A visible light cross-linkable, fibrin-gelatin based bioprinted cardiac construct with human cardiomyocytes and fibroblasts

Shweta Anil Kumar, Matthew Alonzo, Shane C. Allen, Laila Abelseth, Vikram Thakur, Jun Akimoto, Yoshihiro Ito, Stephanie Willerth, Laura Suggs, Munmun Chattopadhyay, Binata Joddar.

Number of pages: 17 including references

Number of figures: 19

Preparation of gel-fu

Porcine gelatin powder, furfuryl glycidyl ether (96%) and DMSO were obtained from Sigma-Aldrich and Duchefa Biochemie (Haarlem, The Netherlands) respectively. Sodium hydroxide (NaOH), hydrochloric acid (HCl), acetone and ether were obtained from Duksan Pure Chemical Co., Ltd (South Korea). For preparation, 2 g of porcine gelatin was dissolved in 80 mL of distilled water and the pH made upto 11 by the addition of 1N NaOH solution. 250 µL of furfuryl glycidyl ether was dissolved in 20 mL of DMSO and added to the gelatin solution at room temperature, and the mixture stirred for 30 h at 65 °C, following which it was made neutral by the addition of 1N HCl solution. The resulting mixture was then dialyzed in DI water for 48 h for the purification of f-gelatin using a dialysis membrane, with a molecular weight cut-off of 1,000 Da (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). After dialysis, the purified gel-fu (furfuryl-gelatin) was isolated through evaporation, washed with acetone and ether and then dried. The derivatization of gelatin into gel-fu was confirmed by the spectra generated by a nuclear magnetic resonance (NMR) spectrometer (Gemini 2000, 300 MHz, Varian Inc., Palo Alto, CA, USA).[1]

Fibrinogen preparation

Fibrinogen solution was prepared at concentrations of 10-60 mg/mL as described in Kolehmainen et al.[2] Briefly, ~1300 mg of lyophilized fibrinogen was dissolved in 20 mL of Tris Buffered Saline (pH 7.4). The solution was incubated at 37 ^{o}C for one hour, then dialyzed overnight in 4 L of Tris Buffered Saline. The solution was sterile filtered with a 0.2 mm syringe filter. The concentration of the solution was measured on a NanoVue Plus spectrophotometer (Biochrom, Holliston, MA) using the Protein A280 assay. The solution was diluted to the final 10-60 mg/mL concentration with sterile Tris Buffered Saline.

Cytotoxicity Assessment of Rose Bengal

F-gelatin (10 wt %) and RB (0.5 wt %) was mixed together and a 50 µl aliquot was drop-pipetted onto a 12-well cell culture plate (Iwaki, Tokyo, Japan). The droplets were exposed to visible light with a white light-emitting diode source (Luminar ace LA-HDF158A, Hayashi Watch Works, Tokyo, Japan) at a distance of 1 cm for 5 min. Crosslinked hydrogels were incubated in Dulbecco's modified phosphate buffered saline for 24 h at 37 °C, and then sterilized with UV light exposure. NIH/3T3 cells (mouse fibroblast) were cultured for this experiment and passaged using Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. These cells (5.0 X 10^4 cells/well) were seeded in 12-well plate with or without the hydrogels. All samples were cultured for 5 days and their culture medium was replaced every other day. After 5 days, the cells were trypsinized using 0.25% Trypsin-EDTA cell number and their numbers determined by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and reported as percent viability. Absorbance assay of spent culture medium which correlated directly with cell growth was measured at 450 nm. Furthermore, bright filed images of cells cultured with or without the hydrogels were tracked at varying time points. In addition, after 24 h of culture, Calcein AM (Thermo Fisher Scientific) was added to the cultured to visually confirm the presence of viable cells in hydrogels and in other samples. Images were acquired using a fluorescent microscope (Olympus, Tokyo, Japan).

Evaluation of the cell orientation in bioprinted constructs

To confirm that the bioprinting process promotes better cell geometric confinement and spatial orientation within the fibers, Z-stack images were processed using ImageJ software. First, the color images (RGB) were processed using the command "Split Channels" which allowed us to obtain the blue channel or DAPI stained nuclear image of all samples, bioprinted and non-bioprinted.

Next, we used Analyze> Plot Profile to create a plot of intensity values across a line drawn through the image in various directions.[3] The average intensity values for one characteristic bioprinted and one non-bioprinted sample are plotted in Figure 6, in the main body of the manuscript. Other supplementary images are reported in Supplementary Figure 5B-I. Images 5B-E represent images from bioprinted samples and images 5F-I represent images from non-bioprinted samples. Supplementary Figure 5B confirms that the image intensity/distance of the plot profile drawn along the direction of cell growth aligned with the 3D printed design had a consistent appearance of peaks, compared to other images (5C-E) where the plot profile was drawn in other areas of the image not aligned with the 3D printed design. On the contrary, in non-bioprinted samples (5F-I) the image intensity/distance did not reveal a consistent appearance of peaks, in any direction confirming the absence of a definite 3D print or design.

Preparation of sections for immunostaining

To perform immunocytochemistry, the cell-laden constructs were fixed with cold methanol (ThermoFisher) for 20 min at room temperature. Fixed samples were immediately converted into cryo-blocks by embedding them in optimal cutting temperature (OCT) embedding compound (ThermoFisher), and frozen at -20 to -80 °C. Next, thick sections ~5-15 μm were isolated using a cryostat and mounted onto gelatin-coated histological slides to enhance adhesion of the tissues. These slides were then processed for immunohistochemistry as described in the main body of the manuscript.

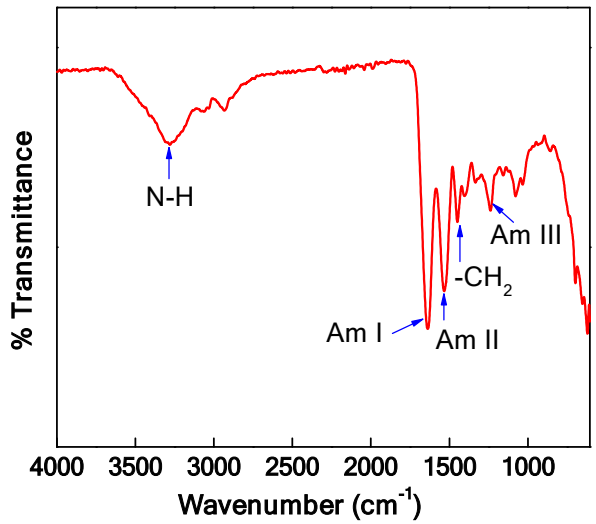


Figure S1A: Characteristic FTIR spectra for the fibrin-gelatin based pattern after a dual step crosslinking process, firstly photocrosslinking by exposure to visible light followed by chemical crosslinking by the addition of a solution of Thrombin-$CaCl_2$.

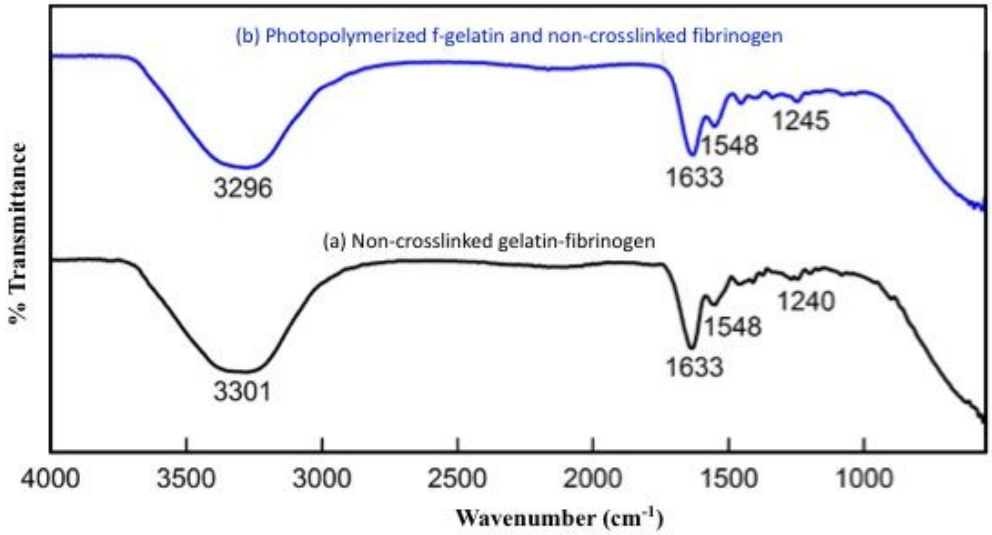
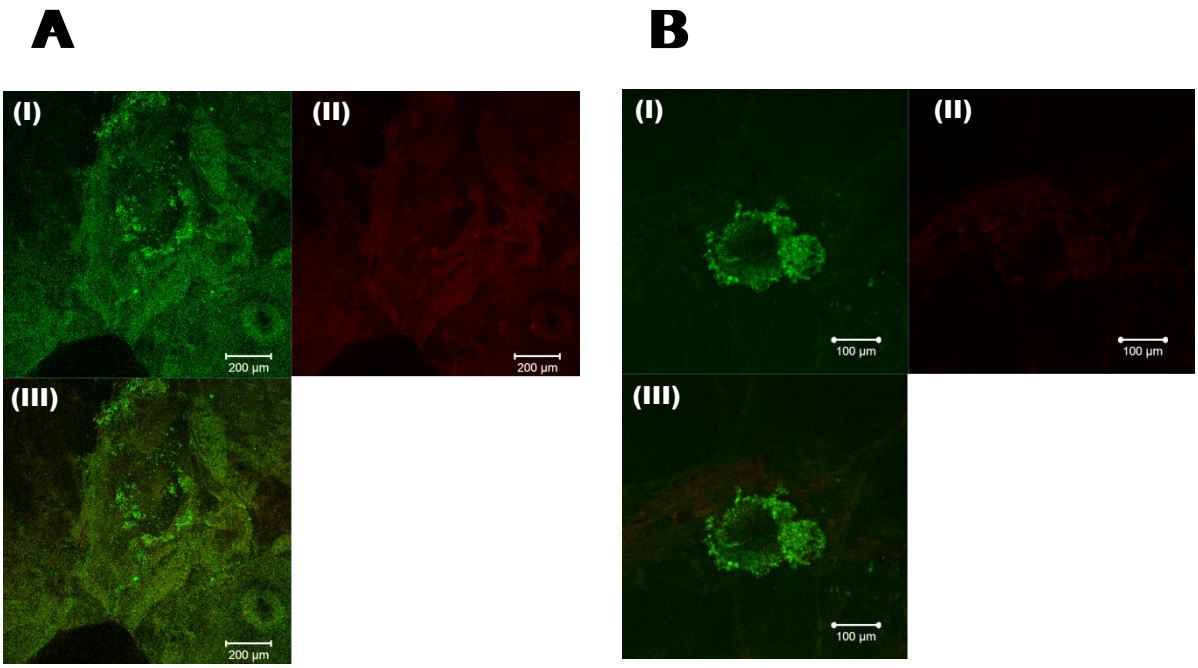


Figure S1B. Shown are characteristic spectra from two controls, (a) showing signatures for the uncrosslinked gelatin-fibrinogen composite and (b) showing only photopolymerization of the f-gelatin component.



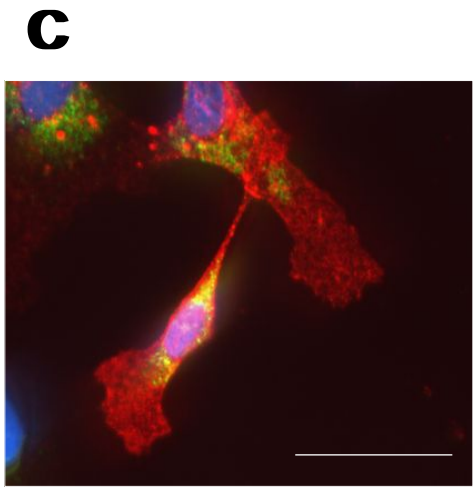


Figure S2: (A) and (B) shows characteristic images of human iPS-CM that were bioprinted within fibrin-gelatin patterns and imaged after 24 h using Live/Dead assay. Calcein AM (live cell stain: green) and Ethidium homodimer (dead cell stain: red) was used to depict live cells retained within the bioprinted and crosslinked construct as shown in A and B-panels (I), and dead cells depicted in panels (II), respectively. Panels (III) in both A and B depict overlaid images for both live and dead cell stained samples. (C) Shown is a characteristic image of Troponin-T staining (red) which confirms the presence of cardiac myocytes responsible for sarcomere contraction. CX43 (green) and DAPI counterstain is depicted in blue. Scale bar is 100 µm.

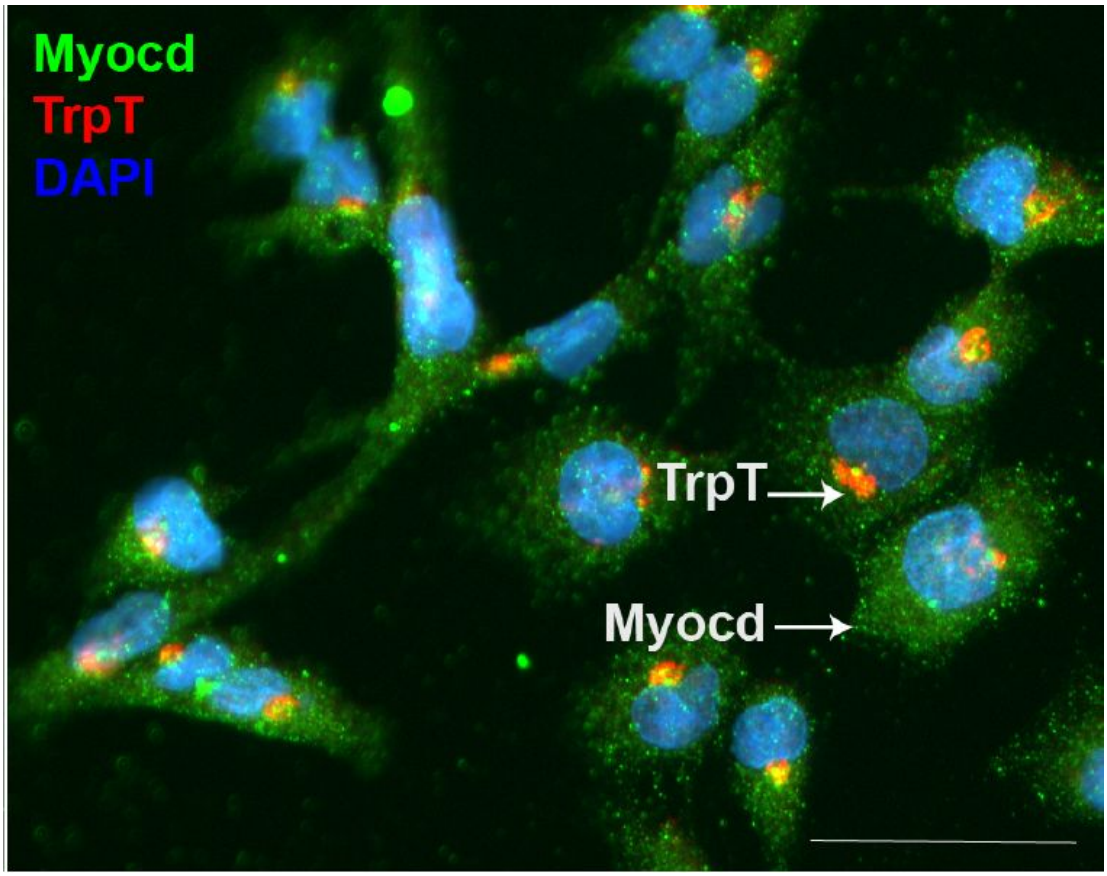
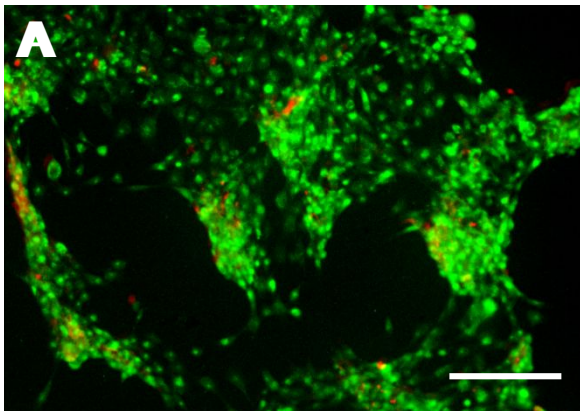
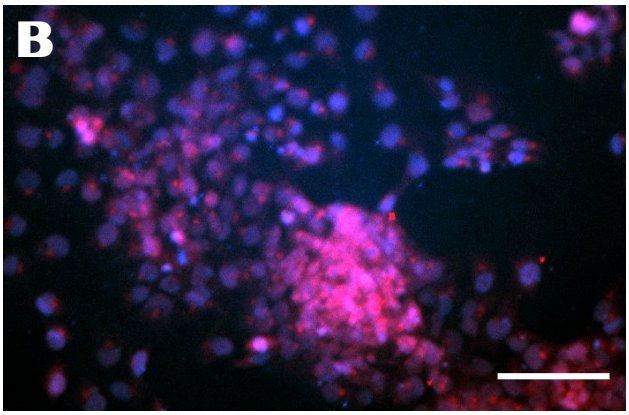


Figure S3: Human CM (AC16 cells) expressed Myocardin (Myocd) when probed using specific antibodies. Myocardin is required for the maintenance of cardiomyocyte structure and sarcomeric organization and its loss is associated with programmed cell death.[4] Shown are human CM (AC16) cells immunostained with MyoCD (green), Troponin (TrpT: red) and DAPI (blue). Scale bar is 100 μm.





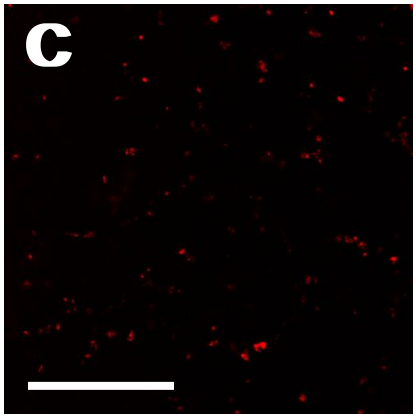


Figure S4. (A) and (B) shows characteristic Live/Dead and Troponin-I stained images of human CM cell lines, which were cultured in tissue culture wells and imaged after 5-days, as controls. Scale bar depicts 200 µm in (A) and 100 µm in (B). (C) Shows viable human CM (pre-stained using PKH26: red) imaged after 24 h of culture within non-printed fibrin-gelatin gels, manually casted and cross-linked. Scale bar depicts 200 µm.

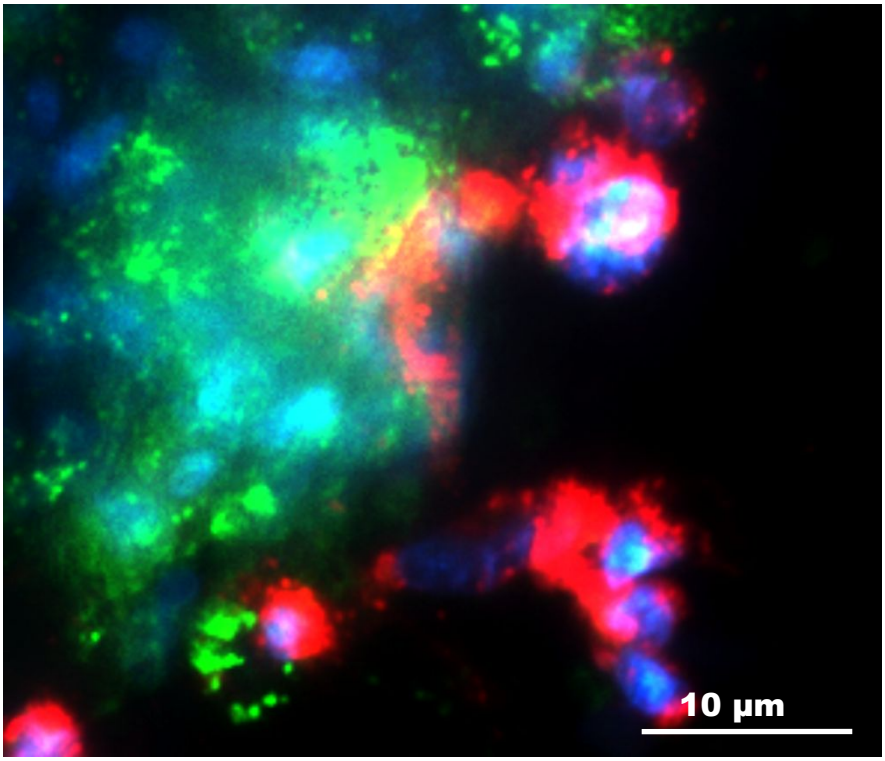


Figure S5A. Human CM (pre-stained using PKH26: red) and CF (pre-stained using PKH67: green) coupled in non-printed 3D gels (fibrin-gelatin). DAPI stained nucleus are in blue.

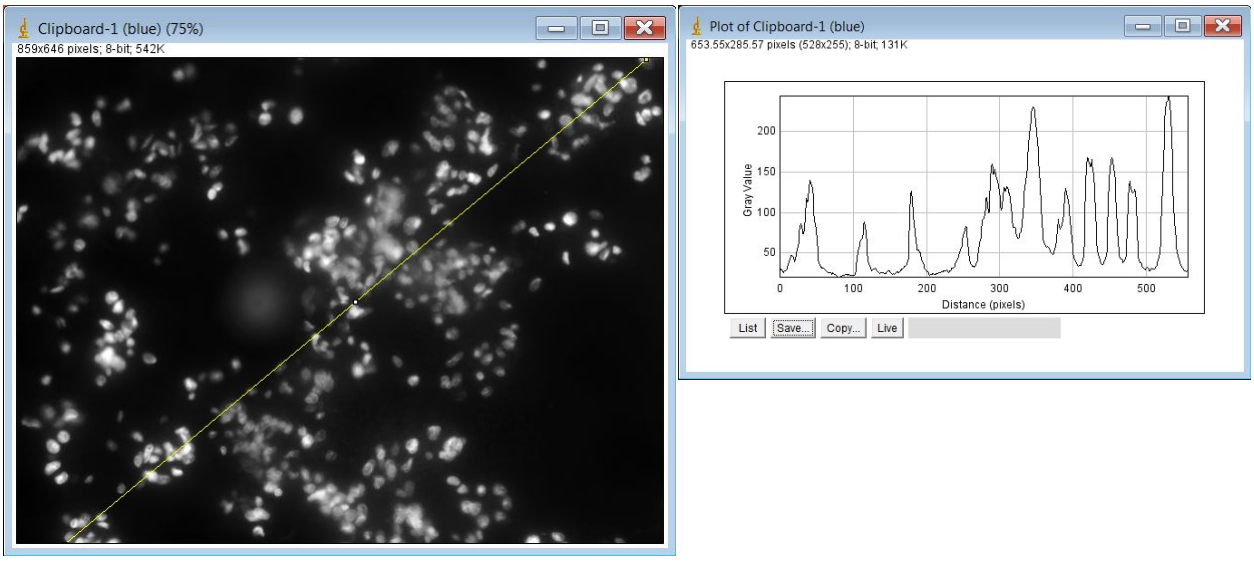


Figure S5B. Plot Profile depicting intensity values across the yellow line in the image representing bioprinted samples.

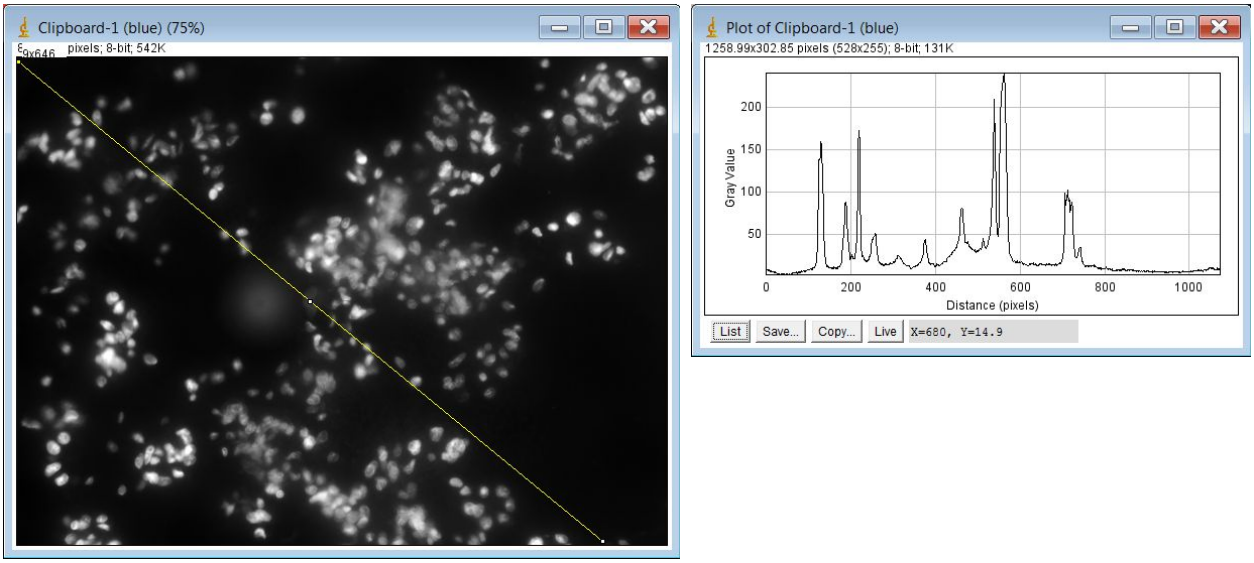


Figure S5C. Plot Profile depicting intensity values across the yellow line in the image representing bioprinted samples.

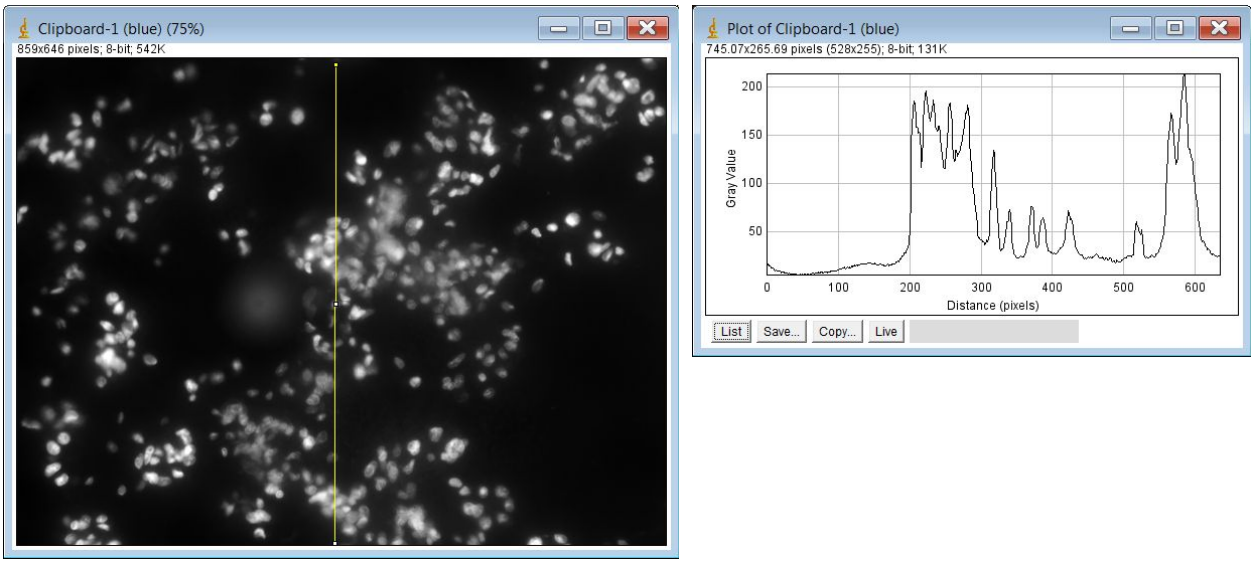


Figure S5D. Plot Profile depicting intensity values across the yellow line in the image representing bioprinted samples.

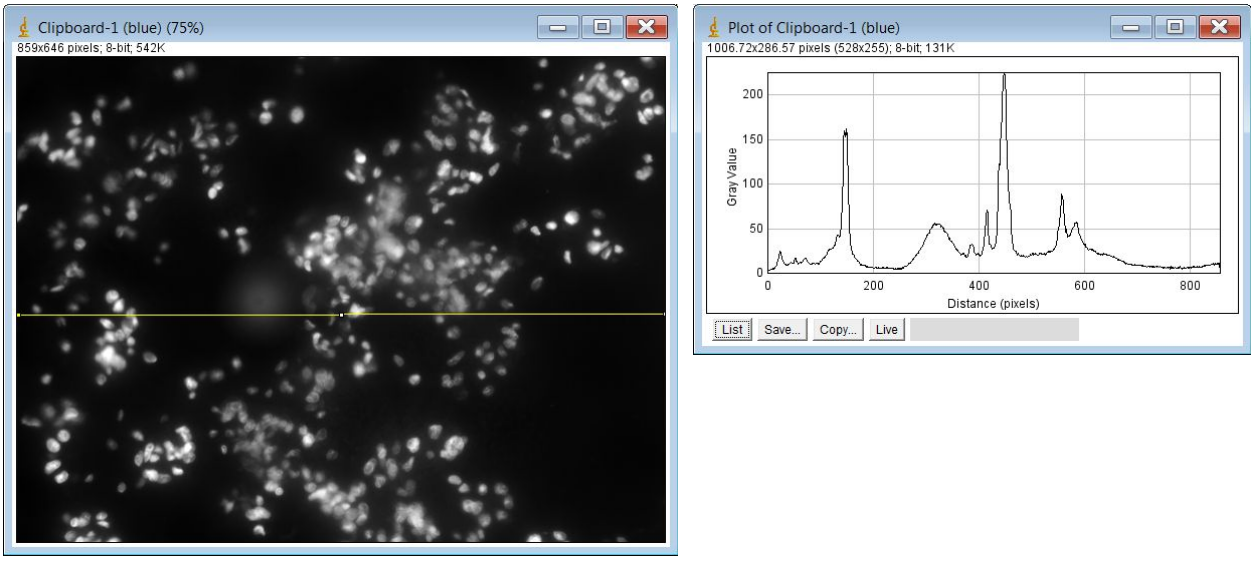


Figure S5E. Plot Profile depicting intensity values across the yellow line in the image representing bioprinted samples.

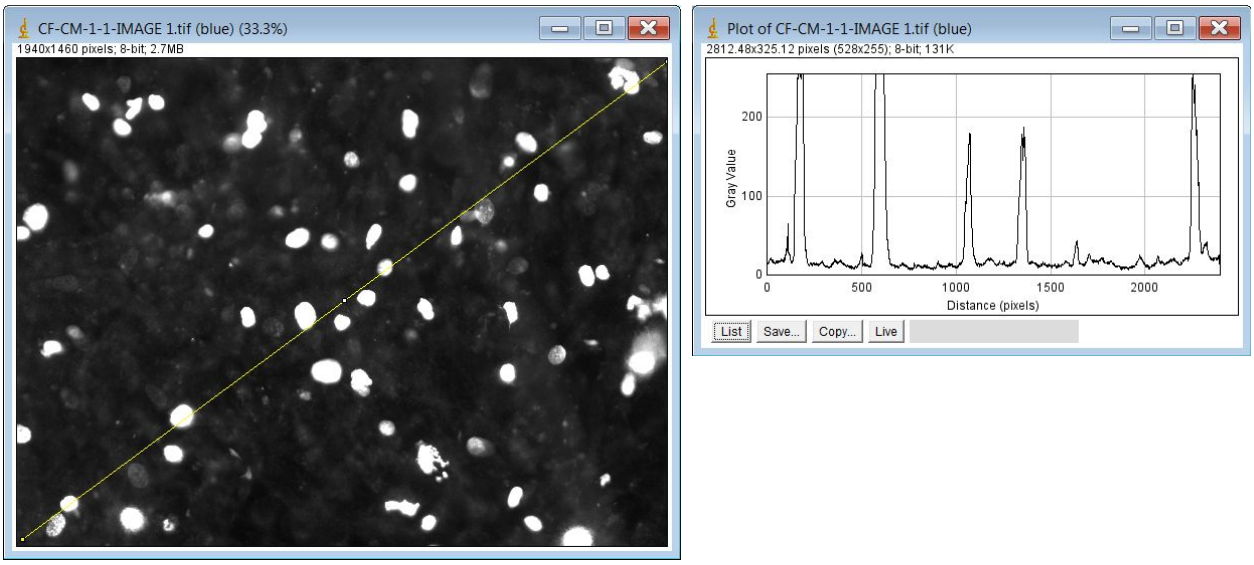


Figure S5F. Plot Profile depicting intensity values across the yellow line in the image representing non-bioprinted samples.

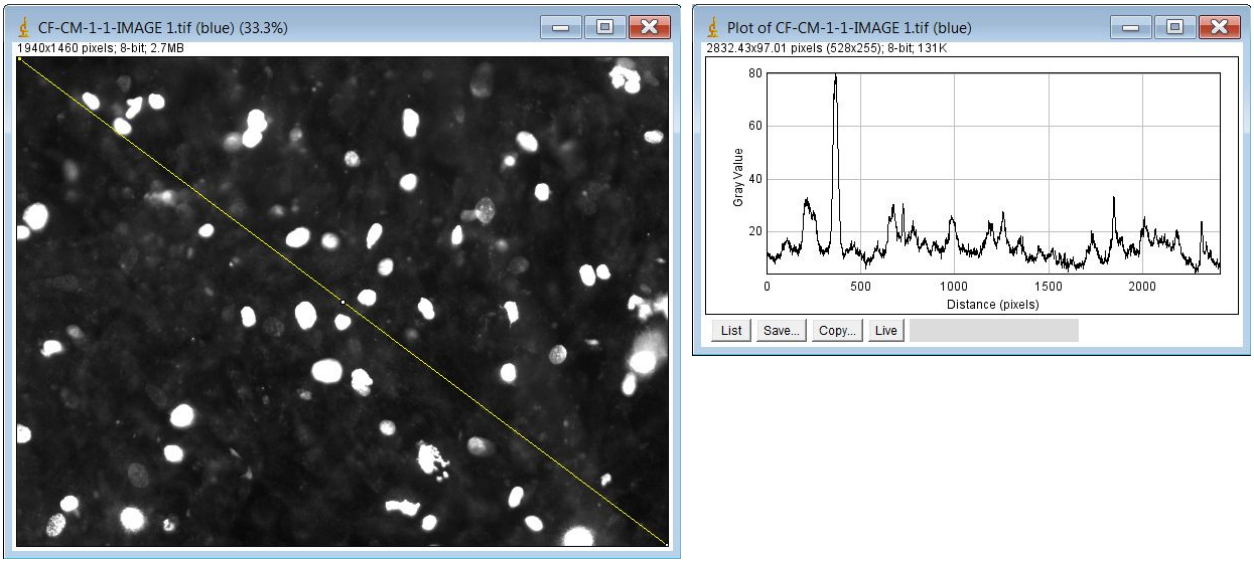


Figure S5G. Plot Profile depicting intensity values across the yellow line in the image representing non-bioprinted samples.

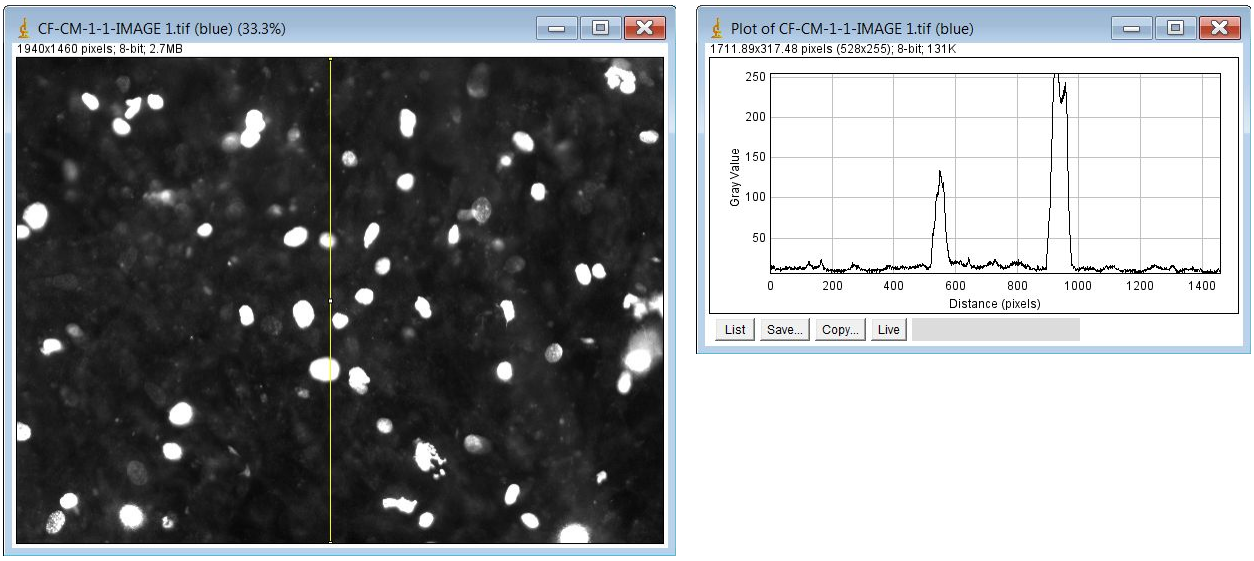


Figure S5H. Plot Profile depicting intensity values across the yellow line in the image representing non-bioprinted samples.

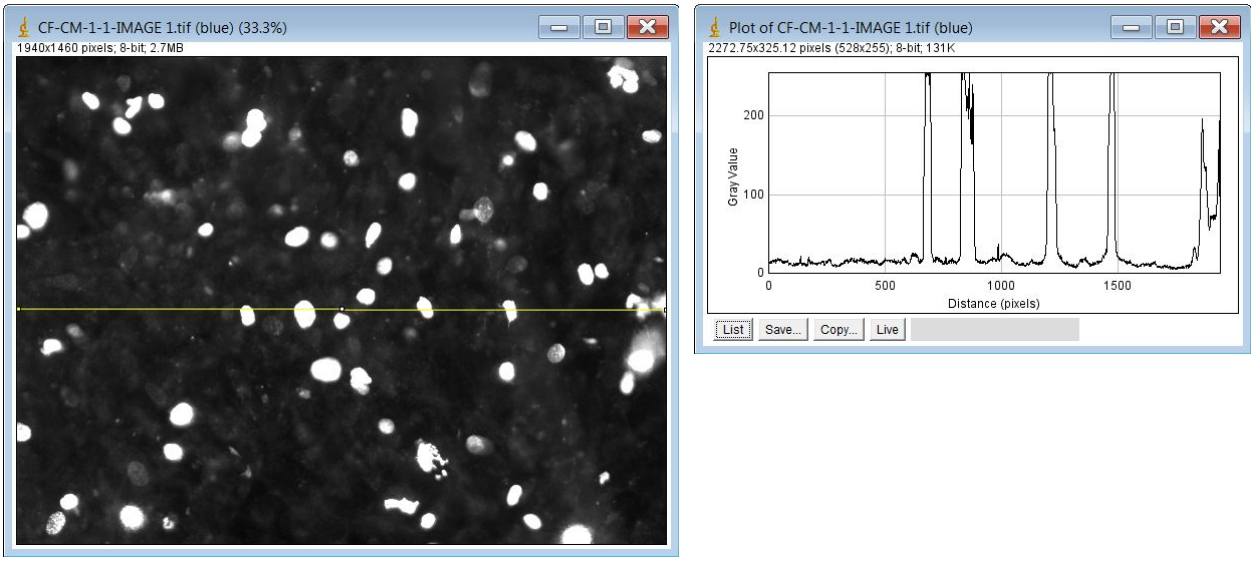


Figure S5I. Plot Profile depicting intensity values across the yellow line in the image representing non-bioprinted samples.

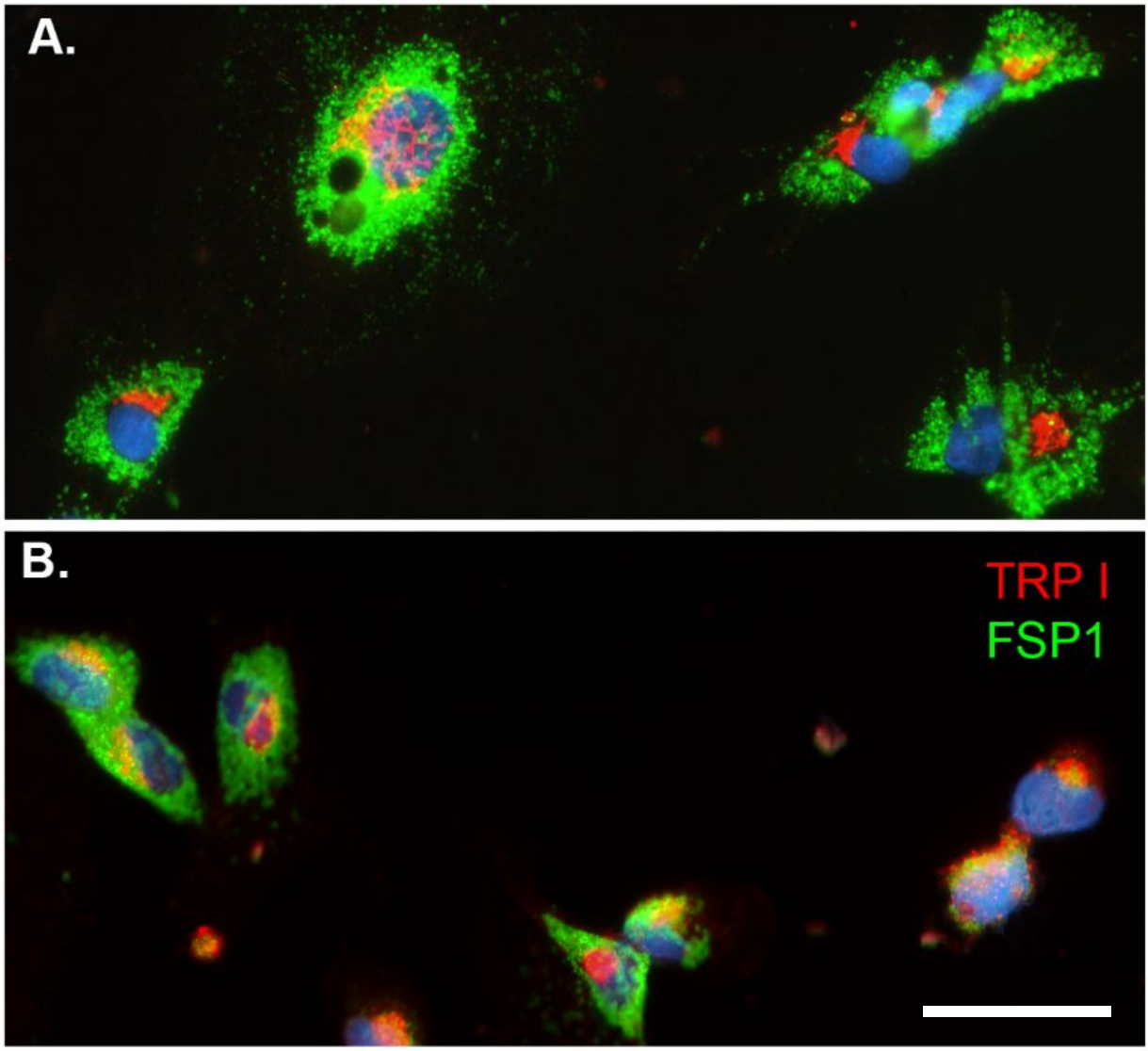


Figure S6. Confirmation of hetero-cellular coupling between CM and CF. Shown in panels, A and B are co-cultured cells immunostained with TRP-I (red), FSP-1 (green) and DAPI (blue). Scale bar corresponds to 50 μm.

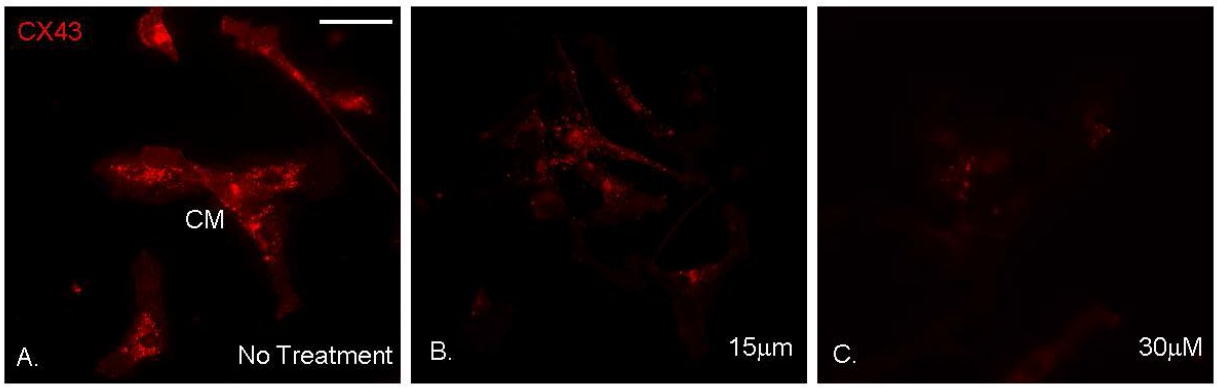


Figure S7. Confirmation of Cx43 (red) expression in CM cultured without Gap26 (A) and with increasing doses of Gap26 in (B) and (C). Scale bar corresponds to 50 μm.

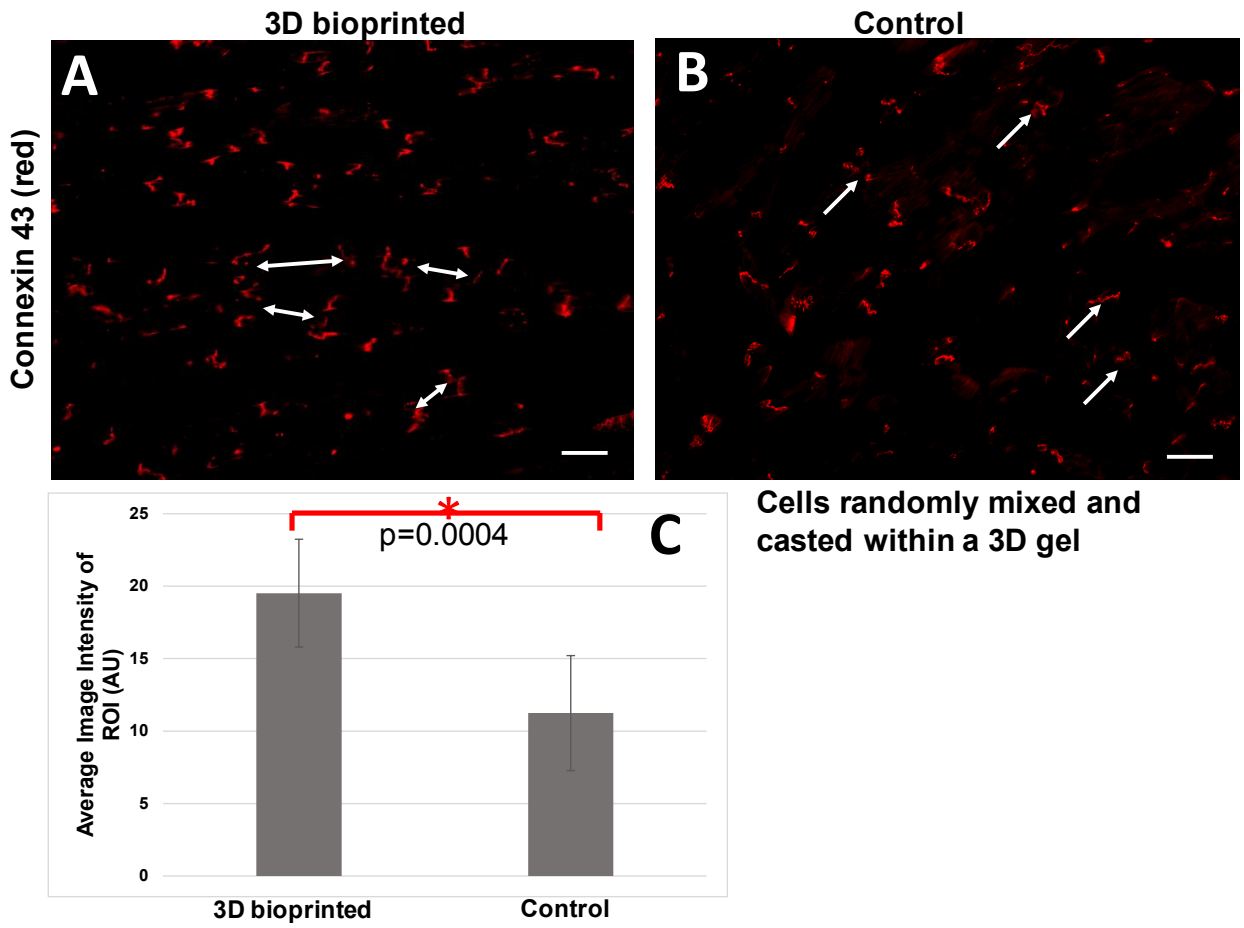


Figure S8. In A and B, a comparison of the overall morphologies of gap junctions of cardiomyocytes (AC16) that were 3D bioprinted versus randomly casted within 3D gels are compared. The gap junctions of the cells stained with Connexin (Cx)-43 are shown. In A, note the conventional distribution of Cx43-positive gap junctions predominantly at the intercalated discs (double arrows). This observation is similar to the expression of Cx-43 shown by native cardiomyocytes in vivo cardiac tissues [5].

Note the disrupted morphology and lack of pattern for Cx-43 expression in B, but not in A. In B, the Cx-43 are sporadically distributed on lateral surfaces (arrows) in the cells. Scale bars are 10 μm in A and B. In C, the quantification of average intensity of the Cx-43 (using Image J) in cells in 3D bioprinted and non bioprinted controls revealed significant differences.

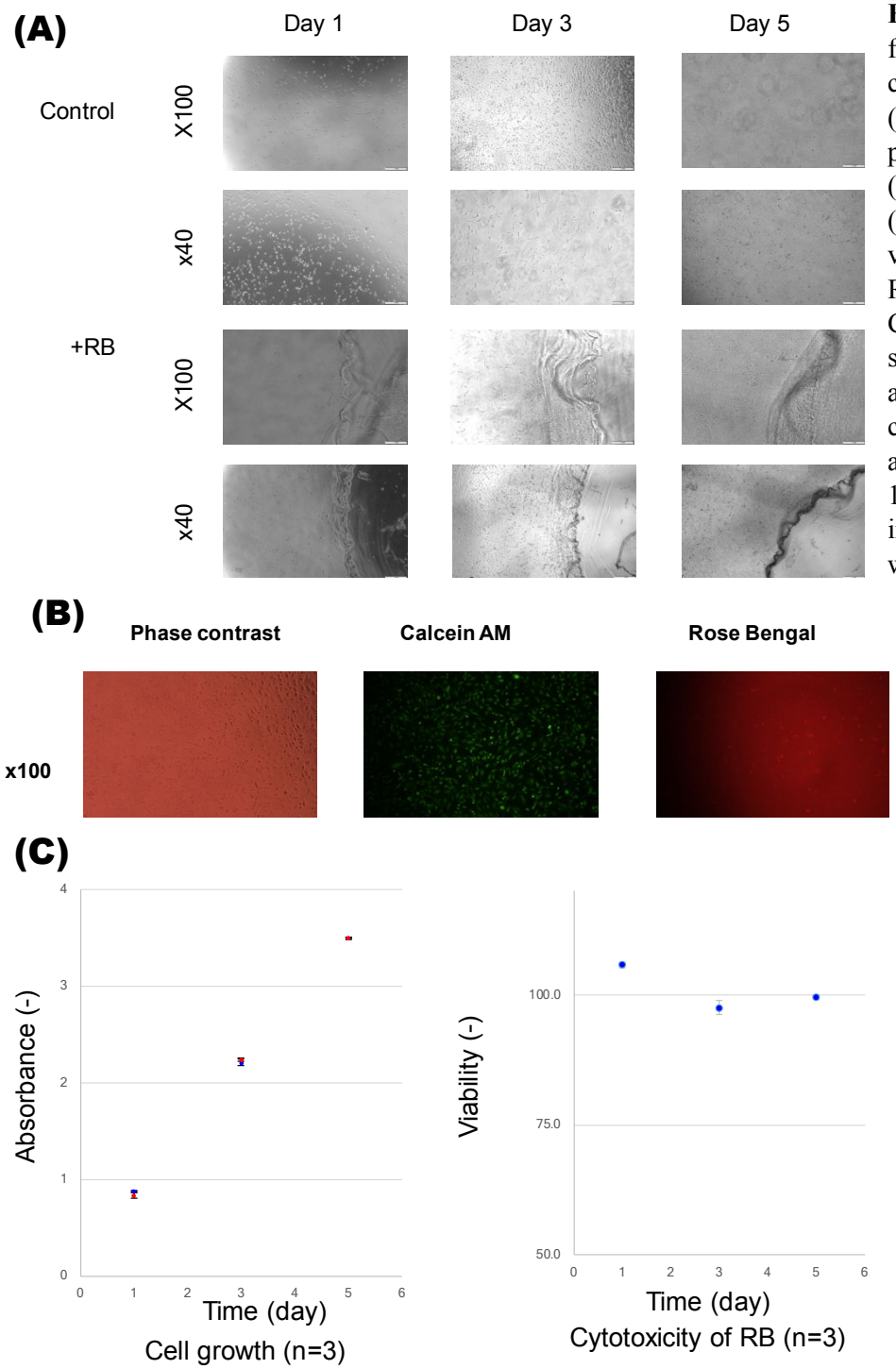


Figure S9. (A) Bright-field images of cells cultured in TCPS (Control) or in the presence of Rose Bengal (+RB) at days 1, 3 and 5. (B) Confirmation of cell viability after addition of Rose Bengal using Calcein AM, which only stains for viable cells after 24 h of in vitro culture. (C) Absorbance assay conducted at days 1, 3 and 5 depict an increase in cell growth while viability was also maintained across samples at different time points.

References

1. AnilKumar, S.; Allen, S. C.; Tasnim, N.; Akter, T.; Park, S.; Kumar, A.; Chattopadhyay, M.; Ito, Y.; Suggs, L. J.; Joddar, B., The applicability of furfuryl-gelatin as a novel bioink for tissue engineering applications. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **2018**.
2. Kolehmainen, K.; Willerth, S. M., Preparation of 3D fibrin scaffolds for stem cell culture applications. *Journal of visualized experiments: JoVE* **2012,** (61).
3. http://rsbweb.nih.gov/ij/docs/guide/userguide-27.html#sec:Analyze-Menu.
4. Huang, J.; Min Lu, M.; Cheng, L.; Yuan, L.-J.; Zhu, X.; Stout, A. L.; Chen, M.; Li, J.; Parmacek, M. S., Myocardin is required for cardiomyocyte survival and maintenance of heart function. *Proceedings of the National Academy of Sciences* **2009,** *106* (44), 18734-18739.
5. Tirziu, D.; Giordano, F. J.; Simons, M., Cell communications in the heart. *Circulation* **2010,** *122* (9), 928-937.