Electronic Supplementary Information

Dynamic Protein Hydrogels with Reversibly Tunable Stiffness Regulate Human Lung Fibroblasts Spreading Reversibly

Linglan Fu,[a] Amanda Haage,[b] Na Kong,[a] Guy Tanentzapf[b] and Hongbin Li[a]*

Materials and methods

Protein engineering

The genes of FGR(G-MEP-R)$_2$ and FRF$_4$RF$_4$R were constructed using standard molecular biology techniques following well-established protocols.[1, 2] Fig. S2 shows the full amino acid sequences of the polyproteins. Protein expression was carried out in *E. coli* strain DH5α. Seeding culture of pQE80L-FGR(G-MEP-R)$_2$ and pQE80L-FRF$_4$RF$_4$R were allowed to grow overnight in 10 mL 2.5% Luria-Bertani broth (LB) medium containing 100 mg/L Ampicilin. The overnight culture was then grown in 1L LB medium at 37 °C for 3 hours to reach OD600=0.8. Protein expression was induced with 1 mM isopropyl-1-β-D-thiogalactoside (IPTG) and continued at 37℃ for 4 hours. The cells were harvested by centrifugation at 4000 rpm for 10 mins at 4 °C and frozen at -80 °C. Then, cells were thawed and lysed by incubation with 1mg/mL lysozyme for 30 mins. Nucleic acids were removed by adding 0.1 mg/mL DNase and RNase. The supernatant with soluble protein was collected after centrifuging the cell mixture at 12000 rpm for 60 mins. The soluble protein was purified by Co^{2+} affinity column. Yields of FGR(G-MEP-R)$_2$ are approximately 45 mg per liter of bacterial culture. Purified proteins were dialyzed against deionized water for 2 days to remove NaCl, imidazole, and phosphate. Then the protein solution was filtered and lyophilized.

Hydrogel preparation for cell spreading and tensile test

Gelation of the polyprotein based on photochemical crosslinking strategy[3], was done near a flame or in a biocabinet. To prepare 12% FGR(G-MEP-R)$_2$ (120 mg/mL) and 7% FRF$_4$RF$_4$R (70 mg/mL) hydrogels with flat, large surface areas for cell spreading and imaging, lyophilized proteins were re-dissolved in sterile PBS mixed with ammonium persulfate (APS, 1M) and $[Ru(II)(bpy)3]^{2+}$ (20 mM) with final concentrations of 50 mM and 260 uM respectively. The mixed protein solution was quickly transferred onto a clean hydrophobic surface and sandwiched by a sterile glass coverslip. The thickness of hydrogels was ~0.8 mm, and the area of the hydrogel matrices was ~0.5 cm^2. Then, the sample was exposed under 200W fiber optic white light source from the height of 10 cm above the coverslip for 10 min. The hydrogel was gently taken off of the mold and soaked in sterile PBS to remove unconsumed crosslinking reagents. Then the washed gels were transferred to 6-well plates in fresh DMEM medium with desired redox conditions. Throughout the experiments, oxidized hydrogels were immersed in regular DMEM oxidized by air, but reduced hydrogels were treated with additional 10 mM Glutathione (GSH), which is a reductant generally used for cell studies.

To prepare a ring-shaped 12% FGR(G-MEP-R)$_2$ hydrogel for tensile tests, 12 mg lyophilized protein was dissolved in 93.7 μL phosphate-buffered saline (PBS), with 5 μL ammonium persulfate (APS, 1M) and 1.3 μL (20 mM) with final concentrations of 50 mM and

260 μM respectively. The protein solution was quickly transferred to a custom-made plexiglass ring-shaped mold (d_{in}=8 mm, d_{out}=10mm, h= 3mm), and was exposed under a 200W fiber optic white light source from the height of 10 cm above the mold for 10 min. The ring sample was carefully taken out of the mold. The ring shaped, oxidized samples were stored in filtered PBS (with air oxygen as oxidizing agent) over night to achieve equilibrium swelling.

To obtain reduced samples, the oxidized ring samples stored in PBS were transferred and immersed in PBS with additional 10 mM GSH for over 6 hours to allow complete oxidization reaction and to achieve swelling equilibrium.

Characterize dityrosine cross-links in hydrogel by acid hydrolysis-fluorescence method

The degree of crosslinking of the FGR(G-MEP-R)$_2$ hydrogels was characterized following a well-established fluoremetry method.[4, 5] Dityrosine has an emission at 410 nm when excited at 315 nm. For quantification of the dityrosine and dityrosine-like compounds generated in FGR(G-MEP-R)$_2$ hydrogels, dityrosine was prepared as a standard compound by one step oxidation from L-tyrosine. A standard fluorescence-concentration curve of dityrosine was obtained in our previous work. The protein hydrogel was first hydrolyzed by acid followed by fluoremetry measurements of the content of dityrosine-like peptide. Typically, a 12 % hydrogel sample (~10 mg) reacted with 100 μL HCl (6 N) in a sealed 1.5 mL centrifuge tube in the metal heat block at 105 °C for 2 h to achive full hydrolysis of the peptide bonds. Then, 100 μL of acid hydrolysis product was transferred into a new 1.5 mL centrifuge tube and neutralized by 10 uL NaOH (5 M). Next, 100 mM Na_2CO_3-$NaHCO_3$ buffer (pH 9.9) was added to the tube to 1 mL final volume. Fluorescence spectra of the samples were measured. According to the fluorescence-concentration standard calibration curve of dityrosine, the yield of dityrosine and dityrosine-like products in the hydrogel was then determined.

Cell spreading and redox switching

HLF cells were first cultured in T75 tissue culture flasks with Dulbecco's Modified Eagle's medium (DMEM) supplemented with10% (v/v) fetal bovine serum (FBS) and 1% (v/v) Penicillin/Streptomycin mix. Since the main purpose of this study is to investigate the effect of cyclic change of Young's modulus on the spreading/adhesion behaviors of HLF, only standard DMEM medium and 37 °C were used (with the exception that the reduced medium is DMEM supplemented with 10 mM GSH).

For cell spreading experiments, 1.4x10^5 HLF cells were seeded on top of the pre-treated oxidized and reduced FGR(G-MEP-R)$_2$ hydrogels in each well of a 6-well plate at 37 °C for 24 hours, with the FRF_4RF_4R hydrogels serving as controls. The hydrogels from DAY1 (Ox, Rd) without redox switching were fixed and stained after 24 hours incubation, using the protocol described below. The rest of hydrogels underwent the first redox switching cycle by removing the old oxidized and reduced media and replacing with fresh reduced DMEM (10 mM GSH) and oxidized DMEM respectively (DAY2: Ox-Rd, Rd-Ox), and then incubated at 37 °C for another 24 hours. This was then repeated to obtain the second redox switching, DAY3 samples (Ox-Rd-Ox, Rd-Ox-Rd).

To stain and image HLF cells, samples were first washed twice with pre-warmed sterilized PBS (pH 7.2, 37 °C) to remove non-adherent cells before cell fixing. Adherent cells were stained with Alexa Fluor 488 phalloidin and 4′,6-diamidino-2-phenylindole (DAPI) following the manufacturer's protocol. Briefly, cells were fixed with a 4% paraformaldehyde solution in PBS for 15 mins at room temperature. Fixed cells were permeabilized by exposure to 0.1% Triton X-

100 in PBS for 5 mins. Samples were then incubated with phalloidin for 20 mins and DAPI for 5 mins at room temperature. After staining, samples were washed with PBS to remove excess staining dye. Images were taken at ×10 magnifications by a fluorescence microscope (Nikon, Eclipse Ti).

To assess the cytocompatibility of FGR(G-MEP-R)$_2$-based hydrogels, we used a Click-iT EdU assay to assess the hydrogels' cytocompatibility. In this assay, the fluorescently labeled thymidine analogue EdU (5-ethynyl-2'-deoxyuridine) is incorporated into newly synthesized DNA efficiently, allowing for the fluorescently labeling of proliferating cells. HLF cells were plated on 7% FRF$_4$RF$_4$R hydrogels and both oxidized and reduced 12% FGR(G-MEP-R)$_2$ hydrogels which were pre-treated for cell loading. We also cultured HLF on polystyrene tissue culture dishes as a control. The cells were seeded with 50% confluence and allowed to grow on hydrogels and control dishes surfaces for 4 days before sequential treatment. Oxidized/reduced DMEM media mixed with 1X EdU working solution (10 uM EdU in DMSO) were added in the culturing wells to replace the regular DMEM under redox conditions for continuous incubation. Then the hydrogel samples were fixed and staining at two different length of incubation time, 24 hrs and 72 hrs. Staining and EdU detection of samples followed the standard Click-iT EdU Assay protocol. The samples were washed with pre-warmed PBS. The HLF cells were fixed by 3.7% formaldehyde followed by permeabilization in 0.5% TritonX-100, and then washed by 3% BSA in PBS. 200 uL of Click-iT reaction cocktail was added to each sample, incubating for 30 mins before washing with 3% BSA in PBS. For nuclear staining, DAPI staining was proceeded afterwards. Images were collected using an Olympus FV1000 confocal microscope

Tensile Test

Tensile tests were performed on an Instron-5500R tensometer with a custom-made force gauge in PBS or PBS with 10 mM GSH at constant temperature (25 °C). For technical reasons, ring-shaped biomaterial specimens were used. An extension rate of 25 mm/min was used in our experiments. The stress at 15% strain is taken as the Young's modulus of the sample.

Scanning electron microscopy (SEM) imaging

Each hydrogel was imaged using a Hitachi S4700 scanning electron microscope. 12 % hydrogel samples were prepared in Eppendorf tubes, and stored at 4 °C for 24 hours to allow the mixture to completely gelate. The samples were then shock-frozen in -80 °C, and quickly transferred to a freeze drier where they were lyophilized for 12 hours. Lyophilized samples were then carefully fractured in liquid nitrogen, and fixed on aluminum stubs. The sample surface was coated by 5 nm of gold prior to SEM measurements.

References

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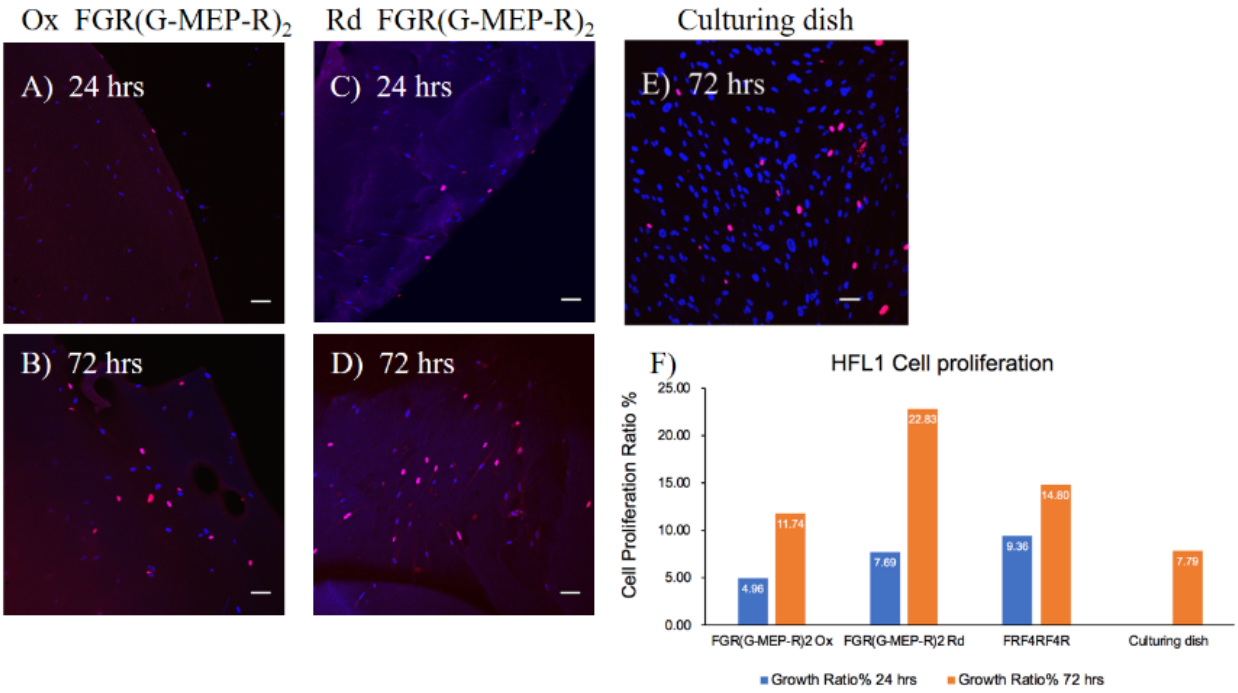

Figure S1. HLF cells can proliferate on MEP hydrogels. All cell nuclei were stained using the blue fluorescent dye DAPI (4′,6-diamidino-2-phenylindole). Cell nuclei, newly grown after Click-iT Edu treatment, were fluorescently labelled by Alexa Fluor azide (red). On reduced FGR(G-MEP-R)$_2$ gels, the ratio of new cells to the initially seeded cell increases from ~8% to 23%. On oxidized FGR(G-MEP-R)$_2$ gels, the cell proliferation ratio increases from ~5.0% to 12.0%, The scale bars represent 50 μm.

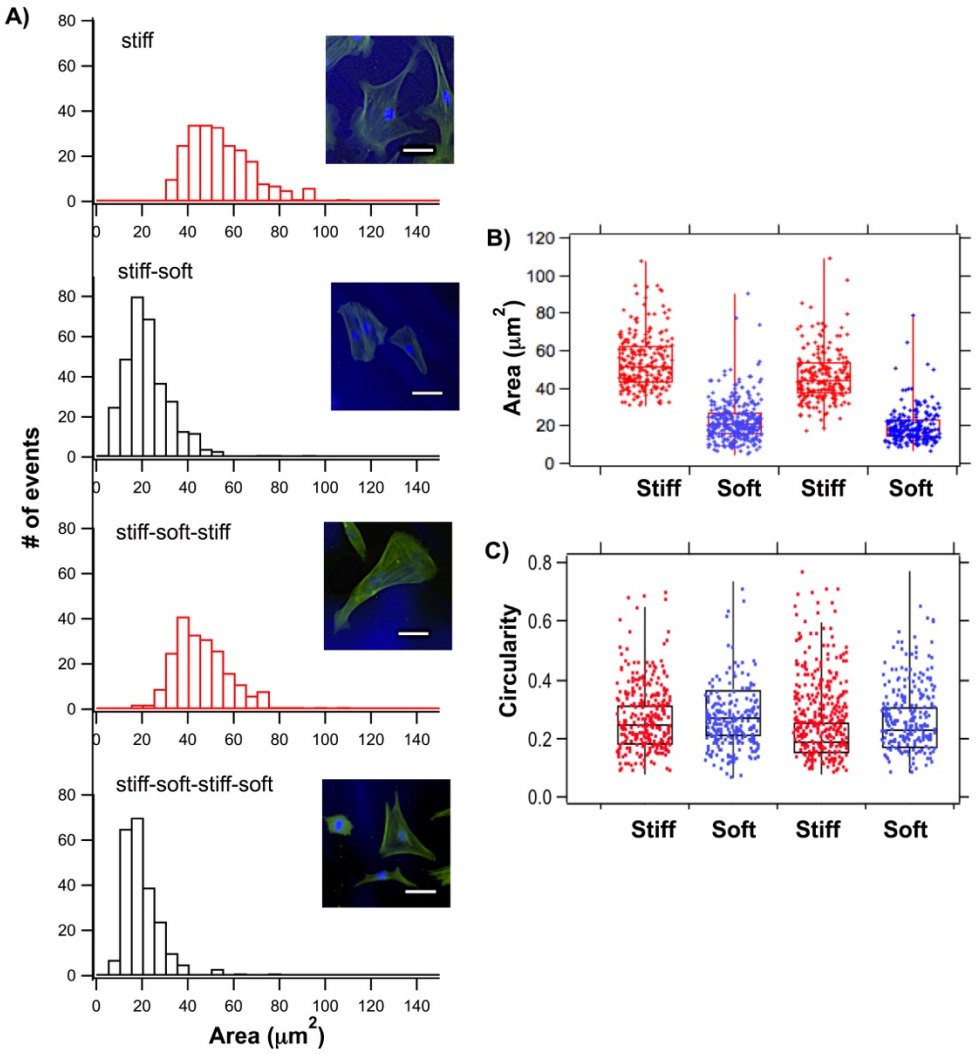

Figure S2. Morphology of human lung fibroblasts can dynamically respond to the change of the hydrogel stiffness. A) Histograms of the cell area of HLF during the two stiff-soft-stiff-soft switching cycles of change the stiffness of the hydrogels. On stiff hydrogels, HLF spread well with large cell areas. Upon switching to soft hydrogel state, HLF dynamically change their morphology and display much small cell area. Inset show fluorescence images of HLF. Actin filaments were labeled by Alexa Fluor 488 Phalloidin and Nuclei DNA were stained by DAPI. The scale bars represent 5 μm. B) Box plot of the cell area of HLF on protein hydrogels. C) Box plot of the circularity of HLF on protein hydrogels with different Young's modulus. In response to the Young's modulus of the hydrogel, HLF only showed a small change in the circularity.

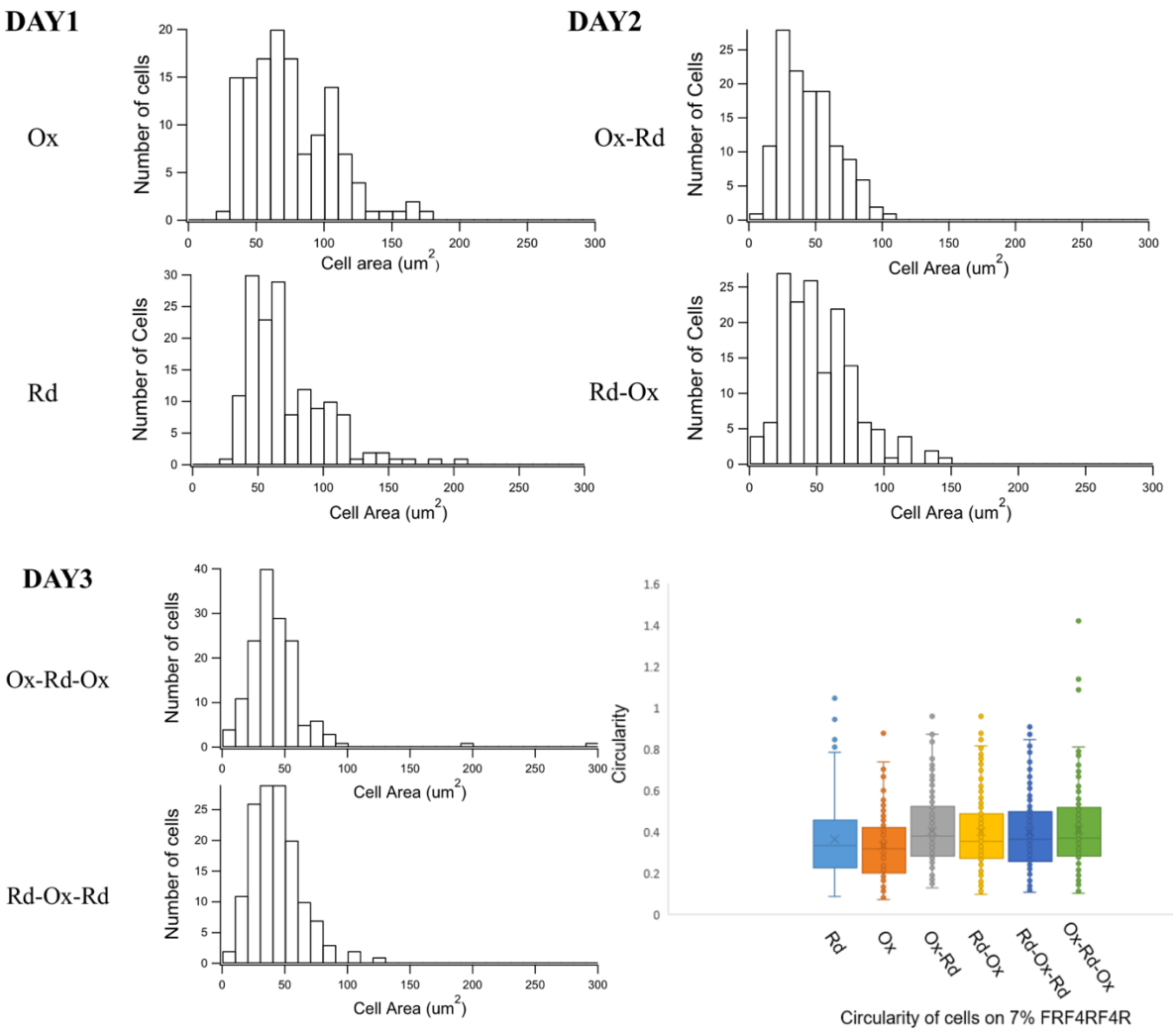

Figure S3. Control experiments were carried out with HFL1 cells spreading on 7% FRF_4RF_4R hydrogels at the same time as the same conditions and procedures as the experiments on 12% $FGR(G\text{-}MEP\text{-}R)_2$. Redox conditions of the culture medium were switched alternatively every 24 hrs. There were no obvious change of cell area and cell circularity on control gels during the redox switching as MEP hydrogels.

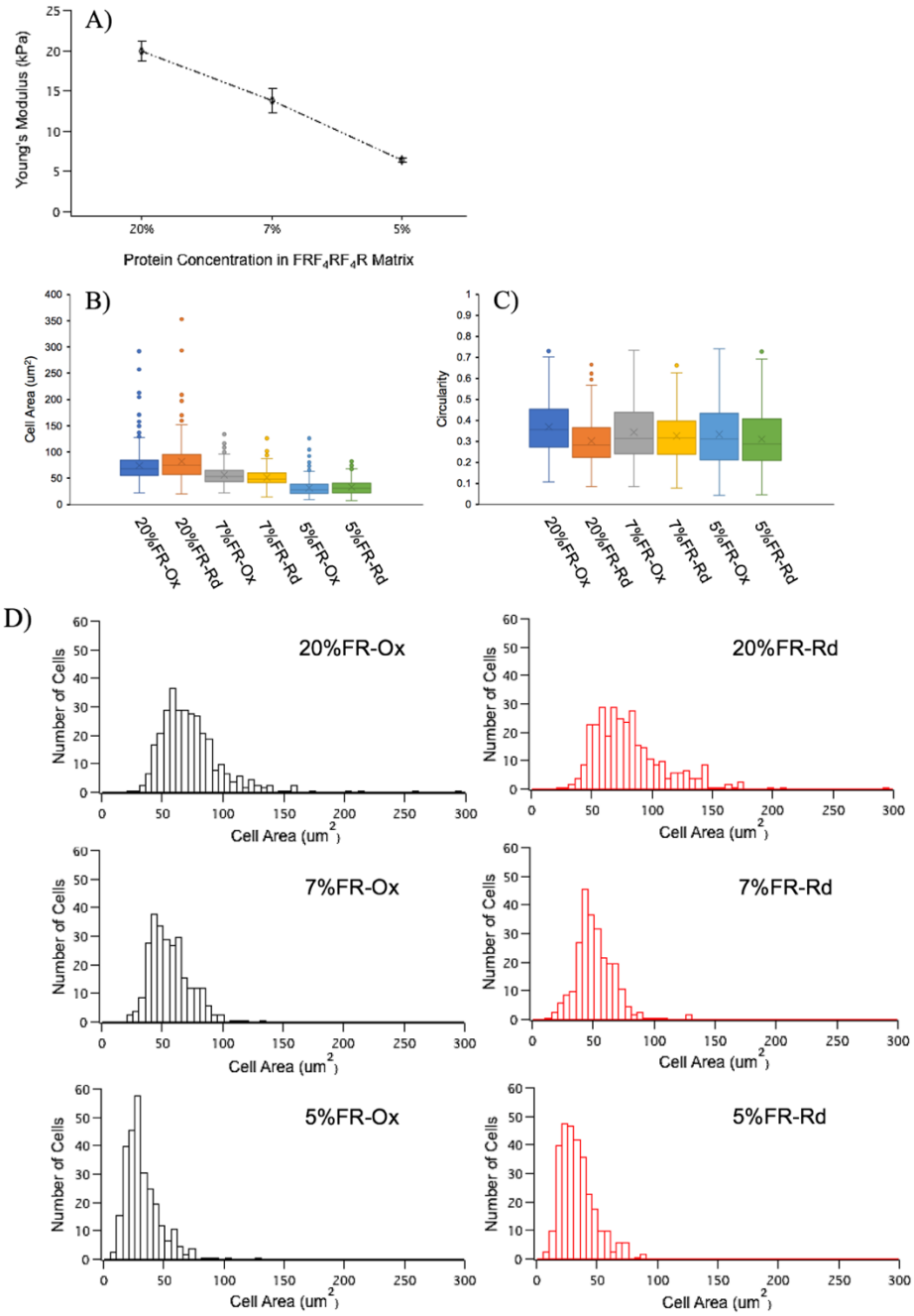

Figure S4. Control HLF culturing experiments on FRF_4RF_4R hydrogels with different Young's modulus. A) Young's modulus of 20%, 7%, 5% FRF_4RF_4R hydrogels. Their Young's moduli are 19.9 kPa, 13.7 kPa and 6.4 kPa respectively. B-C) Cell spreading area and circularity of HLF on the reduced/oxidized FRF_4RF_4R hydrogels with different Young's modulus. Both cell spreading area and circularity of HLF do not display changes in response to redox condition.

FGR(G-MEP-R)$_2$
MRGSHHHHHHGS
RLDAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDRTTIDLTEDENQYSIGNLKPDTEYEVSLISR**RGD**MSSNPAKETFTTRS
MDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTRS
GGRPSDSYGAPGGGNRS
(MDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTRS
*MDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVGCGLG*LIEVEKPLYGVEVFVGETAHFEIELSEPDVHGQFKLKGQPLAAS
PDCEIIEDGKKHILILHNCQLGMTGEVSFQAANTKSAANLKVKEL*CGDGEWTYDDATKTFTVTE*RS
GGRPSDSYGAPGGGNRS)$_2$

FRF$_4$RF$_4$R
MRGSHHHHHHGS
RLDAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDRTTIDLTEDENQYSIGNLKPDTEYEVSLISR**RGD**MSSNPAKETFTTRS
GGRPSDSYGAPGGGNRS
(RLDAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDRTTIDLTEDENQYSIGNLKPDTEYEVSLISR**RGD**MSSNPAKETFTTRS)$_4$
GGRPSDSYGAPGGGNRS
(RLDAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDRTTIDLTEDENQYSIGNLKPDTEYEVSLISR**RGD**MSSNPAKETFTTRS)$_4$
GGRPSDSYGAPGGGNRS

Figure S5. The amino acid sequences of FGR(G-MEP-R)$_2$ and FRF$_4$RF$_4$R proteins. The sequence of Tnfn3 is colored in purple. The sequences of the host and guest domains of GL5CC/I27 (MEP) are colored in blue and green, respectively. The sequence of GB1 and resilin are colored in black.