5'-GTAACCCGTTGAACCCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'

Method S2 – Synthesis of orthogonal sortase-degradable peptide crosslinkers

The peptides H-RGLPETGGRK(dde)-NH_2, H-RGLAETGGRK(dde)-NH_2, H-RGLPESGGRK(dde)-NH_2, H-RGPQGIWGQLPETGGRK(dde)-NH_2, H-RGPQGIWGQLAETGGRK(dde)-NH_2, H-RGPQGIWGQLPESGGRK(dde)-NH_2, and H-RGTEGLPGRK(dde)-NH_2 were synthesized on Rink Amide resin (0.5 mmol scale) using standard Fluorenylmethyloxycarbonyl (Fmoc) microwave-assisted solid-phase peptide synthesis techniques on a Liberty1 Microwave Peptide Synthesizer (CEM; Matthews, NC). Resin-bound peptide was transferred to a manual synthesis vessel and the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (dde) protecting group was removed in 15 mL 2% hydrazine solution in dimethylformamide (DMF; 3 x 10 mins). N-terminal and lysine-attached azides were conjugated by reacting N_3-COOH (2 mmol, 259 mg) and O-(7-Azabenzotriazol-1-YL)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU, 750 mg, 1.97 mmol) and DIEA (1.38 mL, 4 mmol) in 5 mL DMF for 90 min at room temperature (RT). Azide coupling was verified via a Kaiser test to confirm the absence of free amines.[12] Peptide was cleaved in 15 mL trifluoracetic acid (TFA) containing triisopropylsilane (TIS) and water (95:2.5:2.5 vol/vol %). Crude product was precipitated by dilution 10-fold into 150 mL of ice-cold diethyl ether and pelleted by centrifugation. The pellet was washed twice with diethyl ether and dried under nitrogen. Semi-prep scale reversed-phase high-performance liquid chromatography (RP-HPLC) was used to purify product through a C18 column with a linear acetonitrile gradient (5-100%) with 0.1% TFA in water. Pure fractions containing product were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Autoflex II; Billerica, MA) and lyophilized. Pure peptide was reconstituted in PBS containing 20% dimethyl sulfoxide (DMSO), adjusted to pH 7 using 6 M NaOH, and stored in working aliquots at -80 °C. Final diazide- modified peptide crosslinkers were N_3-RGLPETGGRK(N_3)-NH_2 (denoted in the manuscript as LPETG); N_3-RGLAETGGRK(N_3)-NH_2 (LAETG), N_3-RGLPESGGRK(N_3)-NH_2 (LPESG), N_3-RGPQGIWGQLPETGGRK(N_3)-NH_2 (MMP ∨ LPETG, where ∨ is the Boolean logic symbol for an OR-type operation), N_3-RGPQGIWGQLAETGGRK(N_3)-NH_2 (MMP ∨ LAETG), and N_3-RGPQGIWGQLPESGGRK(N_3)-NH_2 (MMP ∨ LPESG), and N_3-RGTEGLPGRK(N_3)-NH_2 (TEGLP sortase-insensitive scramble).

Method S3 – Rheometry

Gel formation kinetics and plateau modulus were measured at 37 °C using 8 mm parallel plate geometry (Gap: 0.5 mm; Strain: 1%; Frequency: 1 Hz) on a Physica MCR301 rheometer (Anton Paar; Graz, AT) for 30 µL hydrogels containing 4 mM PEG-tetraBCN and 8 mM di-azide peptide crosslinker. Frequency and strain were verified to fall within the linear viscoelastic regime for this material by frequency and amplitude sweep. Plateau modulus was estimated as the storage modulus after 60 mins of gelation for each sample. Experiments were performed in triplicate.

Method S4 – Cell encapsulation and viability

HS5 immortalized human bone marrow stromal cells (HS5, HS5-mTagBFP2, HS5-eGFP, and HS5-mCherry) were a gift from Dr. Brian Hayes at the Fred Hutchison Cancer Research Center. Prior to encapsulation, cell lines were maintained in high glucose DMEM (Corning; Corning, NY) with 10% FBS (Thermo Fisher; Waltham, MA) and passaged 1:5 when confluent. At 80% confluence, HS5 cells were encapsulated at 10^7 cells mL^{-1} in 5 µL droplet hydrogels containing 4 mM PEG-tetraBCN, 8 mM di-azide peptide crosslinker, and 1 mM RGD-azide. After gelation for 30 mins at 37 °C, media was added and cells were cultured in the gels for 7 days. DMEM with 50 µM sortase and 18mM GGG was added to each gel to induce dissolution. One hour following dissolution, cell viability was analyzed using a LIVE/DEAD cell viability assay (calcein AM / ethidium homodimer) according to manufacturer's instructions (Thermo Fisher; Waltham, MA). Gels and released cells were imaged on a Leica SP8 confocal microscope at 10x magnification.

Method S5 – Fluorophore release experiments

PEG-tetraBCN was pre-reacted with AFDye 568 azide (Click Chemistry Tools; Scottsdale, AZ) at a molar ratio of 40:1 to create fluorescent PEG macromer. 10 µL hydrogels were synthesized in Eppendorf microcentrifuge tubes with a final PEG-tetraBCN concentration of 4 mM and 2:1 molar ratio of crosslink to PEG-tetraBCN (8 mM peptide). Following gelation at 37 °C for 60 mins, gels were swollen overnight in sortase reaction buffer (20 mM Tris-base, 50 mM NaCl, pH 7.5). To initiate dissolution, first 200 µL of reaction buffer containing 50 µM sortase enzyme was added to the equilibrated hydrogel for one hour, then replaced by 200 µL of 50 µM sortase and 18 mM GGG (Sigma Aldrich; St. Louis, MO) in buffer. Gels were incubated throughout the dissolution process at 37 °C under agitation at 200 rpm in a shaker incubator. At designated timepoints, 5 µL of the supernatant was transferred to a 96-well plate and diluted in 50 µL buffer. Supernatant fluorescence was measured on a plate reader ($\lambda_{excitation}$ = 570nm; $\lambda_{emission}$ = 610nm; λ_{cutoff} = 590nm) (Molecular Devices; San Jose, CA). Fluorescence readings were normalized to standard curves created for each individual experiment from the supernatant of fully dissolved gels.

Method S6 – Fluorescent sortase diffusion experiments

To fluorescently tag sortase and track its diffusion through PEG-tetraBCN gels, 10 µL of Alexa Fluor 488 NHS (10 mM in DMSO) were added to 1 mL 5M, 2A9, or 4S9 stock (100 µM) and allowed to react at room temperature for one hour. To remove unreacted dye, tagged sortases were then spin concentrated using an Amicon Ultra 15 centrifugation filter and diluted to a final concentration of 100 µM in 1X PBS with 20% glycerol. 5 µL hydrogels were synthesized with a final PEG-tetraBCN concentration of 4 mM, using AFDye 568-tagged PEG-tetraBCN and the sortase-insensitive TEGLP scramble peptide crosslinker (final concentration 8 mM). Gels were swollen overnight in 1X PBS, which was then replaced with 50 µM fluorescent sortase in 1X PBS. Confocal images were acquired at 0, 60, and 120 minutes after sortase addition on a Leica Stellaris 5 confocal microscope, with z-position set to the bottom of the gel.

Method S7 – Patterning device design and fabrication

Patterning devices used to create patterned gels in Figures 4 and 5 were designed in Solidworks 2017 (Dassault Systémes; Vélizy-Villacoublay, FR) and 3D printed out of clear resin (RS-F2-GPCL-04; Formlabs; Somerville, MA)

using a Form 2 stereolithography 3D printer (Formlabs; Somerville, MA). Design files for all patterning devices are included in the Supporting Information and a schematic for the devices are found in Figure S7. All patterning rails were sonicated in isopropyl alcohol (IPA) for 10 mins and again in clean IPA for an additional 5 mins. They were then dried with compressed air and cured under a 395-405 nm 20W UV lamp (Quans) for 1 hour. Devices were exposed to UV in a biohood for 1 hr for sterilization before use with cell culture. To prevent hydrogel or cell adhesion to the patterning rails, devices were incubated in 1% bovine serum albumin (BSA) for 1-2 hrs and allowed to fully air dry prior to use.

Method S8 – Multilayer hydrogel encapsulation and release

Multilayer hydrogels containing layers of HS5-mTagBFP2, HS5-eGFP, and HS5-mCherry cells were formed either by sequential casting of hydrogels between glass slides to form a bullseye pattern (Flow Cytometry Experiments) or by pipetting gel precursor into the patterning devices for sequential casting steps (Imaging Experiments). Gels were rinsed in Hank's Balanced Salt Solution (HBSS) for 5 mins between casting steps to wash away any unencapsulated cells. Composite gels were then sequentially treated with sortases as described above, with each dissolution step proceeding for 1 hr. Released cells were collected and fixed in 4% paraformaldehyde in PBS (10 mins, RT) prior to analysis on a FACSCanto RUO cytometer (BD Bioscience; San Jose, CA). Compensation was set using pure HS5 mTagBFP2/eGFP/mCherry populations. Singlets were gated using forward and side scattering, and fluorescence was measured using the BV421 ($\lambda_{excitation}$ = 405nm; $\lambda_{emission}$ = 405±50nm), EGFP ($\lambda_{excitation}$ = 488 nm; $\lambda_{emission}$ = 530±30nm), and Texas Red ($\lambda_{excitation}$ = 561nm; $\lambda_{emission}$ = 610±20nm) detector configurations (Figure S5). Fixed multilayer gels were also imaged after sequential dissolution steps on a Nikon Ti microscope with a Yokogawa W1 spinning disk head under 10x magnification.

Method S9 – Dual color cardiac fibroblast bullseye experiment

Postn lineage reporter (Postn-m^T/m^G) mice were generated by crossing mice bearing a tamoxifen-inducible Cre cassette knocked into the *Postn* locus ($Postn^{MerCreMer}$) with mice expressing a membrane targeted conditional dual color fluorescent reporter (m^T/m^G) knocked in to *Rosa26* genomic locus.[13,14]

Three days prior to encapsulation in hydrogel bullseyes, primary cardiac fibroblasts from *Postn*-m^T/m^G mice to be used for activated regions were switched to activation media (DMEM containing 2% FBS) containing 10 ng mL^{-1} bovine TGFβ1 (R&D Systems; Minneapolis, MN). Activation media was replenished daily prior to encapsulation at a density of 10 million cells mL^{-1} in gels containing 3 mM PEG tetraBCN, 1.5 mM sortase/MMP degradable crosslink, and 1 mM N_3-GRGDS-NH_2. After 30 mins of polymerization, gels were cultured in DMEM containing 2% FBS and 2.5 μM 4-hydroxytamoxifen (Tocris Bioscience; Bristol, UK) for 7 days, with media changed every other day.

At the experimental endpoint, gels were imaged live on a Leica Stellaris 5 confocal microscope under 10x magnification. Cells expressing tdTomato and eGFP were segmented using the 3D Analysis Module in LASX, and a custom Python script was then used to normalize cell coordinates to position in the bullseye, exclude detected cells more than two standard deviations from the centroid, and generate histograms displaying mean cell count across 3 technical replicates per condition.

For flow cytometry analysis, gels were sequentially degraded with 4S9, 2A9, and 5M under the same conditions as for the multilayer hydrogel release experiment. Following each sortase treatment, the supernatant was collected, and the gels were rinsed once with 1X PBS, which was also pooled with the supernatant. The released cells were collected by centrifugation and fixed in 4% PFA for 10 mins, then rinsed twice with 1X PBS prior to cytometry for tdTomato and eGFP on a BD FACSAria (BD Bioscience; San Jose, CA).

Results and Discussion

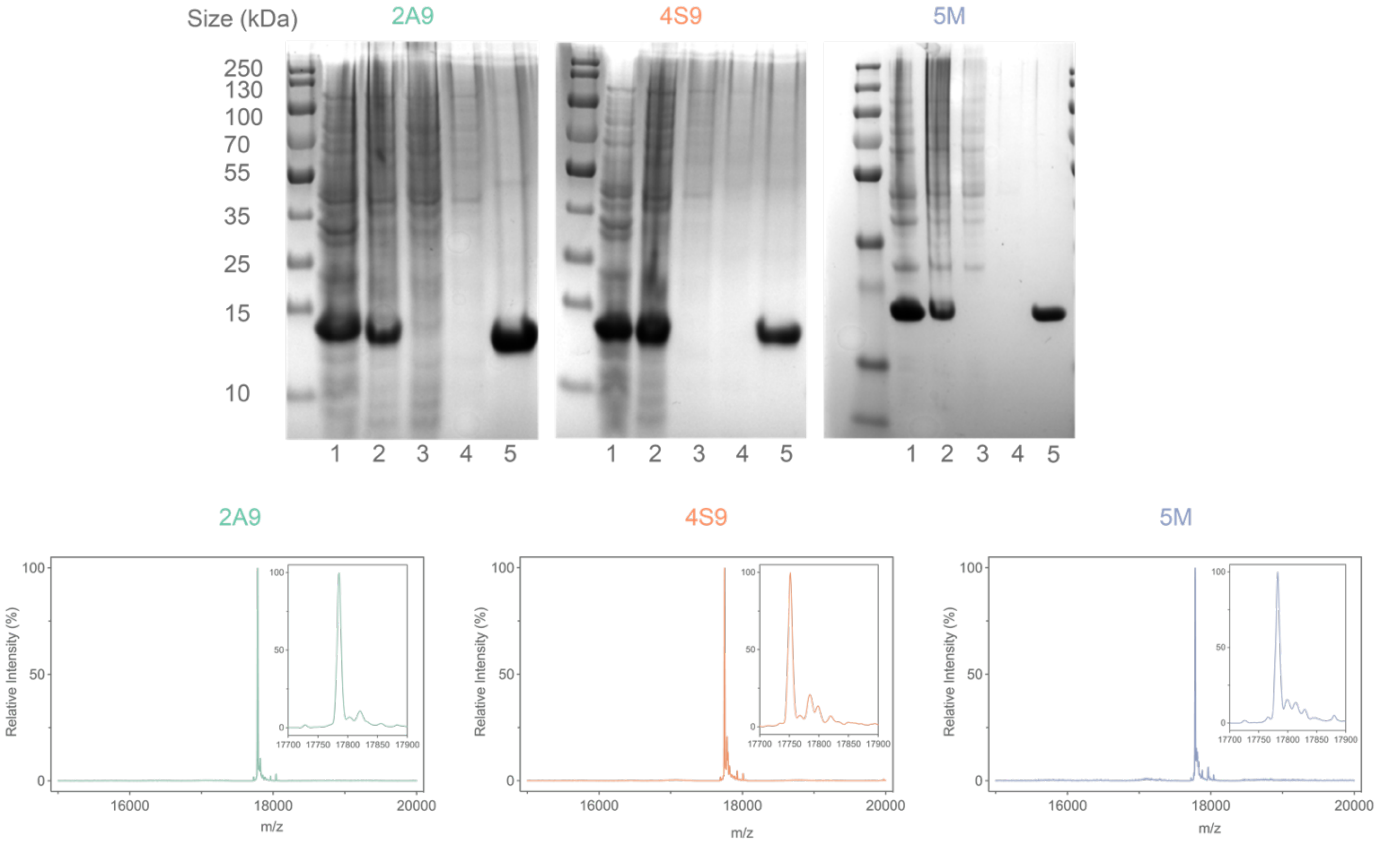


Figure S1 – SDS-PAGE and whole-protein mass spectrometry of evolved sortase enzymes. Samples of whole bacterial cells (1), soluble lysate (2), endotoxin removal wash (3), final wash (4), and purified elution (5) were loaded onto a polyacrylamide gel for SDS-PAGE (top). Exact protein molecular weight was verified with electrospray ionization mass spectrometry (bottom). Expected masses (5M: 17.85 kDa; 2A9: 17.79 kDa; 4S9: 17.75 kDa) matched with the observed masses (5M: 17.78 kDa; 2A9: 17.79 kDa; 4S9: 17.75 kDa).

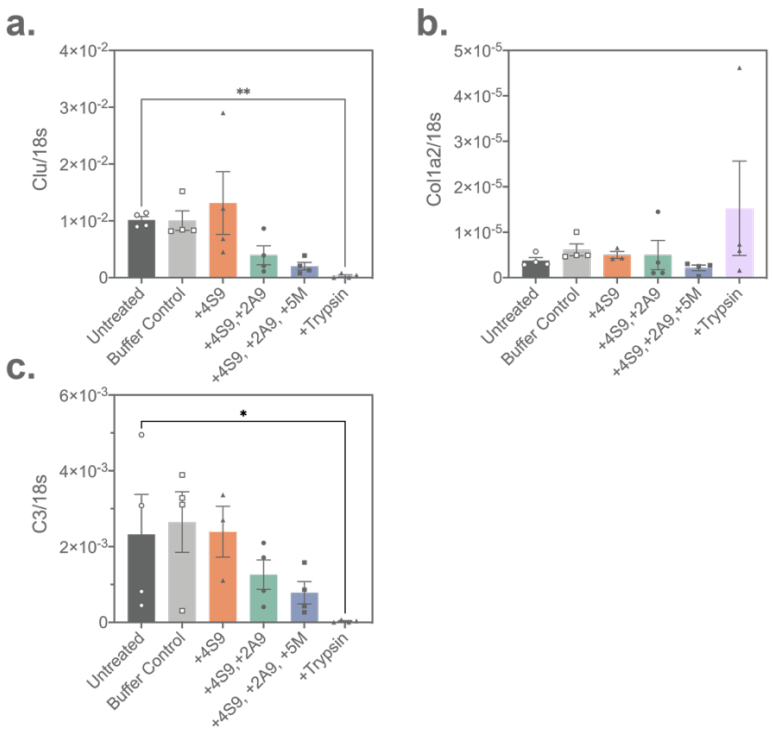


Figure S2 – RT-PCR for Clu, Col1a2, and C3 of primary cardiac fibroblast cultures sequentially treated with sortase variants. **p<0.01, *p<0.5 by Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test. N = 3 biological replicates for 4S9 treated samples, n = 4 for all other conditions except 4S9.

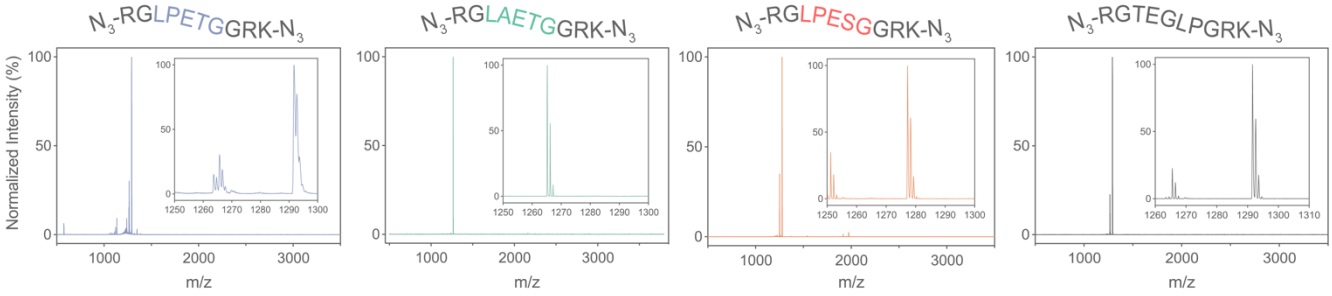


Figure S3 – Matrix-assisted laser desorption/ionization mass spectrometry of sortase-responsive diazide peptide crosslinkers. Expected masses (LPETG: 1291.8 Da, LAETG: 1265.8 Da, LPESG:1277.8 Da; TEGLP [scramble control]: 1291.8 Da) were the dominant observed peaks (LPETG: 1291.7 Da, LAETG: 1265.3 Da, LPESG: 1277.2 Da; TEGLP: 1291.6 Da) for all peptides, with an additional -26 peak (LPETG: 1265.8 Da, LPESG: 1251.2 Da; TEGLP: 1265.6 Da) corresponding to reduction of the azide to an amine during sample ionization.

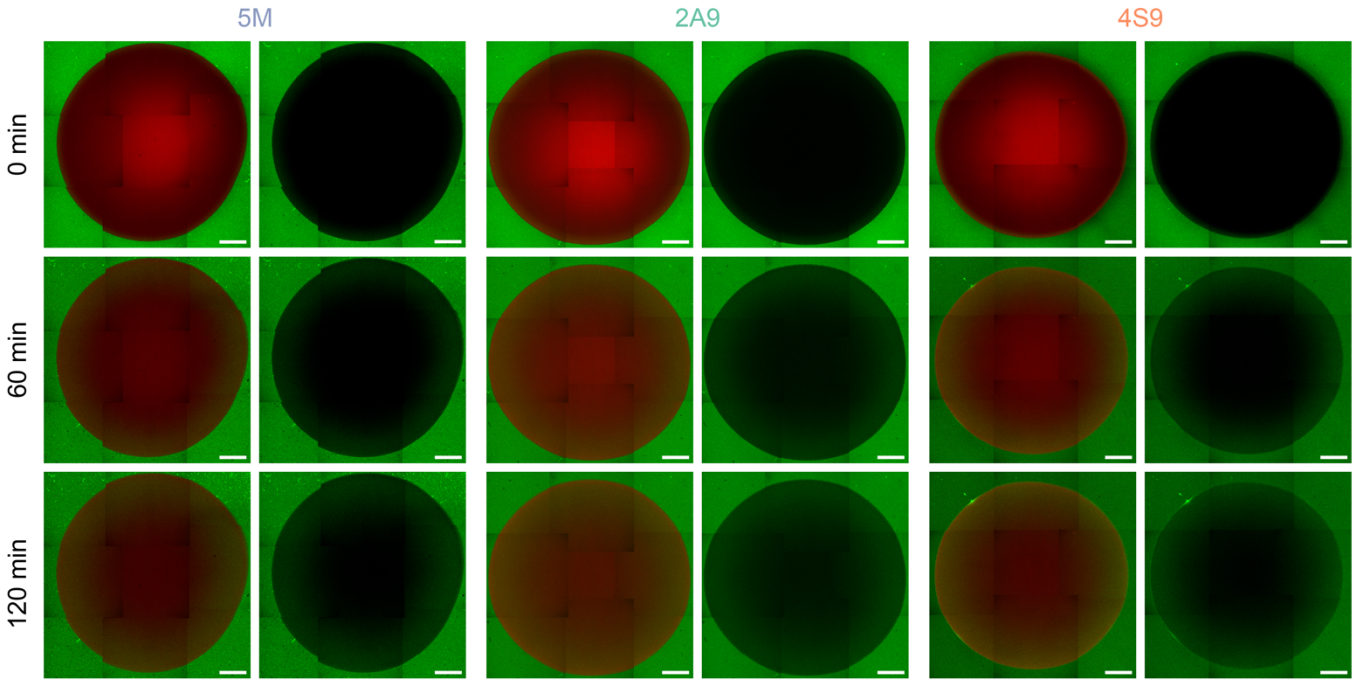


Figure S4 – Sortase diffusion into PEG-tetraBCN hydrogels. Tilescan confocal images showing diffusion of AlexaFluor 488-tagged 5M/2A9/4S9 sortases (green) into AFDye 568 azide-labeled gels (red, 5 µL volume) containing 4 mM PEG-tetraBCN and 8 mM TEGLP sortase-insensitive scramble peptide crosslinker. Images were taken at the glass-bound bottom plane for each gel at 0, 60, and 120 min following sortase addition. Scale bar = 1 mm.

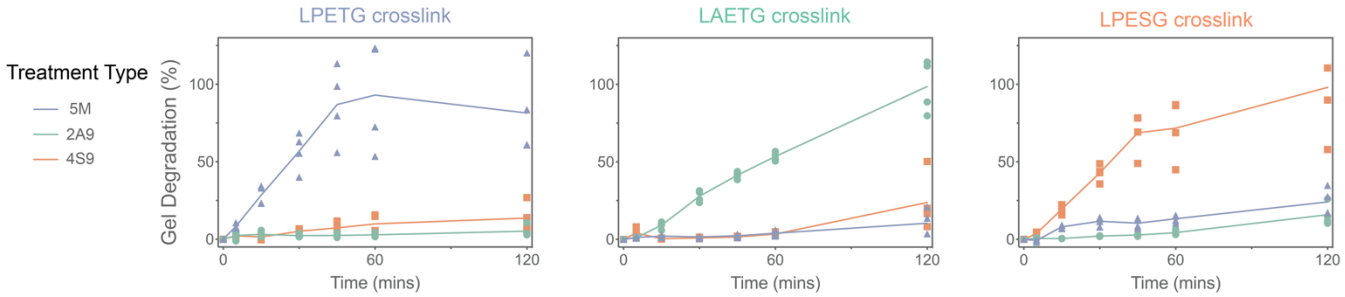


Figure S5 – Hydrogel degradation without evolved sortase preincubation. AFDye 568 azide-labeled gels were simultaneously treated with each evolved sortase (50 μM) and GGG (18 mM) at 0 minutes. Supernatant fluorescence was monitored to assess gel degradation as a function of time.

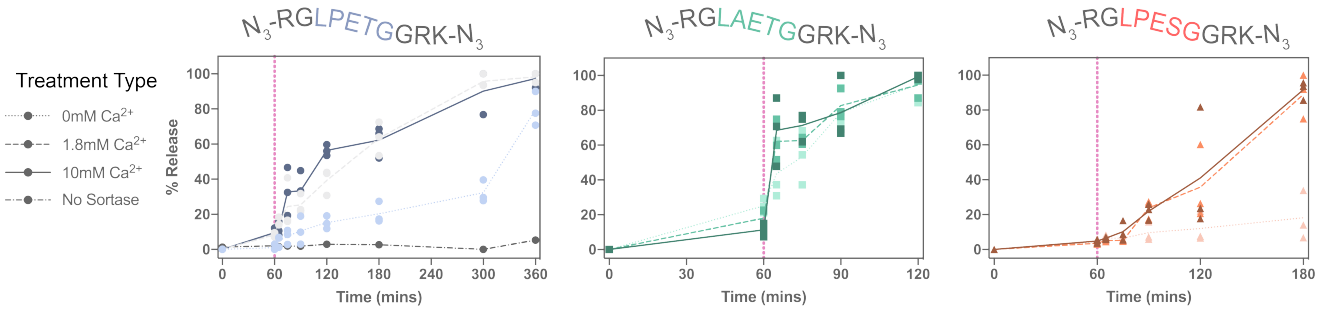


Figure S6 – Calcium-dependence of sortase-based hydrogel degradation. AF568-functionalized sortase-degradable gels were treated with sortase variants in the presence of no calcium (short dashes), 1.8 mM (long dashes), or 10 mM (solid) $CaCl_2$ in sortase reaction buffer. GGG was added at 60 mins, indicated by the dashed vertical line.

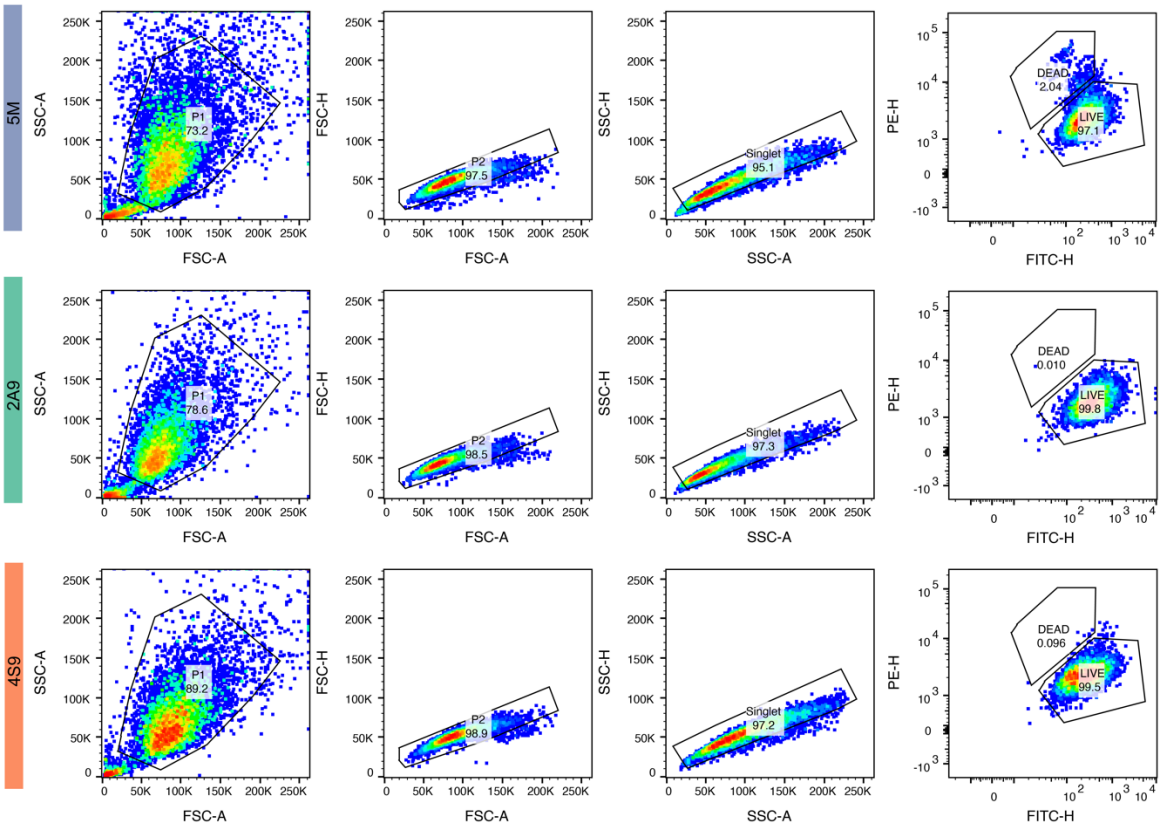


Figure S7 – Example flow cytometry gating for LIVE/DEAD analysis. Cells released from 5M (top), 2A9 (middle), and 4S9 (bottom) degradable hydrogels were first triple-gated for singlets on front and side scatter (P1/P2/Singlet), and then gated for viability based on Calcein AM (FITC-H) and EtHD-1 (PE-H).

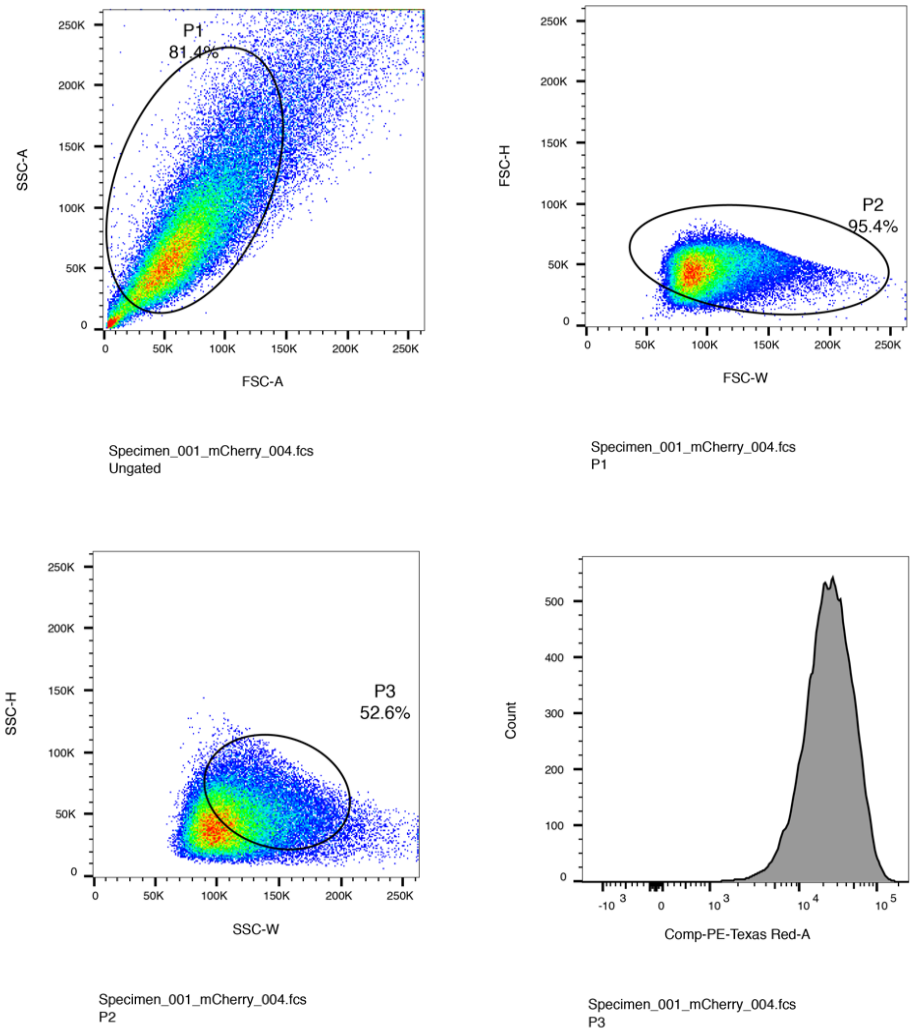


Figure S8 – Gating scheme for flow cytometry experiments shown for an HS5 mCherry control sample. Singlet discrimination was achieved through gating forward and side scatter amplitude, width, and height. Fluorescence gating was set to single population controls of mTagBFP2-, eGFP-, and mCherry-expressing HS5 human stromal cells.

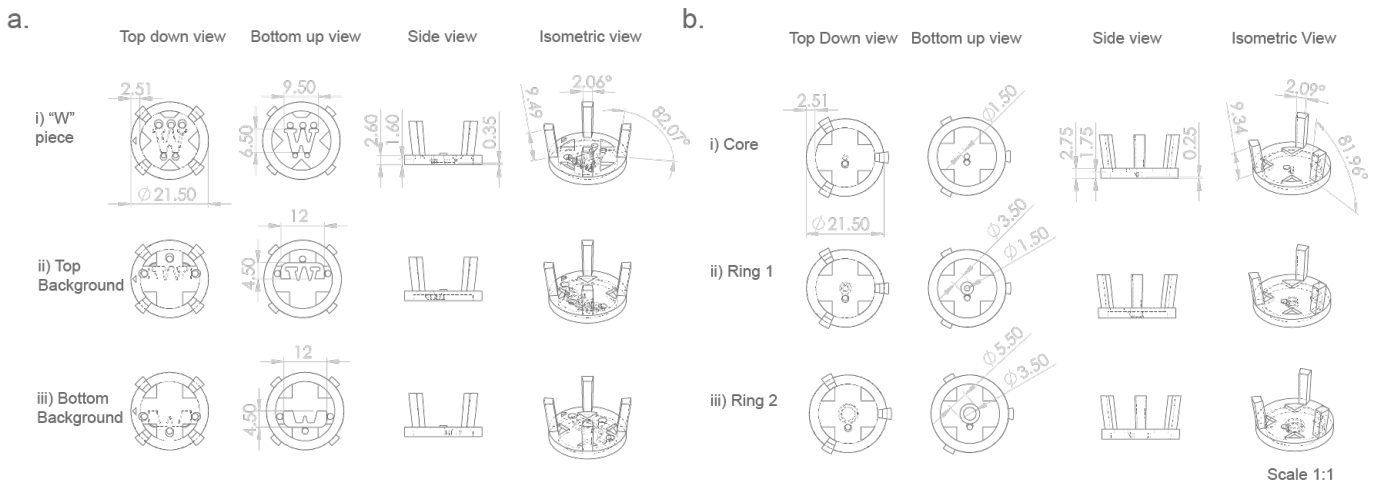


Figure S9 – Schematic diagrams illustrating the dimensions for patterning devices used to create patterned gels. (A) Multiple views of the "W" patterning device used in Figure 4E with all gel layers designed to have a height of 350 μm. (B) Multiple views of the bullseye patterning devices used in Figure 4C, with all gel layers designed to have a height of 250 μm. All dimensions are in mm.

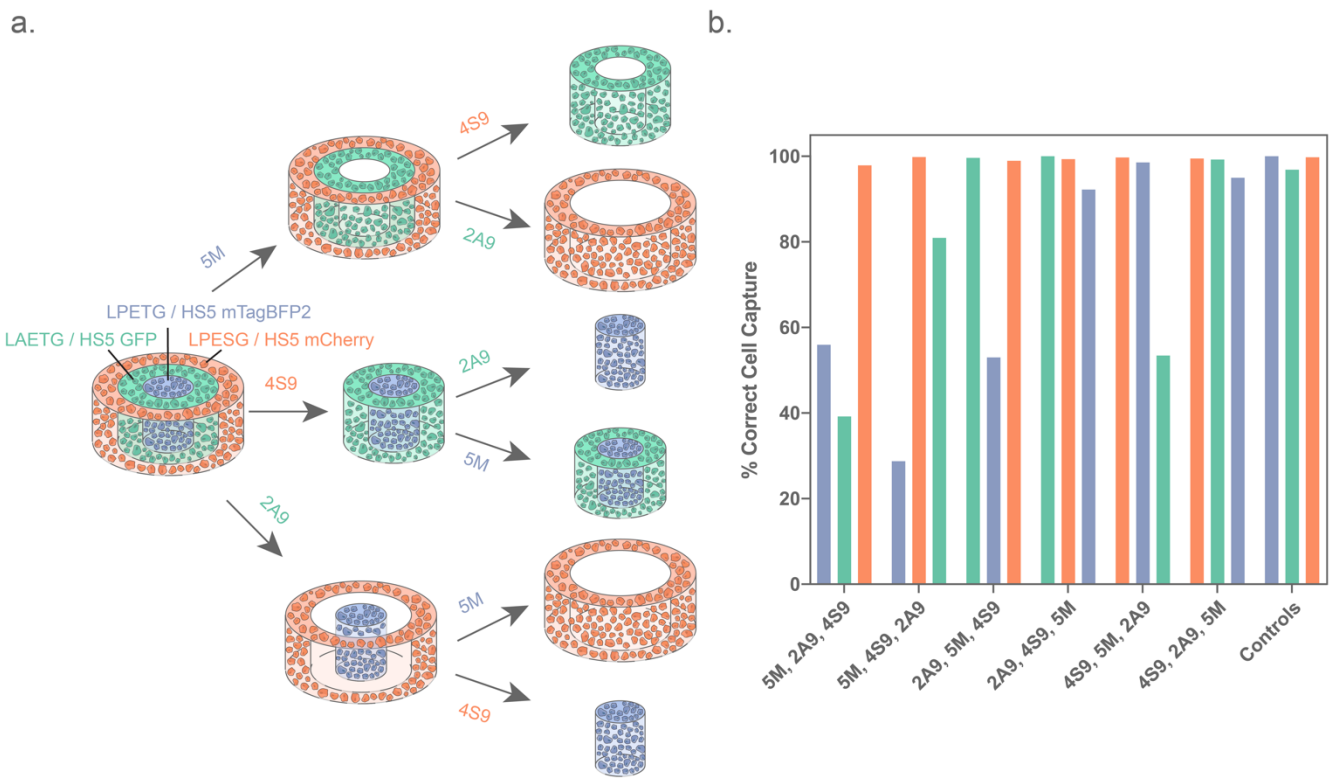


Figure S10 – Varying order of sortase treatment reveals that release specificity requires using 2A9 and 4S9 before 5M. Schematic for varying the order of sortase treatment on a multilayer composite hydrogel (left). Percentage of correct cells captured from each sequential sortase treatment, as measured by flow cytometry (right).

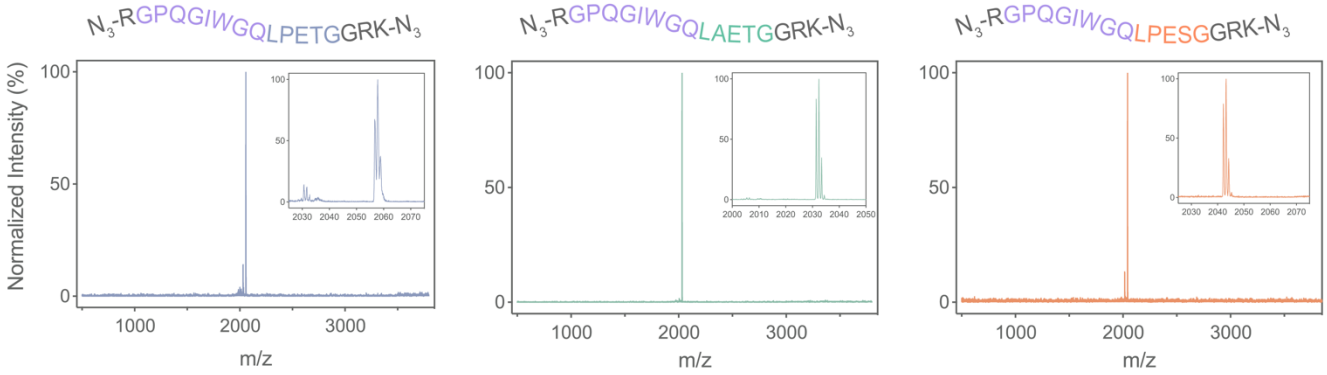


Figure S11 – Matrix-assisted laser desorption/ionization mass spectrometry of matrix metalloproteinase (MMP)-OR-sortase-responsive diazide peptide crosslinks. Expected masses (MMP ∨ LPETG: 2058.3 Da; MMP ∨ LAETG: 2032.2 Da; MMP ∨ LPESG: 2044.2 Da) were the dominant observed peaks (MMP ∨ LPETG: 2056.8 Da; MMP ∨ LAETG: 2031.4 Da; MMP ∨ LPESG: 2042.2 Da) for all peptides.

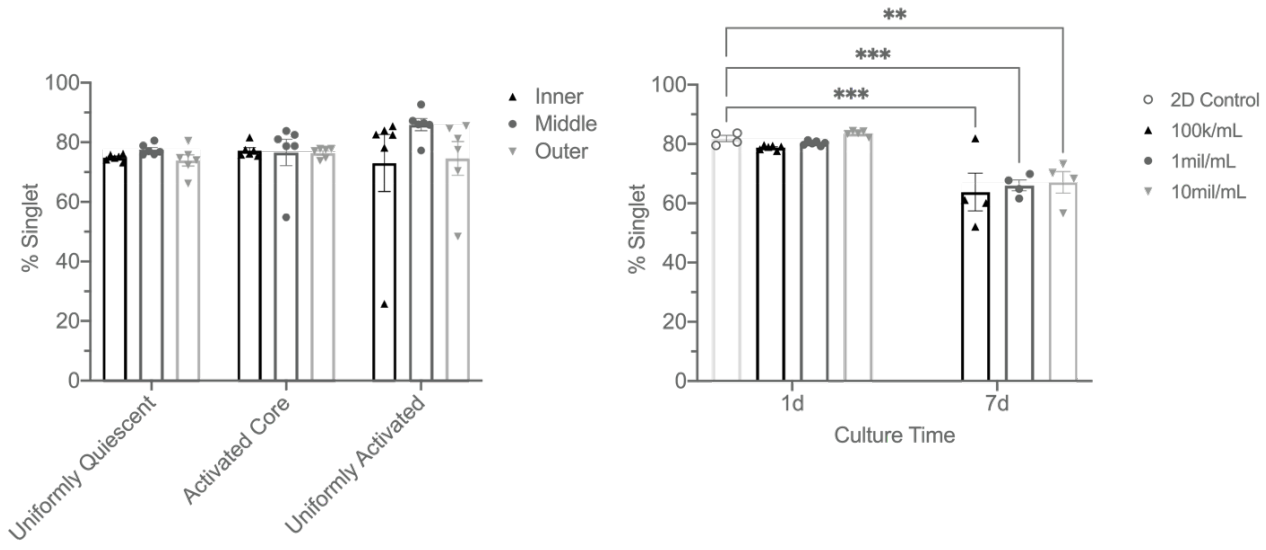


Figure S12 – Cardiac fibroblast culture time, but not seeding density or activation state, affects the efficiency of flow cytometry. Average percentage of singlet events recorded from multimaterial bullseyes (n = 6 bullseyes per condition) did not vary significantly between conditions (left). Singlet efficiencies of recovered fibroblasts (n = 4 gels per condition) did not vary with cell seeding density, but did decrease with cells captured after 7 days of hydrogel culture compared to an acute 1-day timepoint (right). Error bars represent ±SEM. "***" indicates $p < 0.001$, "**" indicates $p < 0.01$ by two-way ANOVA with Tukey's post-hoc test.

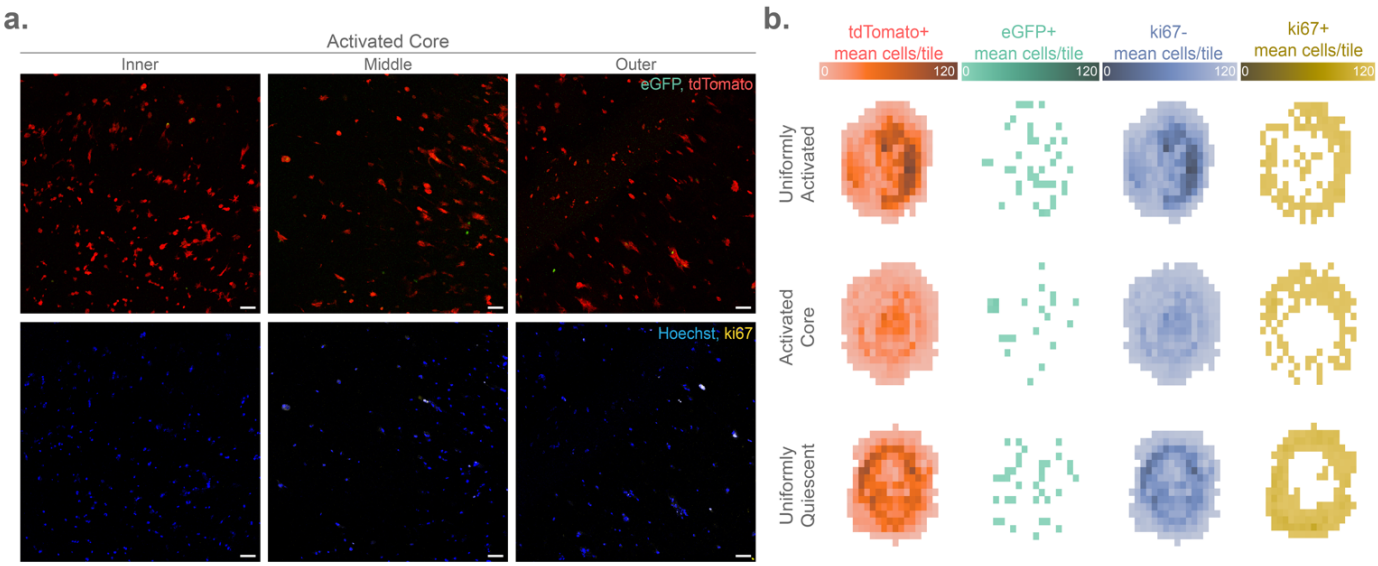


Figure S13 – Cardiac fibroblast proliferation is not affected by activation state after one day in gel culture. (A) Confocal maximum intensity projections from the inner (left), middle (center) and outer (right) regions of a gel bullseye with activated fibroblasts seeded in the center, immunofluorescently stained for Ki67 as a cell cycle marker fixed one day after encapsulation. Scale bar = 100 μm. (B) 2D histograms representing mean cell density (n = 3 bullseyes) of tdTomato+, eGFP+, Ki67-, and Ki67+ show that the cell density differences after one week of culture shown in Figure 5E are not explained by elevated proliferation in the activated core or uniformly activated conditions.

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