

Supporting Information for

Paper-Supported Three-Dimensional Cell Culture for Tissue-Based Bioassays

Ratmir Derda, Anna Laromaine, Akiko Mammoto, Sindy K. Y. Tang, Tadanori

Mammoto, Donald E. Ingber and George M. Whitesides

Methods.

Cells and reagents We purchased MDA-MB-231 cells from ATCC and primary IMR-90, HDF and primary HUVEC cells from Lonza. MDA-MB-231, IMR-90, HDF and LLC cells were cultured in Eagle's minimal Essential medium (MEM) from Lonza supplemented with 10% fetal bovine serum (FBS) and 1% GlutaMax™ (both from Invitrogen). Primary HUVEC cells were cultured in endothelial growth medium (EGM-2, Lonza). Matrigel (growth factor-free) was purchased from BD-Bioscience.

General protocol for plating and culture of cells on paper substrates

To prepare paper substrates, we cut chromatography paper (Millipore) using scissors or a laser cutter (Versa Laser -Universal Laser VL-300, 50 Watt, using the settings for 200 μm deep-engraving plastic). The papers were rinsed them with deionized water, autoclaved, and dried them in a laminar flow hood.

To plate the cells on paper substrates, we detached the cells from growth flasks using treatment with trypsin-EDTA (3-5 min); washed the cells with serum-containing media and pelleted them by centrifugation at 1,000 rpm for four minutes. The cells were re-

suspended in cold Matrigel (free of growth factor) to yield a concentration of 10^7 cells/mL. We spotted this suspension of cells on the specific areas in the paper with a hand-held Gilson P10 pipette man or Eppendorf Repeater Plus pipette, placed the spotted paper into a Petri dish filled with warm (36 °C) growth media and cultured for 24 hours in an incubator at 36 °C and atmosphere of 5% CO₂. Subsequently, we folded the paper using sterile tweezers and we pressed them together using custom made plastic or stainless steel holders. The stacks were cultured in 25 mm-deep Petri dish for the desired time at 36 °C, in a 5% CO₂ incubator, on an orbital shaker set to 40-60 rpm. We exchanged the media every two days. At a chosen time, each construct was processed (pre-treatment, fixation, permeabilization, staining) according to the Table 1. The paper containing labeled cells was scanned using a Typhoon gel scanner with the proper setting for each stain. Table 1 lists scanner settings—the laser excitation, emission wavelength, resolution, and value for the photo multiplier tube (PMT)—for each stain. The images obtained after scanning were analyzed using ImageJ or MatLab.

Table 1. Pre-treatment and staining procedures.

Quantification/ Staining	Pre-treatment	Fixation, permeabilization ^a	Staining protocol (time)	Scanner settings (Excitation/ emission (nm); resolution (μm); PMT (V))
Quantification of number of cells in paper using actin stain	N	Y, Y	Incubate with 1:500 dilution of Alexa Fluor 633 phalloidin in TB-PBS (30 min)	red /670; 50; 450
			Incubated with 1:300 dilution of Texas Red phalloidin in TB-PBS (30 min)	green/610; 50; 500
			Incubate with 1:300 dilution of Alexa Fluor 488 phalloidin in TB-PBS (30 min)	blue/520; 50; 300
Quantification of S-phase cells using Click-iT EdU stain	Supplement the media with 4 μM EdU for 4 or 24 hours at 36 °C and 5% CO ₂	Y, Y	1) Click-iT EdU reaction (12 h) 2) Wash with TB-PBS (3x5 min). 3) Incubate with 1 μM SYTOX green in TB-PBS. (15 min)	EdU: red/670; 50; 400 SYTOX: green/526; 50; 300
Quantification of DNA damage using Click-iT TUNEL stain	N	Y, Y	1) Incorporate TdT (Invitrogen) 2) Click-iT reaction to conjugate azido-Alexa Fluor 633 (Invitrogen). 2) Wash with TB-PBS (3x5 min) 3) Incubate with 1 μM SYTOX green in TB-PBS (15 min)	TUNEL: red/670; 50; 400 SYTOX: green/526; 50; 300
Quantification of metabolically active cells using calcein	1) Rinse samples with HBSS (20min). 2) Incubate in calcein solution (4 μM in HBSS).	Y, N	N	blue/520; 50; 300 ^b

Abbreviations used: EdU - 5-ethynyl-2'-deoxyuridine, HBSS - Hanck's balanced salt solution, TdT - Terminal Deoxynucleotidyl Transferase, PBS - phosphate buffered saline, Y - Yes, N - No.

^a To fix the samples, we incubated them in the solution of formaldehyde (4% PFA in PBS) for 15 min. To permeabilize cell membrane, we incubated them with solution of 0.1% triton X-100 and 0.2% bovine serum albumin (TB-PBS) for 10 min.

^b We noticed that prolonged incubation in PFA solution (>24 hours) lead to significant loss of fluorescence due to diffusion of the calcein out of the cells. The samples, hence, were scanned using Typhoon gel scanner within a few hours after fixation.

Quantification of metabolically active cells in eight layers stacks using calcein. To

investigate diffusion of calcein in eight-layer stacked samples, we separated or stacked

the paper layers immediately before the incubation step. See **Fig. S3** for results.

1. “Stack → non-stack”: We cultured the stacked layers for nine days, then washed the samples with warm HBSS, separated the layers using sterile tweezers and placed them within one minute in a pre-warmed solution of calcein AM (4 μ M in HBSS). The separated layers were rocked for ten minutes at 36 °C, and fixed with cold PFA solution.
2. “Non-stack → stack”: The non-stacked layers were cultured for nine days, washed with warm HBSS, stacked, pressed together with a plastic holder and placed them within one minute in the pre-warmed solution of calcein AM (4 μ M in HBSS). The separated layers were rocked for ten minutes at 36 °C, and fixed with cold PFA solution.
3. “Stack → flip”: After nine days of culture, we washed the stacks with warm HBSS media, removed the cellulose acetate layer from the bottom layer (L8), and placed it atop (L1). The resulting stack was placed in a pre-warmed solution of calcein AM (4 μ M in HBSS) within one minute. The separated layers were rocked for ten minutes at 36 °C, and fixed with cold PFA solution.

Quantification of gene expression using real-time PRC. After nine days of culture, we placed the cell-containing area in a 2-mL Eppendorf tube containing 500 μ L of lysis buffer (buffer RLT, RNAeasy Kit, Quiagen). Vortexing the tubes at 4 °C for twenty minutes turned the paper substrate to a homogenous pulp which was centrifuged at 14,000 rpm for two minutes. Both supernatant and the pellet were transferred to QiaShredder tubes (Quiagen) and centrifuged them at 14,000 rpm for two minutes. The flow-through solution was processed using RNAeasy kit according to the manufacturer’s procedure

(Quiagen). Concentrations of RNA were determined using NanoDrop (Thermo Scientific). The yield of RNA from the multi-layered samples correlated with the number of cells in the layers, and ranged from 80 to 2000 ng/ μ L (A_{250}/A_{280} ratio of >1.9). We detected nearly no degradation in the samples as judged by 28S:18S rRNA bands on agarose gels.

We synthesized cDNA from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814) using 100 ng of total RNA in 20 μ L of the reaction volume. The resulting cDNA solution was diluted 10 fold and stored at -80 °C. For PCR, we mixed 5.1 μ L of cDNA solution with 7.5 μ L of Power SYBR® Green PCR Master mix and 1.2 μ L of 10 μ M solution of forward and reverse primers for human VEGF (forward: 5'-TGFA GTACATCTTCAAGCCATCCTGTGTGC-3' and reverse 5'-CCTATGTGCTGGCCTTGGTGAGGTTTGAT-3'), human IGFBP3 (forward: 5'-TCTGCGTCAACGCTAGTGC-3', reverse 5'-GCTCTGAGACTCGTAGTCAACT-3'), human beta-2 microglobulin (forward: 5'-GAATGGAGAGAGAATTGAAAAAGTGGAGCA-3', reverse 5'-CAATCCAAATGCGGCATCTTCAAAC-3'), human actin (forward: 5-CATGTACGTTGCTATCCAGGC-3' and reverse 5'-CTCCTTAATGTCACGCACGAT-3'). We performed the reaction on an Applied Biosystems 7900HT Real-Time PRC system using 40 cycles of a two-step cycling protocol (60 °C for one min, 96 °C, 15 seconds).^a The concentration for VEGF, IGFBP3 transcripts was estimated using the $\Delta\Delta C_t$ approach using C_t of beta-2 microglobulin (human cells) from the same sample. We performed a pair-wise significance test for the

^a The above conditions were tested with 100 fold serial dilution of total cDNA and yielded linear 2^{C_t} vs. Log(concentration) plots and slopes corresponding to efficiency of DNA synthesis equal to 1.9-2.0.

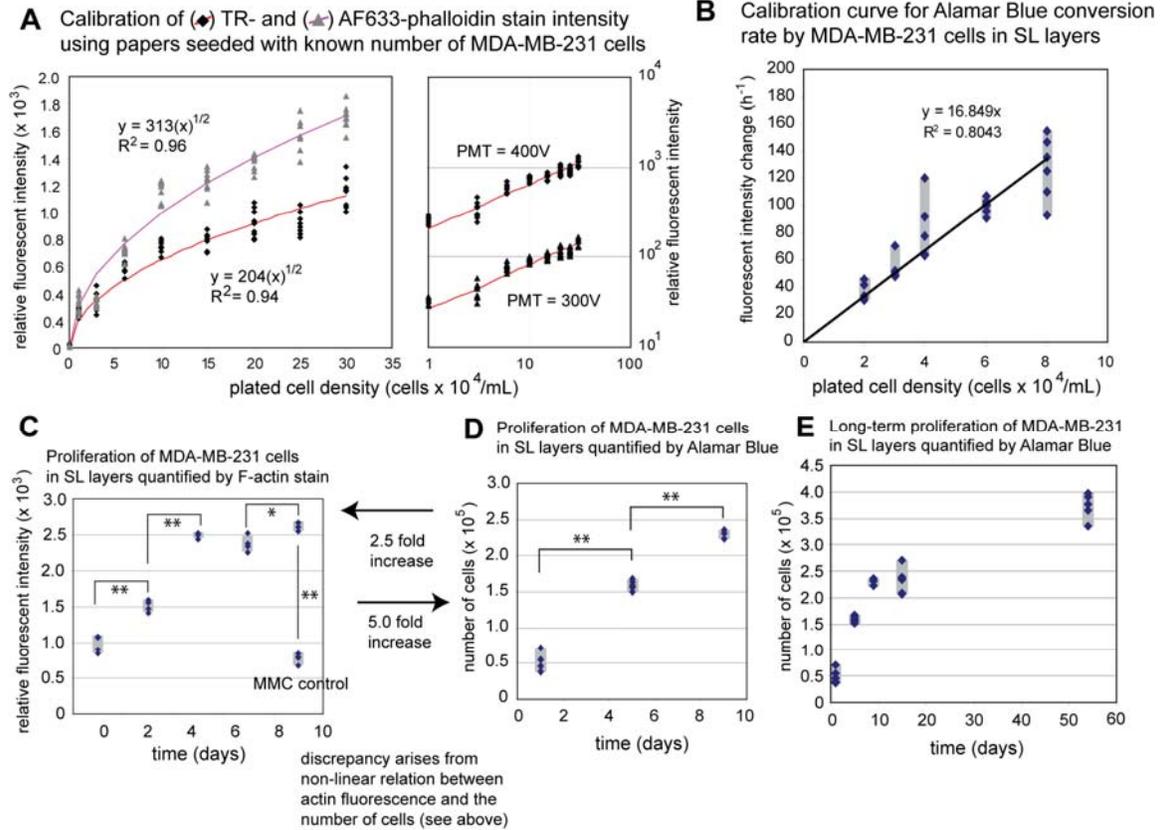
samples calculating P-values for $2^{\Delta Ct}$ values using the Wilcoxon rank sum test. To simplify presentation, all $2^{\Delta Ct}$ were divided by an average $2^{\Delta Ct}$ of L1.

***In vivo* implantation assay.** All animal studies were reviewed and approved by the Animal Care and Use Committee of Children's Hospital Boston. We mixed LLC cells with 1:1 vol/vol mixture of Matrigel and growth medium (10^7 cells/mL), spotted 6 μ L of this suspension onto the patterned chromatography paper (size 8 mm x 8 mm, for SL sample) or 8 x 6 μ L onto the patterned strip of paper (64 mm x 8 mm, for L1L8 sample) (Fig. S4D). The samples were incubated in the growth medium at 37 °C overnight, folded and implanted subcutaneously on the backs of C57BL/6 mice (left and right side). As controls, we cultured the same samples in growth medium *in vitro*. After three days, we injected rhodamine-conjugated ConA into the retro orbital sinus, excised the papers surgically (Fig. S4E), fixed with 4% PFA for 24 hours, and de-stacked the layers for analysis. For sectioning, excised and fixed samples were incubated in 30% sucrose in PBS for 24 hours, embedded in optimal cutting temperature (OCT) compound; each samples was cryosectioned from the edge to the middle of the sample (Fig. S4F, G). We thawed the remaining halves of the samples, rinsed with PBS and de-stacked the layers for further analysis. Sections and de-stacked layers were imaged using fluorescent gel scanner (Fig. 6C-G).

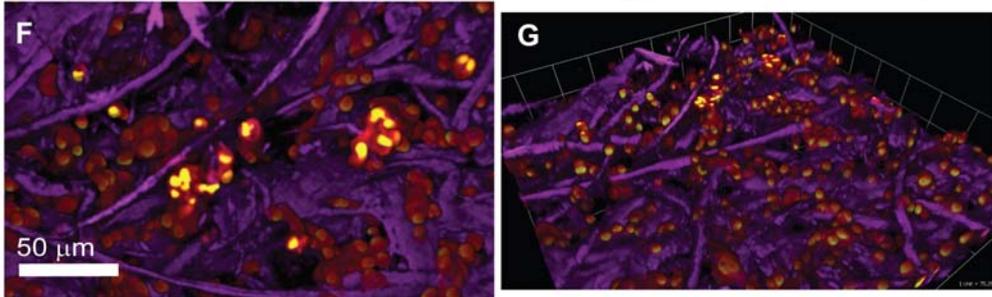
We stained the sections (18- μ m thick) with hematoxylin and eosin (H&E) (Fig. 6H-K) as described previously(1, 2). For immunostaining, the sections were hydrated with PBS (10 min), permeabilized with PBS containing 0.1% Triton-X100 (30 min), blocked with PBS containing 10% fetal calf serum (FCS) and 0.1% triton X-100 (30 min). We used 1:100

dilution of primary antibody (anti-CD31 or anti-CD45, overnight at 4 °C), washed with PBS (3 x 5 min), and incubated with 1:100 dilution of Alexa Fluor 633-conjugated secondary antibody. We mounted the samples using Vectashield+DAPI and performed four-color imaging (blue: DAPI; green: GFP; red Rhodamine ConA; far red: immunostaining) using Carl Zeiss ApoTome or Leica laser scanning confocal microscope (Fig. 6I-P, Fig. S4).

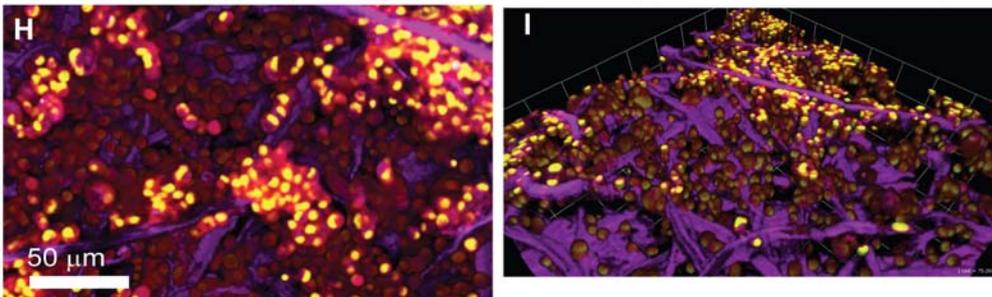
Supplementary Figure S1.



Confocal microscopy, day 1; top view (e) and side view (f) of 3D reconstructed images



Confocal microscopy, day 1; top view (g) and side view (h) of 3D reconstructed images

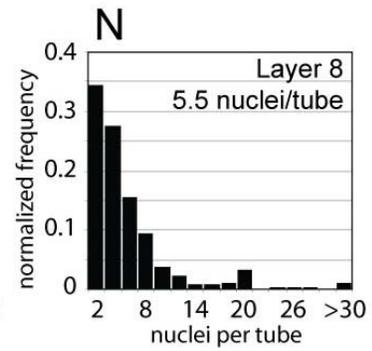
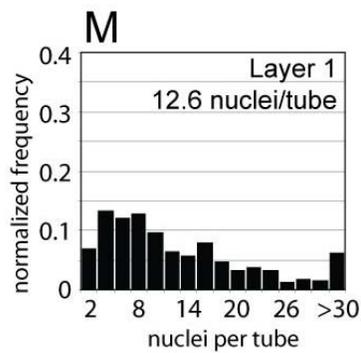
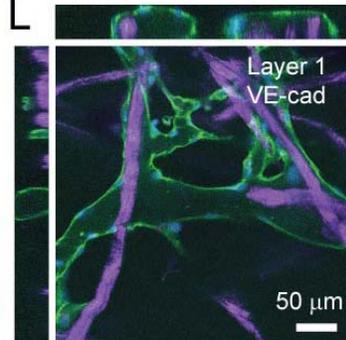
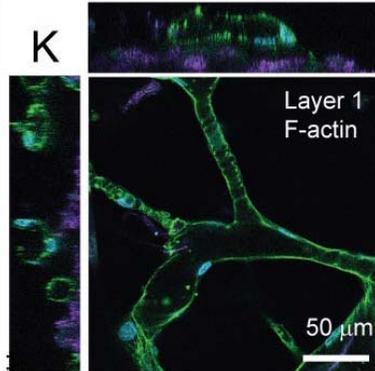
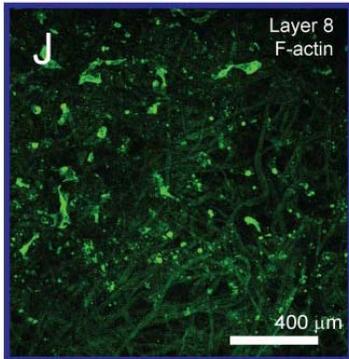
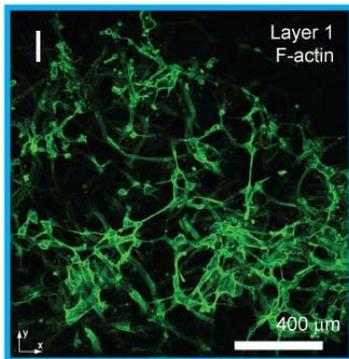
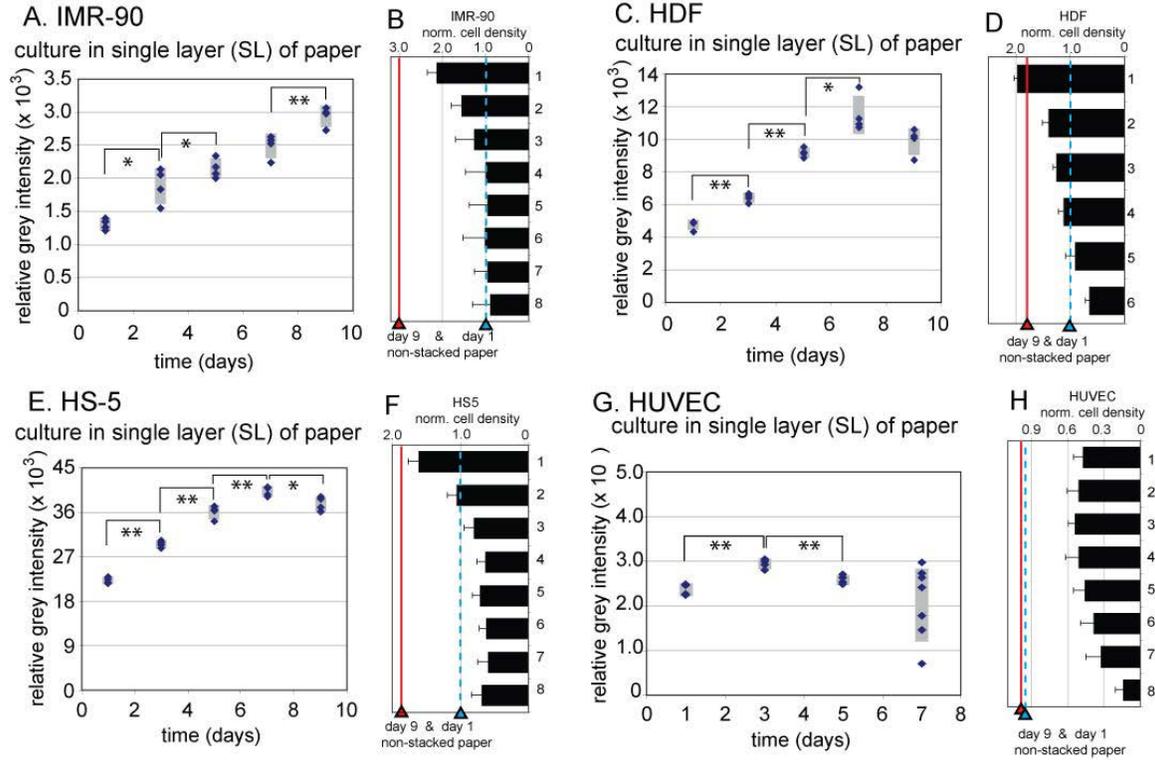


Supplementary Figure S1.

(A) To relate the fluorescent intensity of F-actin to the number of MDA-MB-231 cells inside the paper, we spotted 4 μL of a suspension of MDA-MB-231 cells in Matrigel ($10^6 - 3 \times 10^7$ cells/mL) onto filter paper, incubated the samples for two hours in growth medium, fixed them, and stained them with Texas Red- or Alexa Fluor 633-conjugated phalloidin. Similarly to the previous results (Fig. 1I), we observed a non-linear relation between F-actin fluorescence and the number of cells in paper. The nature of the dependence ($y=ax^{1/2}$) was similar for the two dyes we tested. Non-linearity is not the result of the detector saturation, because we observed similar trends with different photomultiplier (PMT) voltages applied to the detector. Both data sets on log-log plot fit a line with slope equal to 0.5. (B) We also quantified the number of MDA-MB-231 cells in paper using the rate of turnover of metabolic reagent (Alamar BlueTM). We incubated papers presenting different number of cells with growth medium containing 10% of Alamar Blue. Growth medium was sampled at different times (one, two, three and six hours); fluorescence of the media at these times was determined, and the rate of change of fluorescent intensity (units per hour) was plotted against the number of cells. The observed linear correlation between Alamar Blue turnover, and the number of cells, was used as a calibration curve in Fig. 3 and 5. (C) We cultured MDA-MB-231 cells in paper-Matrigel matrix for one, three, five, seven or nine days and at those days, we removed one sample from the medium and fixed it with 4% PFA solution. At the end of the experiment, samples from different days were stained with fluorescently labeled phalloidin and quantified using a fluorescent gel scanner and ImageJ. (D) We cultured MDA-MB-231 cells in paper-Matrigel matrix for nine days; on days 1, 5 or 9, the media

was supplemented with Alamar Blue. After measuring the reagent turnover, the media was changed back to normal growth media. Both F-actin stain intensity (*C*) and Alamar Blue turnover rate (*D*) increased over time, but the results from F-actin stain did not match that of Alamar Blue due to the non-linear relation between F-actin fluorescence and the number of cells. The discrepancy can be resolved when F-actin fluorescence is converted to the number of cells using $y=x^{1/2}$ calibration. The resulting $(2.5)^2 \sim 6$ fold increase of F-actin stain over nine days resembles the result obtained by Alamar Blue (five fold / nine days). Growth rate obtained from F-actin stain is higher, because this staining method detects both metabolically active and inactive cells. (*F*) Long-term proliferation of MDA-MB-231 cells monitored by Alamar Blue. (*F-I*) Confocal microscopy images of MDA-MB-231 cells on day 1 (*F-G*) and day 9 (*H-I*) confirm the results from Alamar Blue, and F-actin, and show that the number of cells in paper indeed increase by 5-6 fold over nine days. We fixed the cells with PFA, stained with AF-633 phalloidin and Sytox to visualize F-actin (red) and nuclei (green), and imaged them using a laser scanning confocal microscope (Leica) with 20x objective. Cellulose fibers were detected via reflection (purple). 3D reconstruction was performed using Volocity. The inside of the cell appear yellow due to overlap of red (F-actin) and green (nucleus) channels in the 3D reconstructed images.

Supplementary Figure S2.

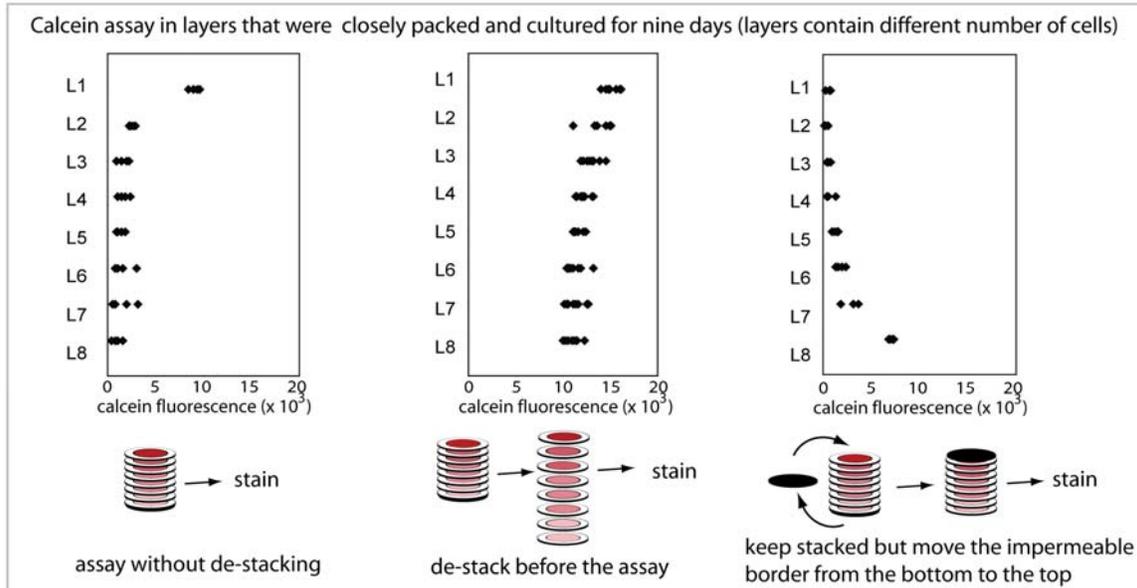
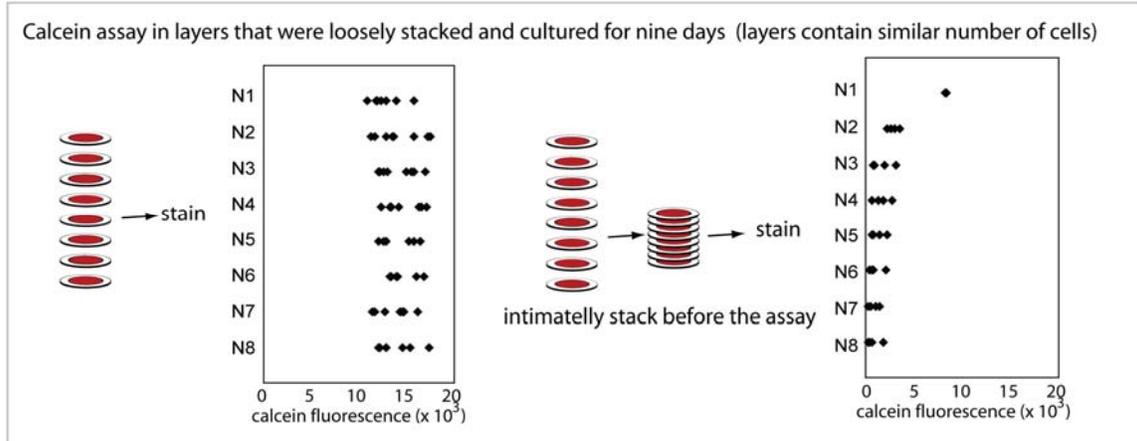
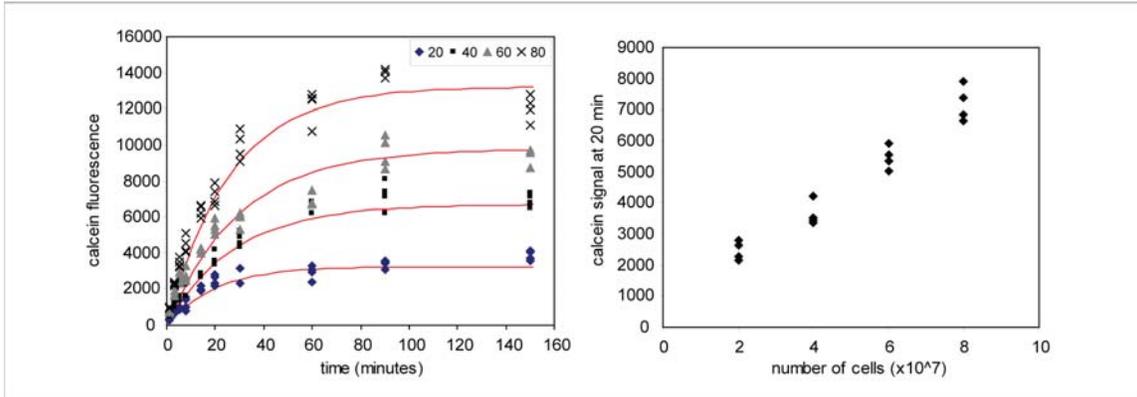


Supplementary Figure S2.

Investigation of the rate of proliferation of various cell types in a paper-supported Matrigel matrix. (A-H) We spotted a suspension of cells in Matrigel (4 μ L, 10^7 cells/mL) onto filter paper, and allowed the cells to equilibrate for 24 hours in growth media. The stacks of eight or six layers were cultured nine days. SL controls were cultured for one, three, five, seven, or nine days and on those days, we removed one sample from the growth medium and fixed it with 4% PFA. At the end of the experiment, L1-L8 samples and SL samples from different days were stained with fluorescently labeled phalloidin, and quantified using a fluorescent gel scanner and ImageJ. The blue line represents actin stain intensity in SL samples on day one; the red line represents stain intensity in SL on day nine. In B, D, F, H, an average from four to six measurements are presented and the error bar is one standard deviation. In A, C, E, G, all data are presented, and the overlaying grey bar is equal to 2x (standard deviation). We calculated p values (* $p < 0.05$, ** $p < 0.01$) using the two-tailed, two-sample unequal variance t-test. (I, L) Confocal images and 3D-reconstruction of the confocal images of the endothelial lumens formed on day seven by HUVEC cells in the top (L1) layer, and the small lumens in the bottom (L8) layer of the eight-layered stack of HUVECs. (I-K) Cells were stained with AF488-phalloidin or anti-VE-cadherin antibody (L) and imaged using laser scanning confocal microscope. Color schemes are the following: purple: cellulose fibers (detected via reflection), blue – nuclei, green – F-actin (I-K) or VE-cadherin (J). (L-M) The number of nuclei per tube was counted in 11 images for “Layer 1” and six images for “Layer 8”. Counting was performed manually by three independent participants. “Layer 1” has a

significantly higher fraction of large tubes, with more cells per tube (3-30 nuclei per tube); whereas much smaller tubes (1-5 nuclei per tube) are observed in “Layer 8”.

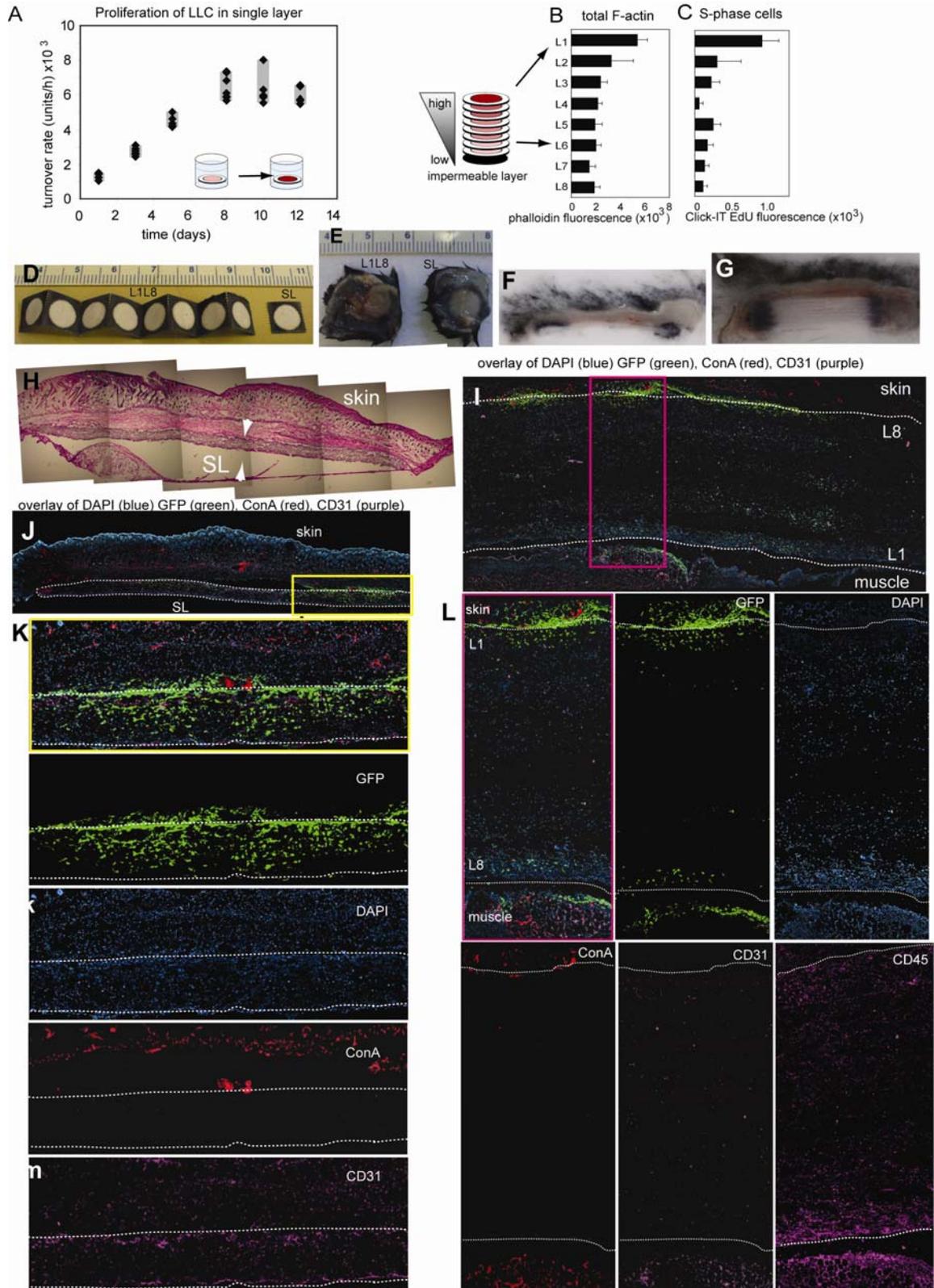
Supplementary Figure S3.



Supplementary Figure S3.

Quantification of metabolically active cells using calcein dye. The outcome of the calcein assay depended on the time and the concentration of cells, and could be described using first order kinetics. The red line demonstrates the fit to the curve $C=C_0(1-e^{-kt})$. At a defined time point (e.g. 20 minutes), the signal was proportional to the number of cells. In 3D cultures, however, the outcome of calcein analysis was also determined by the balance of diffusion rate and reaction rate. To address this issue, we investigated several stacked and non-stacked geometries and determined that staining of thick 3D cultures led to development of calcein signal only in layers exposed to medium (even though live cells reside in every single layer). This discrepancy was resolved when the layers were separated prior to the assay, to allow for unrestricted diffusion of calcein to cells in each layer.

Supplementary Figure S4

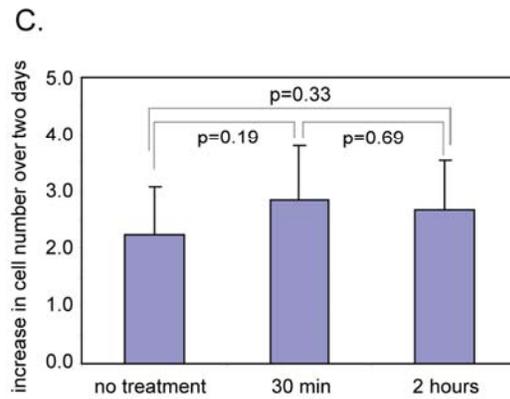
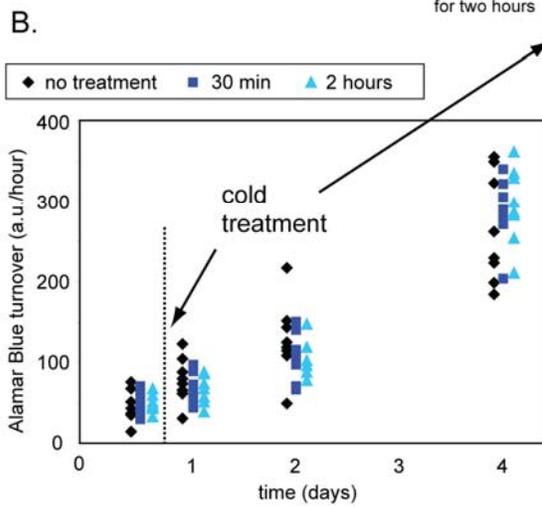
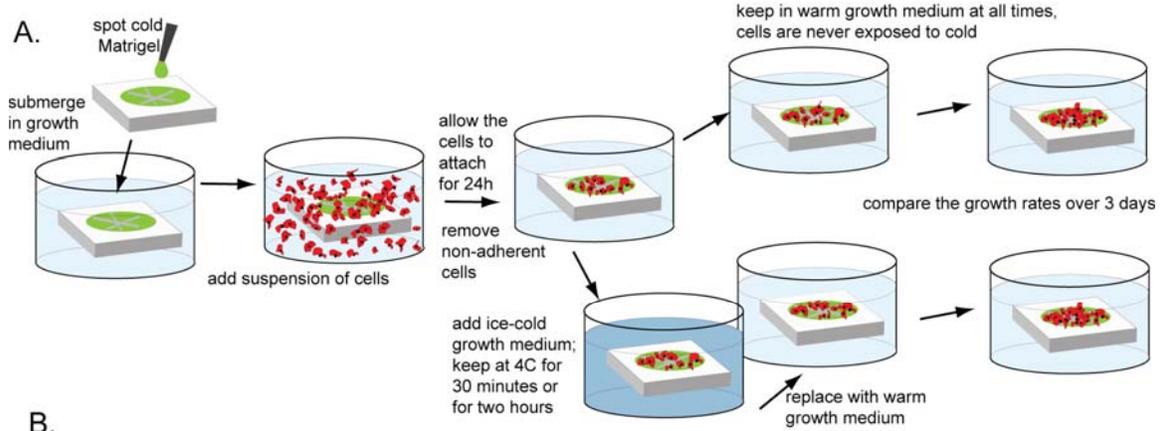


Supplementary Figure S4

(A) LLC cells proliferate normally when cultured inside paper-Matrigel substrates *in vitro* as demonstrated by Alamar Blue. In this experiment, we generated suspension of LLC cells in 1:1 vol/vol mixture of Matrigel and growth medium (10^7 cells/mL) and spotted 4 μ L of this suspension onto the 200 μ L-thick chromatography paper. At designated time, we supplemented the medium with 10% of Alamar Blue, measured rate of change of fluorescence, and substituted back to the complete growth medium. (B-C) After nine days of culture in eight layers of paper, distribution of cells (as judged by F-actin stain, B) and distribution of S-phase cell (as determined by Click-iT EdU stain, C) resembles that of MDA-MB-231 cells (Fig. 2B, D) and other cell lines (Fig. S2). (D) Patterned chromatography paper before cell seeding and implantation. Black areas are patterned with printer ink(3) and form hydrophobic, impermeable border; white areas are unmodified paper. (E) Samples after three days implantation and excision. (F-G) Light microscopy (2x) image of the OCT-embedded samples sectioned to the middle of the sample. (H) 18 micron-thick sections of implanted SL sample stained with Hematoxylin and Eosin (HE). (I-L) We stained the sections from SL samples (J, K) and L1L8 samples (I, L) with anti-CD31 to detect endothelial cells or anti-CD45 antibody to detect differentiated hematopoietic cells, and appropriate secondary antibody conjugated to AlexaFluor633. Functional vasculature was labeled by intravenously injected Rhodamine-ConA. Confocal imaging was performed using Carl Zeiss ApoTome equipped with 10x objective, automated stage and mosaic imaging capability. An average of 20 (SL) to 80 (L1L8) images were collected and stitched to obtain the overview

images. We used the following color scheme: green – GFP, red – Rhodamine ConA, blue – DAPI, purple – CD31 or CD45 (as noted).

Supplementary Figure S5



Supplementary Figure S5

(A) To assess the effect of brief cold treatment on long-term proliferation of cells, we plated the LLC cells atop paper permeated with Matrigel. We allowed the cells to adhere for 24 hours and exposed one set of samples to ice-cold media (4 °C) for 30 min, another set of samples to ice cold-media for two hours, and the third, control set, we never exposed to any cold media (N=8 in each set). We monitored the number of cells in all samples over the next three days using Alamar Blue. (B) A plot of reagent turnover, which is proportional to the number of metabolically active cells, before, and after the treatment. All data from all samples is presented. (C) Increase in number of cells from day two to day four in cold-treated samples is similar to that of control, untreated samples. We calculated ratio of reagent turnover on day four to that on day two for each sample and averaged the resulting values (N=8); error bar is one standard deviation. Growth rates are statistically insignificant ($p > 0.05$) as indicated by two-tailed unequal variance t-test. We conclude that 24 hour incubation in 36 °C-warm media is sufficient to alleviate the effect of brief exposure (<2 h) of cells to cold medium.

References:

1. Yung CW, et al. (2007) Transglutaminase crosslinked gelatin as a tissue engineering scaffold. *J Biomed Mater Res Part A* 83A:1039-1046.
2. Mammoto A, Huang S, Moore K, Oh P, Ingber DE (2004) Role of RhoA, mDia, and ROCK in cell shape-dependent control of the Skp2-p27(kip1) pathway and the G(1)/S transition. *J Biol Chem* 279:26323-26330.
3. Carrilho E, Martinez AW, Whitesides GM (2009) Understanding Wax Printing: A Simple Micropatterning Process for Paper-Based Microfluidics. *Anal Chem*:DOI: 10.1021/ac901071p.