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Electronic Supplementary Information

Patterning microscale extracellular matrices to study endothelial and cancer cell interactions *in vitro*

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Supplementary Figure S1. Growth of cell lines in various co-culture media compositions.
(A) Light microscopy images of ECFCs after 36 hrs culture; and BCC lines (MCF10A, MCF7, and MDA-MB-231) after 96 hrs in varying compositions of co-culture media as indicated. (B) Graphs represent the percent change in cell number after culture of (i) BCCs and (ii) ECFCs. Values shown are mean +/- SD. Scale bars = 100μm.

Supplementary Figure S2. Silane treatment and ECM molecule specificity.
(A) APTMS and (B) OTS support the attachment of both Fn (red) and HA (green). (C) Separate channel images of **Fig. 1B**, right panel. Scale bars = 100 μm.

Supplementary Figure S3. Breast cancer cell viability in HA hydrogels.
Light microscopy images of BCCs encapsulated in HA hydrogels after 1 and 3 days in culture. Inserts are IF images of BCCs stained with calcein (green-live staining) and ethidium bromide (red-dead staining). MDA-MB-231 BCC spreading is indicated by arrows. Significance levels were set at: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Values shown are mean ± SD. Scale bars = 100μm.

Supplementary Figure S4. PDMS micromold dimensions.
Depth of PDMS micromold wells were determined by obtaining a sequence of z-stack images using LSM software. Depths were visualized using orthogonal sections. Top and bottom views of a single well of a PDMS micromold (well dimensions: 200μm x 200μm). Different well depths are achieved by varying parameters during the silicon master fabrication. (A) PDMS micromold well with a depth of 100 μm. (B) PDMS micromold well with a depth of 210μm. Scale bar is 100 μm.

Supplementary Table S1. Polymerization of micromolded HA hydrogels.

Supplementary Experimental

1.1 Fabrication of silicon master and polydimethylsiloxane (PDMS) stamps and micromolds

Standard photolithography techniques were used to fabricate silicon masters patterned with micron-sized features for both stamp and micromold applications as previously described [1-2]. Before use, silicon wafers were rinsed with ethanol and air-dried. To create silicon masters specifically for PDMS stamp fabrication, and for subsequent use in microcontact printing, we followed the previously described protocol [1-2]. To create silicon masters specifically for PDMS micromold fabrication, the same protocol was followed with a slight adjustment. An SU-2050 epoxy negative photoresist was applied by spin coating and the spin coating parameters adjusted accordingly to achieve final desired photoresist heights (50-200mm) via manufacturer's instructions. To create a complementary elastomeric stamps or micromolds, PDMS prepolymer (Sylgard 184) was mixed with a curing agent (Dow Corning, Midland, MI) in a 10 : 1 weight ratio and cast onto the silicon master. The depths of PDMS micromolds were measured using confocal microscopy (LSM 510 Meta, Carl Zeiss Inc., Thornwood, NY). Briefly, a sequence of z-stack images was obtained using LSM software and depths were visualized using orthogonal sections (ESI, Fig. S4).

1.2 Cell culture

1.2.1 MCF10A cell line

The breast cancer cell (BCC) line MCF10A was kindly provided by the laboratory of Dr. Thea Tlsty (University of California San Francisco) associated with the Physical Sciences-Oncology Center (PSOC, National Institutes of Health; Bethesda, MD). MCF10A cells were cultured in Dulbecco's Modified Eagles Medium F12 (DMEM-F12; Gibco Carlsbad, CA) supplemented with 5% v/v horse serum (Gibco), 0.5µg/ml hydrocortisone, 20ng/ml human epidermal growth factor, 10µg/ml insulin (Sigma; Allentown, PA), 100ng/ml cholera toxin (Sigma) and 100 units/ml penicillin and 100µg/ml streptomycin (Sigma).

1.2.2 MCF7 cell line

The BCC line MCF7 (ATCC, Manassas, VA) was maintained in DMEM (Gibco) supplemented with 10% v/v heat inactivated fetal bovine serum (FBS; Gibco) and 100 units/ml penicillin and 100µg/ml streptomycin.

1.2.3 MDA-MB-231 cell line

The BCC line MDA-MB-231 (ATCC, Manassas, VA) was maintained in DMEM supplemented with 10% v/v heat inactivated fetal bovine serum.

1.2.4 Human endothelial colony forming cells (ECFCs)

Human endothelial colony forming cells (ECFCs, passages 1-10, Lonza, Walkersville, MD) were expanded in endothelial growth medium (EGM-2, Lonza) according to manufacturer's instructions.

1.3 Determination of co-culture media composition

All cell types were individually seeded at a cell density of 10,500 cells/cm^2 (BCCs: MCF10A, MCF7 and MDA-231) or 15,625 cells/cm^2 (ECFCs). A series of five different co-culture media compositions with varying ratios of cancer media:ECFC media were investigated

as follows: 100:0 (pure ECFC media), 75:25, 50:50, 25:75, 0:100 (pure cancer cell media). Each cell type was cultured in the co-culture media compositions for 36-96 hours. For the BCC trials, the BCC media corresponding to the cell line being investigated was used in the co-culture media compositions. For the ECFC trials, MDA-MB-231 BCC media was used as the representative cancer media in the co-culture media composition. Experiments were performed in triplicate. Cell growth was visualized and imaged every 12 hours using an inverted microscope (Olympus 1 x 50) (ESI, Fig. S1). Trials investigating BCC growth were stopped after 96 hours, and trials investigating ECFC growth were terminated after 36 hours, when confluence was reached, for further analysis. All cell types were trypsinized, collected and counted (ESI, Fig. S1). The optimal media for co-culture experiments was determined to be 100% ECFC media. All BCC lines maintained at least the same growth rate in 100% ECFC media compared to respective cancer cell media, whereas ECFC growth was significantly impaired during culture containing any cancer cell media constituents. Therefore, we continued all co-culture studies using ECFC media.

1.4 Co-culture of ECFCs and cancer cells on HA and Fn patterned substrates (2-D)

1.4.1 Development of HA and Fn patterned surfaces

To allow the discrete molecular presentation of two ECM components, Fn and HA, adjacent to cell resistant PEG-ylated regions a dual patterning approach using microcontact printing (μCP) and silane chemistry was developed. Hyaluronic acid was the first molecule to be patterned, as previously described [2-3]. Briefly, a fresh 3 percent v/v solution of 3-aminopropyl-trimethoxysilane (APTMS) (Fluka Chemical Corp., Milwaukee, WI) in 95 percent v/v ethanol was prepared, reacted for 5 min, and applied by spin coating onto a PDMS stamp with square features, at 3500 rpm for 35 s. A PDMS stamp with square features transferred the APTMS solution to a plasma-treated glass substrate. Glass substrates were rinsed with ethanol, air-dried, and heated for 1 h at 115 °C. An aqueous HA solution (800 kDa, fluorescein FL-labeled; Sigma, St. Louis, MO; 50 mg/ml) was prepared with 10 mM of EDC (Pierce Biotechnology, Inc., Rockford, IL) and 5 mM of NHS (Pierce Biotechnology) and incubated on the glass substrates for 16 h, protected from light. Excess FL-HA solution was rinsed from the glass substrate with de-ionized (DI) water. To pattern Fn, a fresh 0.5 percent v/v solution of octadecyltrichlorosilane (OTS, Sigma) was prepared in n-hexane (Sigma-Aldrich, Sheboygan,WI) and was applied by spin coating as previously described[1]. A PDMS stamp with stripe features transferred the OTS solution to HA patterned substrates. Substrates were immediately rinsed with hexane, DI water, and then air-dried. To provide a non-adhesive region surrounding the patterned regions, substrates were immersed for 45 minutes at 90 °C in a polyethylene glycol (PEG)-silane solution (prepared fresh daily) of 20 percent v/v2-[methoxy(polyethyleneoxy)propyl]-trimethoxysilane (Mw 300–350, Gelest Inc., Morrisville, PA) in toluene with 1 percent triethylamine. Substrates were rinsed with toluene, DI water and then sterilized for 30 min under UV light in a tissue culture hood. Approximately 200 μl of 30 μg/ml Fn solution was pipetted onto each substrate and reacted for 1 hour. Excess Fn solution was rinsed with phosphate-buffered saline (PBS), followed by incubation with 1 percent bovine serum albumin (BSA) in PBS for one hour at RT to reduce the nonspecific binding of proteins.

1.4.2 Cell culture on HA and Fn patterned substrates

For monoculture studies, human ECFCs, MCF10A, MCF7 or MDA-MB-231 were grown to confluence, washed with PBS, and digested using 0.05 or 0.25 percent trypsin/0.1 percent EDTA (Invitrogen).

For monoculture, cell densities of 1-2x10^3 cells/cm^2 were seeded onto each dual patterned substrate and imaged at indicated time points using inverted microscopy (Olympus 1 x 50). Monoculture studies were analyzed after 24 hours.

For co-culture experiments, human ECFCs were seeded on our dual patterned substrate as described above. After 12 hours of culture, MCF10A, MCF7, or MDA-MB-231 BCCs were seeded and incubated for an additional 24 hours culture. All co-culture experiments were performed in ECFC culture media (ESI†, Fig. S1).

1.4.3 Fibronectin and collagen I substrates

To create a surface presenting both Fn and collagen I, two glass coverslips were coated with Fn (30μg/ml) or collagen I (50μg/ml; Roche) for 1 hour at RT. Coverslips were split into halves and set adjacent to each other so that half of the coverslip was coated with Fn and half with collagen I. Endothelial colony forming cells were uniformly seeded at a concentration of 3x10^5 cells and cultured for 12 hours.

1.5 Three dimensional co-culture of cancer cells and ECFCs

1.5.1 Synthesis of AHA hydrogels

AHA hydrogels were prepared as previously reported.[4] Briefly, AHA was synthesized using a 2-step protocol: (1) sodium hyaluronate reacted with the highly acidic ion exchange resin Dowex-100and neutralizing with 0.2M TBA-OH to synthesize tetrabutylammonium salt of HA(HA-TBA); (2) acrylic acid was coupled to (2.5/equivalent [Eq]) HA-TBA (1 Eq, repeat unit) in the presence of dimethylaminopyridine (DMAP; 0.075 Eq) and di-tert-butyl dicarbonate (1.5 Eq) in DMSO, followed by dialysis and lyophilization; ^{1}H NMR spectrum confirmed the final percent modification of the AHA.

1.5.2 Micromolded HA hydrogels

AHA polymer (3 weight percent [wt%]) was dissolved in a sodium phosphate buffer (Na_3PO_4 buffer: 0.2M Na_3PO_4, pH 8.0) overnight with agitation. The cell-adhesive peptide (GCGYGRGDSPG; molecular weight [MW]: 1025.1 Da; RGDS indicates RGD integrin-binding domain; GenScript) were dissolved in sodium phosphate buffer and added to the AHA solution at final peptide concentrations of 4.7mM (corresponding to ~13% of available acrylate groups within 3 wt% AHA) and allowed to react for 1 hour with gentle shaking. This occurs via conjugate addition reactions between thiol groups on the RGD-containing peptide and acrylate functional groups along the HA backbone.[4] To encapsulate BCCs in HA hydrogels, human BCCs (MCF10A, MCF7, and MDA-MB-231) of cell densities of 3-7 x 10^6 cells/mL were resuspended in the polymer solution. After preliminary viability studies, only MDA-MB-231 BCCs were used in micromolded HA hydrogels at a cell concentration of 6x10^6 cells/ml. Next, the MMP-cleavable peptide crosslinker (MMP; GCRDGPQG↓IWGQDRCG; MW: 1754.0 Da; down arrow indicates the site of proteolytic cleavage; GenScript), dissolved in sodium phosphate buffer, was added at 5.83mM (corresponding to the 30% of available acrylate groups within 3

wt% AHA). Thiol groups on each end of the peptide crosslinker react with acrylate groups of the HA backbone. Immediately after adding the MMP-cleavable crosslinker, ~50 μL of this mixture was pipetted into either sterile molds (5 mm diameter, 2 mm height; for initial studies), or the sterile PDMS micromolds. A cell scraper was used to remove excess polymer solution from the PDMS micromolds before inversion on acrylated glass substrates. PDMS molds were inverted onto acrylated glass substrates and the HA polymer solution reacted for 10-20 minutes at room temperature inside the laminar flow hood. The HA hydrogels were cultured for up to 3 days in the appropriate cell media. To prepare acrylated glass substrates, substrates were plasma treated for 5 minutes, coated with a 98 percent v/v solution of 3-(trimethoxysilyl)propyl acrylate (Sigma), and heated for 30 minutes at 100°C followed by 10 min at 110°C. Substrates were rinsed in 100 percent ethanol and air dried. Visualization and image acquisition were performed using an inverted light microscope (Olympus IX50) at various time intervals along the culture period.

1.5.3 3-D co-culture of cancer cells and ECFCs

Micromolded HA hydrogels encapsulated with MDA-MB-231 BCCs were prepared as described above and cultured for 12 hours in MDA-MB-231 cell media. During this time, glass substrates patterned with Fn were prepared as previously described.[1] ECFC densities of 12,500 cells/cm^2 were seeded onto Fn patterned substrates and cultured for 12 hours. At 12 hours, MDA-MB-231 cell media was aspirated from substrates with micromolded HA hydrogels, and a fibrinogen/thrombin solution (3:2 ratio, Millipore, Bedford, MA) was added and polymerized in an incubator at 37 °C for 20 minutes to form a fibrin gel. ECFC patterned substrates were then inverted on top of fibrin/micromolded HA hydrogels and substrates were cultured in ECFC media for an additional 24 hours[1](Fig. 5B).

1.5.4 Cancer cell viability

Cell viability after incorporation into HA hydrogels was determined with a live/dead cytotoxicity kit (Molecular Probes), using a calcein and ethidium bromide stain. Cells encapsulated in HA hydrogels for 1-3 days were washed once with PBS, and replaced with the appropriate cancer cell media containing calcein and ethidium bromide dye solution (at 2 μM) and incubated for 30 min at 37°C. Substrates were then washed twice with PBS and immediately imaged using fluorescent microscopy (Olympus BX60). ImageJ software (NIH) was used to calculate the percentage of viable cells by quantifying the number of viable (green) cells and the total cells [viable and non-viable (red) cells] in each fluorescent image. Merged fluorescent images were converted to grayscale (8-bit) images and separated into viable (green) and non-viable (red) panels. All particles, representing nuclei, were counted via the 'Analyze'-> 'Analyze Particles' function in ImageJ, and the percent of viable cells calculated by the equation #of viable cells/ total # of cells. Results are presented $^{+}$/-SD.

1.6 Immunofluorescence of 2-D and 3-D substrates

Two dimensional HA and Fn patterned substrates were fixed using 3.7 percent formaldehyde (Fisher Chemical, Fairlawn, NJ) in PBS for 20 minutes and washed with PBS. Substrates used for cell culture with MCF7, MCF10A, MDA-MB-231 and/or ECFCs were permeabilized with a solution of 0.1 percent Triton-X (Sigma) for ten minutes and washed with PBS. Substrates were incubated with either primary anti-human Fn (1:200, Sigma), FITC-conjugated phalloidin (1:40, Molecular Probes, Eugene, OR), anti-human CD-44 (1:100, Sigma), or anti-human CD31 (1:200, Sigma) for 1 hr. Samples incubated with primary anti-mouse

antibodies were washed twice with PBS and incubated with anti-mouse IgG Cy3 conjugated secondary antibody (1:50, Sigma) for 1 h. After rinsing twice with PBS, samples were counterstained with DAPI (1:1000, Roche Diagnostics, Basel, Switzerland) for an additional ten minutes. The immunolabeled cells were visualized using fluorescence microscopy (Olympus BX60).

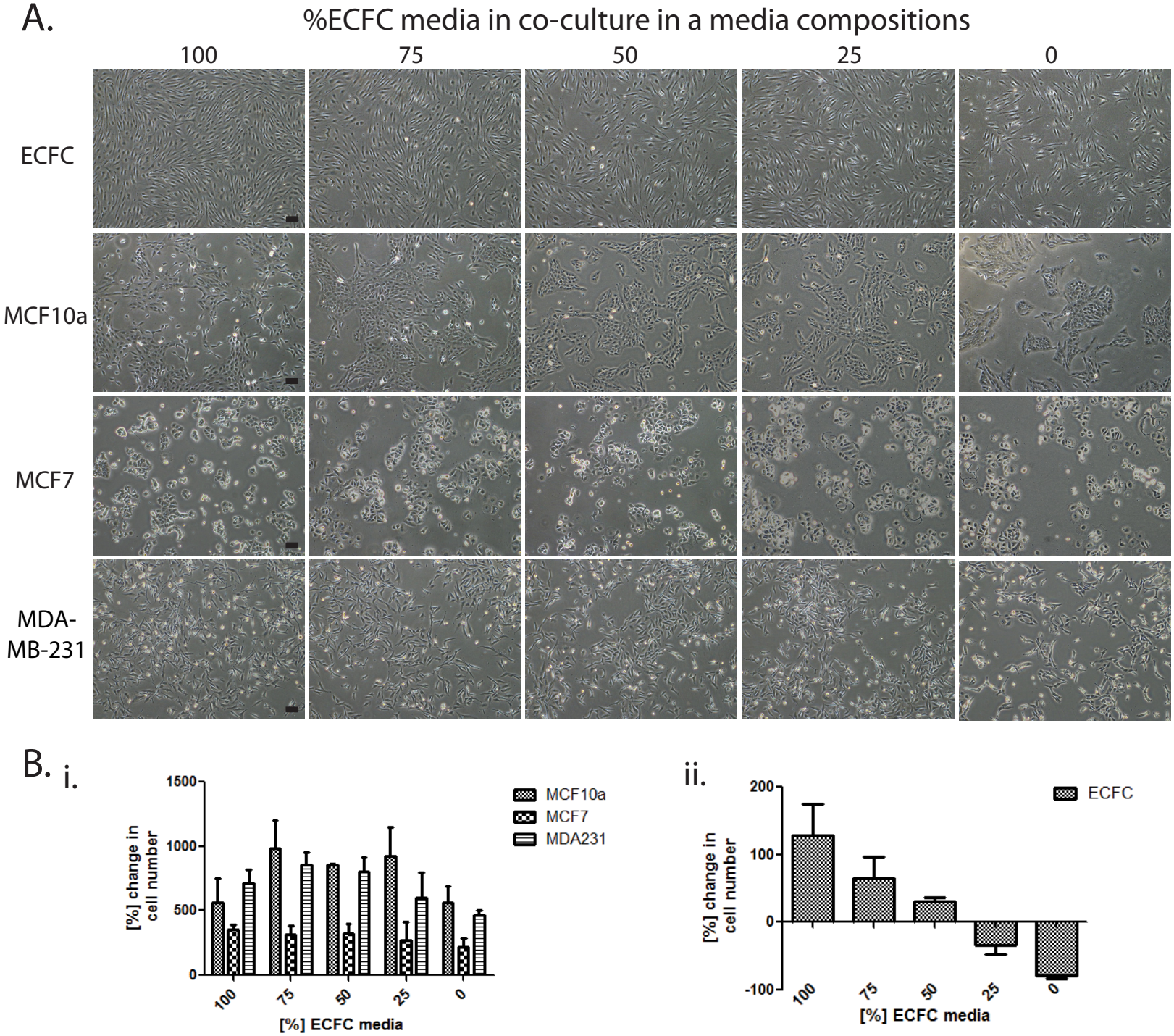
MDA-MB-231 BCCs encapsulated in 3-D micropatterned HA hydrogels were fixed using 3.7 percent formaldehyde (Fisher Chemical, Fairlawn, NJ) in PBS for 20 minutes and washed with PBS. Substrates were stained with DAPI for 10 minutes, followed by incubation with FITC-conjugated anti-human Ki67 (1:50, Invitrogen) for 1 h. Samples were immediately rinsed twice with PBS and visualized using fluorescence microscopy (Olympus BX60). The percentage of proliferating cells was determined by quantifying the number of Ki67+ (green) cells and the total cells in each fluorescent image. Merged fluorescent images were converted to grayscale (8-bit) images and separated into Ki67+ (green) and all cells (blue-dapi) panels. All particles, representing nuclei, were counted via the 'Analyze'-> 'Analyze Particles' function in ImageJ, and the percent of proliferating cells calculated by the equation # of Ki67+ cells/ total # of cells. Results are presented $^{+}$/-SD.

1.7 Graphs and Statistics

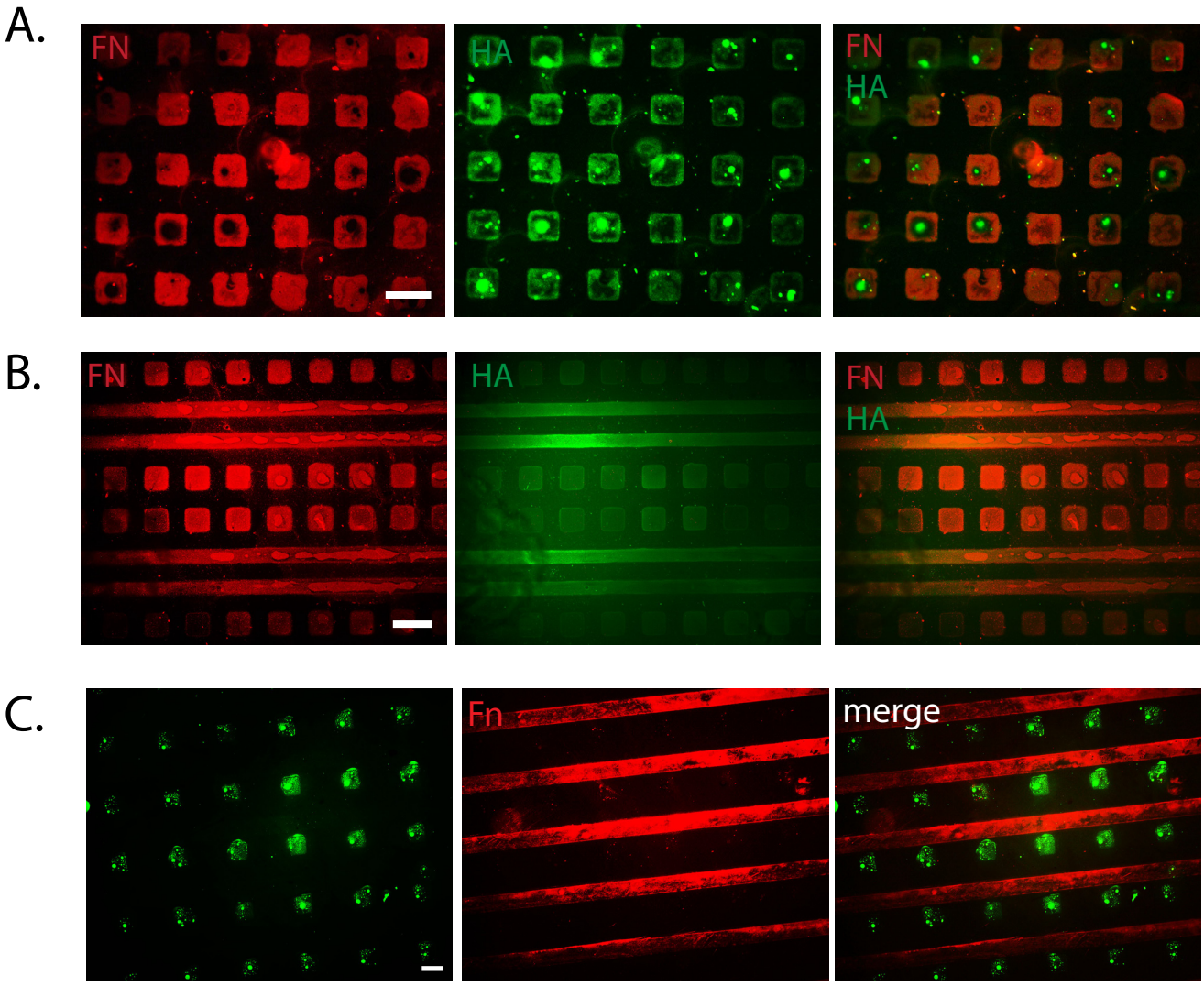
Graphs of cell number and viability were plotted as ± standard deviation (SD). Unpaired two tailed T-tests and parametric two-way ANOVA tests were performed where appropriate (GraphPad Prism 4.02, GraphPad Software, San Diego, CA). Significance levels were determined between samples examined (as described in figure legends), and were set at: *$p<0.05$, **$p<0.01$, and ***$p<0.001$. All graphical data were reported.

1. L. E. Dickinson, M. E. Moura and S. Gerecht, *Soft Matter*, 2010, **6**, 5109-5119.
2. L. E. Dickinson, C. C. Ho, G. M. Wang, K. J. Stebe and S. Gerecht, *Biomaterials*, 2010, **31**, 5472-5478.
3. L. E. Dickinson and S. Gerecht, *Journal of Visualized Experiments*, 2010, **46**, 2413.
4. S. Khetan, J. S. Katz and J. A. Burdick, *Soft Matter*, 2009, **5**, 1601-1606.

Supplementary Figure 1



Supplementary Figure 2



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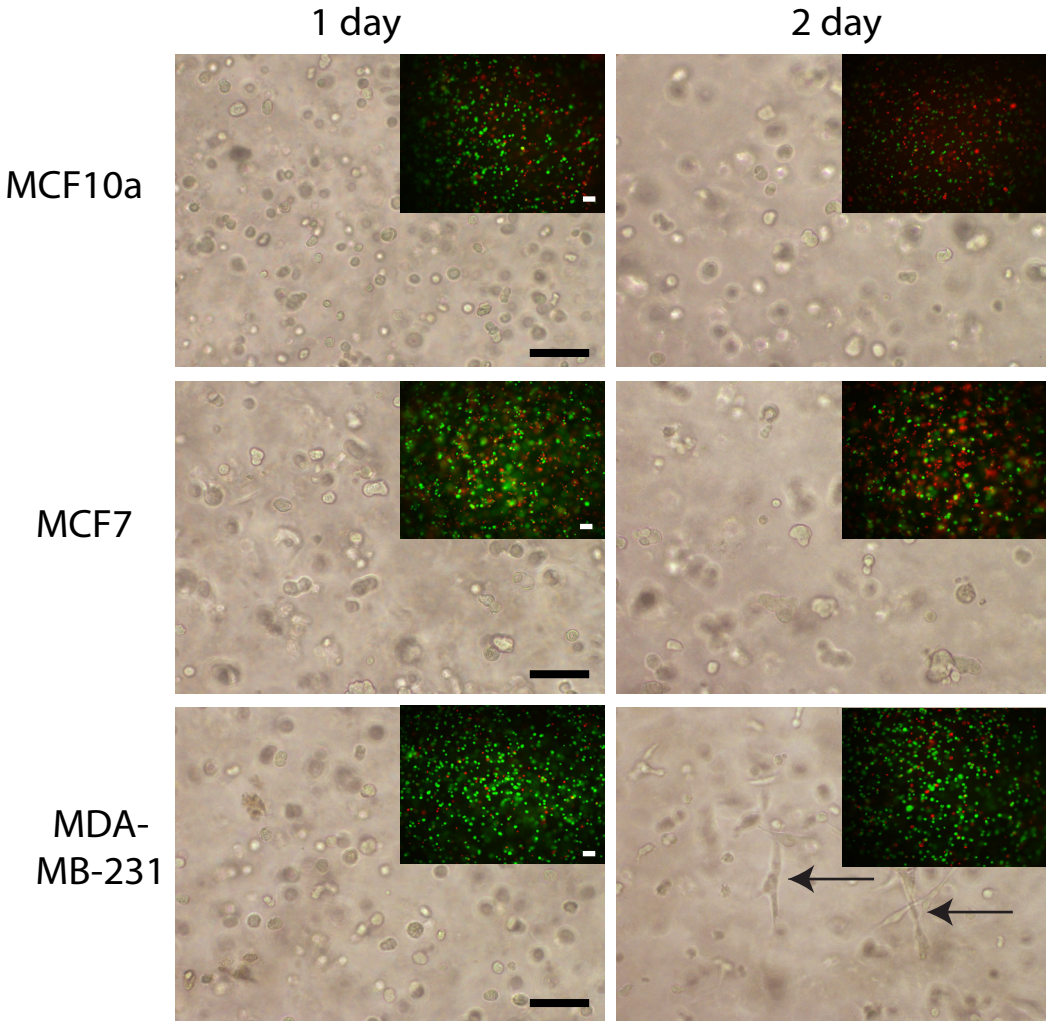
Supplementary Figure 3



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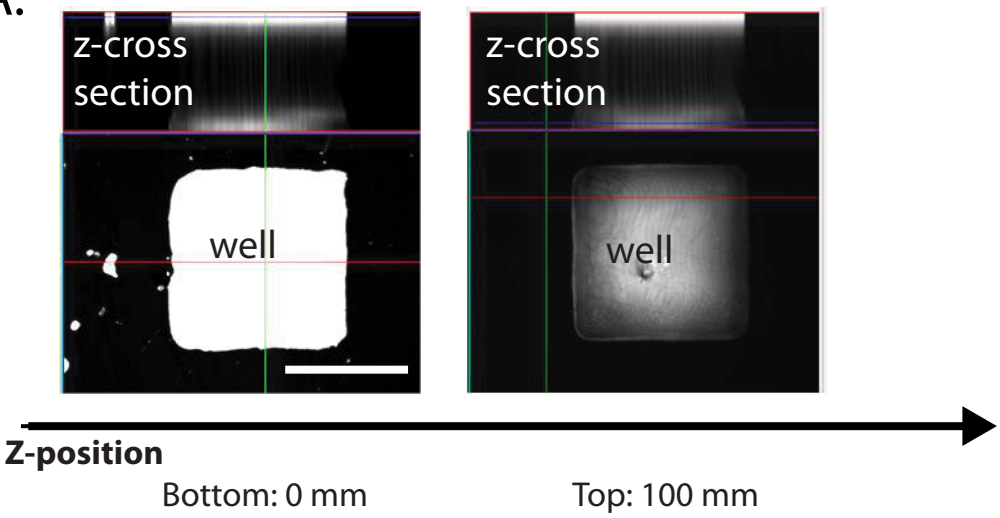
Supplementary Figure 4

A.



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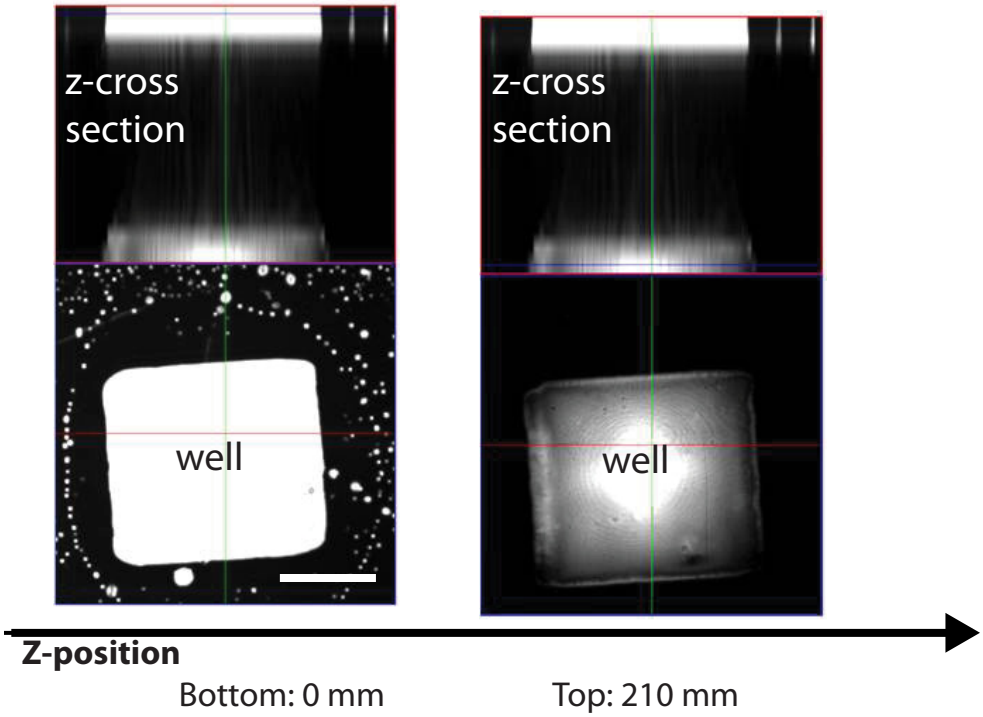

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Table 1

% modification	polymer wt %	Polymerization of micromolded HA hydrogels
25%	1 wt%	HA hydrogel unable to form micromolded structures - too soft and collapsed
	2 wt%	
	3 wt%	
30%	1 wt%	HA hydrogel unable to form micromolded structures - too soft and collapsed
	2 wt%	
	3 wt%	***HA hydrogel able to form micromolded structures of various dimensions***
50%	1 wt%	HA hydrogel unable to form micromolded structures - too viscous
	2 wt%	
	3 wt%	
70%	1 wt%	HA hydrogel unable to form micromolded structures - too viscous
	2 wt%	
	3 wt%	