Biophysical Journal, Volume 99

Supporting Material

Mechanical Force affects expression of an *In vitro* metastasis-like phenotype in HCT-8 cells

Xin Tang, Theresa B. Kuhlenschmidt, Jiaxi Zhou, Philip Bell, Fei Wang, Mark S. Kuhlenschmidt and Taher A. Saif

Supporting Materials:

1. Supporting Materials and Methods:

Cell culture

Normal bovine endothelial cells (ATCC, Manassas, VA) were cultured in a medium with 90% Dulbecco's Modified Eagle's Medium (DMEM; ATCC) and 10% fetal bovine serum (FBS; ATCC). Cells were treated with blebbistatin (diluted from 100 mM stock solution in dimethylsulfoxide; Calbiochem) after plating on substrates and incubating for 12 hrs.

Gel preparation and AFM calibration

Polyacrylamide (PA) gels were prepared following the protocols described elsewhere (9). The PA gels are made with varied relative concentrations of acrylamide (Bio-Rad) and N, N'-methylene bis-acrylamide (Bio-Rad) to obtain different cross-link extents accompanied with different Elastic moduli (Table S1). All the substrates were covalently coated with either 25μg/mL fibronectin or 4 μg/mL laminin (BD). Atomic force microscopy (Asylum) with silicon-nitride cantilever having a spring constant k = 148.14 pN×nm^{-1} (Veeco) was used to characterize the stiffness of the PA gels as well as HCT-8 cell monolayer. A conical-tip approximation (Eqn. S1) was used to extract the substrates Elastic modulus (10).

Atomic force microscopy (Asylum) with silicon-nitride cantilever having a spring constant k = 148.14 pN×nm^{-1} (Veeco) was used to characterize the stiffness of the PA gels as well as HCT-8 cell monolayer. A conical tip approximation (Eqn. S1) for the AFM tip was used to extract the substrates Elastic modulus using:

$$z - z_0 = (d - d_0) + \sqrt{\frac{k(d - d_0)}{\frac{2}{\pi} E(1 - \nu^2)\tan(\alpha)}} \qquad \text{(Eqn. S1)}$$

where z and d are cantilever's base PZT displacement and the cantilever tip deflection, respectively. z_0 is the piezo-controller's vertical position as the AFM tip touches the substrate surface, and d_0 is the initial cantilever deflection prior to bending. ν is the Possion's ratio of hydrated substrates (ν = 0.5 in present study). $\alpha = 35^o$ is the half open-angle of cantilever tip. Fig. S1 shows the curves of force versus substrates indentation and the determined modulus distribution histogram.

Immunofluorescent staining

The live-dead assay to determine cell viability was performed using kit No. L7012 (Molecular Probes, Invitrogen). A working solution was prepared with 1 part of kit component dye A (Syto 9) and 1 part kit component dye B (propidium iodide, "DEAD") in 100 parts PBS and applied to cells on the various PA gels. Cultures were fixed with 4% paraformaldehyde at 37^{o}C for 30 minutes and permeabilized in 0.1% Triton ×100 for 15 minutes. Mouse primary antibody anti-E-Cadherin (Invitrogen, SKU No.: 18-0223) and Alexa Fluor® 488 goat anti-mouse IgG (H+L, 495/519, green) (Invitrogen, SKU No.: A-11029) were used to stain E-cadherin molecules. DAPI was used for staining cell nuclei. Rhodamine phalloidin (520/650, red) was used as fluorescent conjugate to bind specifically to F-actin filaments, but not G-actin.

Imaging and analysis

To minimize any change in the environment during continuous imaging, a temperature controller equipped environment chamber (PrecisionControl LLC) was used to maintain controlled condition (appropriate humidity, 5% CO2, and 37^{o}C) throughout the time-lapse video recording. An inverted optical microscopy (Olympus IX81, Olympus America) and a high-speed SPOT camera were used to record cell behavior. The actin structures of HCT-8 cells were imaged with a Leica SP2 confocal microscopy (Leica SP2, Heidelberg, Germany) with Amira

(Advanced3DVisualization and Volume Modeling) software. The E-cadherin of HCT-8 cells was imaged with Z step size of 200 nm using Yocogawa Spinning-disc confocal microscopy with appropriate fluorescent filters and Andor IQ software (Andor technology). The E-cadherin density per unit membrane area was calculated for colony-associated cells and dissociated cells from two sectional confocal images at 2.5 μm and 3.2 μm heights above the substrate as outlined in Fig. S8. Histograms of the density values are plotted in Fig. 6 and compared when the imaging conditions are similar.

Coulter counter assay

The cells were harvested and individualized by trypsin/EDTA treatment and were restored in complete culture medium containing serum to neutralize residual trypsin (Fig. S9). Since fibronectin was used for cell adhesion on substrates and there is no tissue present, collagenase cannot remove cells from culture plates into single cell state and thus was not used. The cell suspensions were placed in 17×100 mm capped polypropylene tubes (Falcon No.: 352059) and were rotated end over end at 7-8 revolutions per minute in a Labquake shaker (Barnstead/Thermolyne Model No.: 41510) for 1 hour at 37 ^{o}C to allow recovery of any surface cell adhesion molecules (CAMs) or other proteins. The recovery of CAMs following trypsinization was guaranteed by identifying the increase in cell aggregate number as incubation duration prolongs. The pre-incubation time is ~~chosen as~~ 1 hour ~~other than longer because~~ over-aggregation ~~should be avoided~~ ~~in adhesion-rate assay in order~~ to effectively differentiate the precise adhesion rate kinetic. Portions of the pre-incubated cells (0.3ml, approximately 5×10^5 cells) were placed in flat bottom 3 dram shell vials (Fisher catalog No.: 0333926D) and rotated in a gyratory water bath shaker (G-76, New Brunswick) at 12 rpm at 37 ^{o}C for 5, 10, 20, 40, 60, 80, 100 and 120 minutes. At the end of each time period, cells were diluted with 8 mL 0.9% saline and placed on ice to stop further cell aggregation. The number of single cells present at each time point was measured in the Coulter counter as described in (1).

Lentiviral infection and Western Blotting

Human E-Cadherin cDNA was cloned into the 2K7/Neo lentivector (Zhou et al., 2009), which was used to package virus with Viralpower Lentivirus Packaging System (Invitrogen) as described in (2). For infection, lentiviruses (MOI, 10–200) were directly added into culture medium with polybrene (6 μg/mL, Sigma) and incubated with cells for ~20 h. Cells were analyzed 48 h after infection. Cells (5×10^6) were lysed directly with laemmli sample buffer (Bio-Rad). 25 μl of each sample were separated by SDS-PAGE and analyzed by western blot. E-cadherin antibody (Cell Signaling) was used at the dilution of 1:1,000. The Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

2. Supporting Results:

Non-specific cell adhesion strength is diminished after transition to MLP

In addition to measuring the rate of specific homotypic cell-cell adhesion, we characterized the non-specific adhesion strength of HCT-8 cells using a novel Bio-MEMS force sensor (Fig. 5c). The sensor was microfabricated from a single-crystal of silicon(3, 4). The force was measured by a micromechanical spring with a spring constant 14.5 nN/μm and calibrated by Atomic Force Microscopy (AFM). During the experiment, the T-shaped sensor probe was allowed to contact the cell membrane for 2 minutes and was then moved away (Fig. 5c, inset). When the cell adhesion force between the probe and the cell surface hinders retraction of the sensor, the spring deforms by x, giving the force F= kx. The deformation was measured optically using a reference probe. Note the probe is not functionalized, and has a thin coating of native SiO_2 on the surface. Therefore, non-specific adhesive interactions were formed between the cell and the SiO_2-coated probe. The adhesion strength was evaluated from the force balance at the moment of cell-probe detachment. We found that HCT-8 cell colonies on 21 kPa PA gels (on 5^{th} culture day) showed a measurable non-specific adhesion strength of 94.9 ± 31.8 nN. However, disassociated cells (on 8^{th} culture day) showed no measurable adhesion, as if the surface is in a lubricated state (Fig. 5c).

Myosin II activity is involved in cell disassociation process

To furthermore test whether intracellular force serves as the mechanical cue for cell state transition, we used blebbistatin, a potent inhibitor of non-muscle myosin II ATPase (5, 6), to remove the intracellular force generated by myosin II in HCT-8 cells on 21 kPa gels. We adjusted the intracellular force levels by varying the dosages (0 μM, 2 μM, 5 μM and 10 μM) of blebbistatin. It is noted that blebbistatin can disrupt directed cell migration, but it does not block cell movement (7, 8). We found, without any blebbistatin or with the lowest dosage of blebbistatin (2 μM), the disassociated cells emerge from the edges of the cell colonies after 6~7 days of culture (Fig. S10). The daily percentages of cell clusters dissociating in dishes without blebbistatin were 5.7 ± 2.3 % (6^{th} day), 13.3 ± 4.9 % (7^{th} day), 17.3 ± 8.2 % (8^{th} day), 31.2 ± 7.2 % (9^{th} day). With 2 μM blebbistatin, the percentages were 4.1 ± 0.7 % (6^{th} day), 9.3 ± 7.3 % (7^{th} day), 6.0 ± 5.9 % (8^{th} day) and 4.6 ± 4.0 % (9^{th} day). However, for dishes with 5 μM and 10 μM of blebbistatin, no dissociation could be identified throughout the entire culture time (Fig. S10). Together, our results suggest the appropriate intracellular force generated by myosin II contributes to triggering the MLP cell state transition.

3. Supporting Figures:

Figure S1| The micromechanical properties of PA gels and HCT-8 cell monolayers measured by Atomic Force Microscopy. (**a**) The plots of AFM tip applied force versus the tip indentation (δ) into the substrates. (**b**) The histogram of microscopic Young's modulus (E) of substrates and HCT-8 cell monolayers. They are obtained by fitting the force versus indentation plots with the appropriate indentation model (see Materials and Methods). The conical-shaped tip model is used where n=20 for each substrate.

Figure S2| HCT-8 cells cultured on very soft PA gels (1 kPa) do not form cell islands or monolayers, but remain rounded and unspread. (**a**) Phase-contrast picture of HCT-8 cells cultured on 1 kPa gels after 4 days. (**b**) Phase-contrast picture of HCT-8 cells cultured on 1 kPa gels after 7 days. They do not show metastasis-like phenotype (MLP). Scale bar: 100 μm.

Figure S3| HCT-8 cells cultured on stiff polystyrene substrates (~ 3.6 GPa) form continuous cell layers after 2~3 culture days, but do not show the MLP cell dissociation process. (**a**) Phase-contrast image of HCT-8 cells cultured on polystyrene substrates after 4 days. (**b**) Phase-contrast image of HCT-8 cells cultured on polystyrene substrates after 10 days. Cells do not show the MLP. Scale bar: 100 μm.

Figure S4| The viability of the disassociated HCT-8 cells is examined by dye exclusion live-dead assays. The green fluorescence (indicated by white arrowheads) indicates the viable cells, while red fluorescence (indicated by black arrowheads) indicates the non-viable cells. Here all single disassociated cells are shown to retain full viability 2 days after disassociation. Scale bar: 100 μm.

Figure S5| The cell shape factors of both the dissociated and original HCT-8 cells. It effectively characterizes the cell circular shape. The dimensionless cell shape factor is calculated as $S=4*Pi*A/P^2$, where A is the spread area of cells and P is the cell perimeters. S varies from 1 for a circular shape to 0 for a highly ruffled shape. (**a**) The phase-contrast pictures for the dissociated HCT-8 cells on 21 kPa gels after 7 days culture (top left), the intact cell colonies on 21 kPa gels after 7 days culture (top right), the harvested and re-cultured dissociated HCT-8 cells on stiff polystyrene substrates after 24 culture days (bottom left) and original HCT-8 cells under same culture condition on polystyrene substrates after 24 days (bottom right). (**b**) The cell shapes of both dissociated and original HCT-8 on 21 kPa gels and stiff polystyrene substrates are characterized (n =35 for each column). Scale bar: 100 μm.

Figure S6| Normal cells cultured under the same condition do not show dissociation phenotype (MLP). (a) Normal MA104 cells (African Green Monkey Kidney) cultured on 21 kPa PA gels and stiff polystyrene substrates (E= 3.6 GPa) after 11 culture days. Scale bar: 100 μm. (b) Normal Bovine endothelial cells cultured on 21 kPa PA gels and stiff polystyrene substrates (E= 3.6 GPa) after 11 culture days. Scale bar: 200 μm. The results are consistent with study reported in (7).

Figure S7| Disassociated HCT-8 cells show less actin filaments than original HCT-8 cells. Both cells are cultured on stiff polystyrene substrates under the same condition. Filamentous actin amounts are quantified by fluorescent light intensity (see Supporting Materials and Methods).

Figure S8| The step-by-step process diagram of E-Cadherin analysis. Confocal microscopy was used to take Z-plane sectional images (Z step size: 200 nm) along the thickness of the cells to obtain the 3D E-cadherin membrane distribution. It consists of following key steps: cell

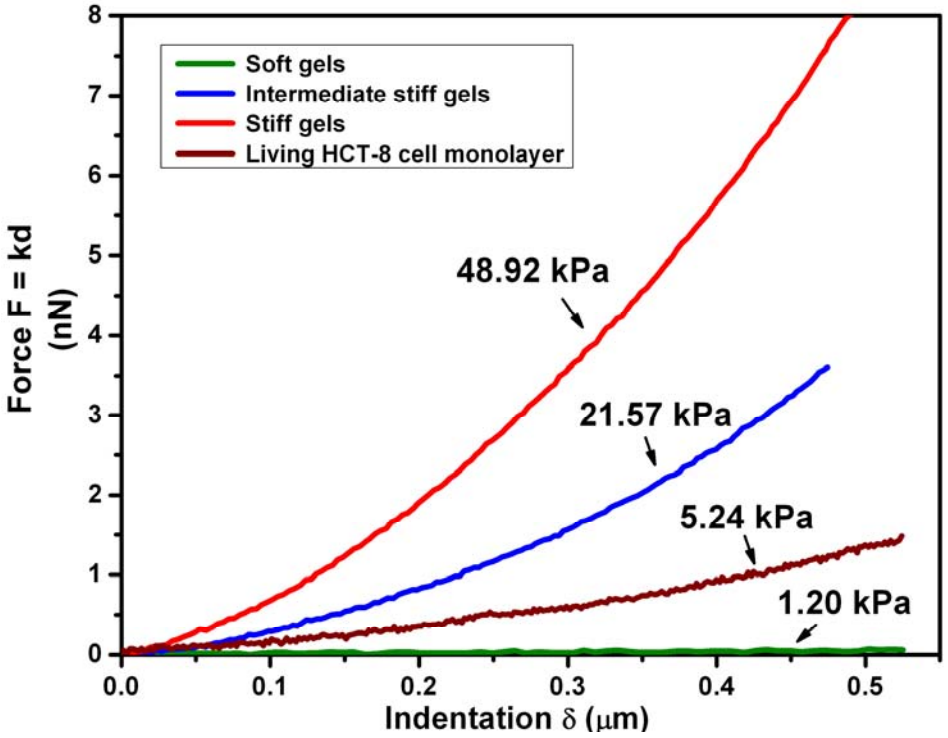
culture and staining, imaging by spinning-disc confocal microscope, the image processing and data analysis.

Figure S9| The step-by-step process diagram of Coulter counter adhesion assay. It consists of following key steps: cell culture and trypsinization, centrifuging to remove Trypsin/ EDTA, 1-hour end over end rotation incubation at 37 ^{o}C to prevent cell aggregation but allow recovery of cell surface adhesion molecules (CAMs), incubation by gyratory rotation at 37 ^{o}C for different time periods to measure the rate of cell adhesion using a Coulter counter to enumerate the number of single cells remaining at each time point.

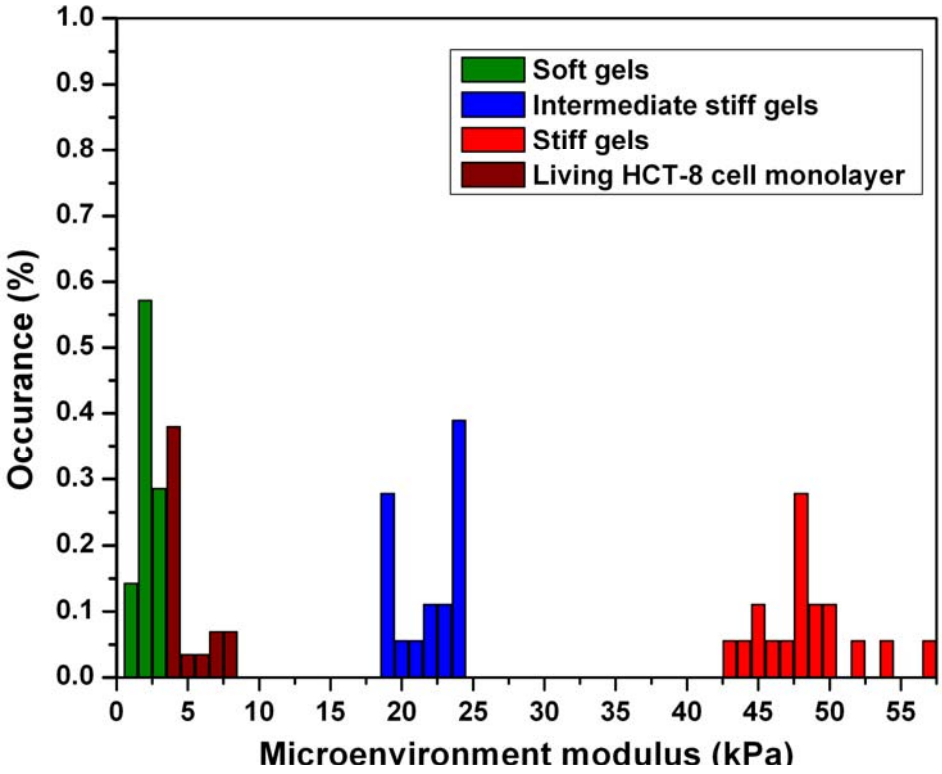
Figure S10| Cell dissociation process is inhibited by Blebbistaten which inactivates Myosin II. (**a**) Various dosages of blebbistatin, 0 μM, 2 μM, 5 μM, and 10 μM, are added to culture dishes on 21 kPa gels to inhibit the myosin II activities and reduce intracellular force. The cells without blebbistatin or with the least amount of blebbistatin (2 μM) show the cell dissociation process after 6-7 days. The cells with higher blebbistatin concentrations (5 μM and 10 μM) do not show cell dissociation. The red arrows point to the dissociated cells. Scale bar: 100 μm. (**b**) Percent of cell clusters dissociated following 6-9 days in culture on PA substrates in the presence and absence of blebbistatin. These data suggest appropriate intracellular forces contribute to triggering of the cell dissociation process.

Figure S11| MLP transition is stable. HCT-8 cells are first cultured on 21 kPa PA gel with surface functionalized by fibronectin. By 14 days of cultured, most of the cell colonies disassociate. These disassociated cells are harvested by trypsinizing the whole PA gel surface and re-cultured on fresh PA gel (E= 21 kPa) with surface functionalized by fibronectin, and hard polystyrene substrates (E = 3.6 GPa). (a1-a3) The re-cultured disassociated cells on 21 kPa PA gels on 2nd, 5th and 8th culture days. (b1-b3) The re-cultured disassociated cells on hard polystyrene substrate (E = 3.6 GPa) on 2nd, 5th and 8th culture days. In both cases, the cells persistently retain their disassociated phenotype (MLP) regardless of the degree of stiffness of the new substrates, in contrast to forming colonies on soft gel and monolayer on hard substrate (Fig. 1a, 1d). These results suggest that exposure of HCT-8 cells to the intermediate soft mechanical microenvironment has stably locked them into a MLP that is more characteristic of a dissociative rather than associative (monolayer) or anchorage-dependent cell growth. While culture on hard substrates (polystyrene) preserved the original HCT-8 cells' associative cell growth phenotype typical of in vitro epithelial monolayers for more than 50 passages, a single exposure to intermediate soft substrate triggered these cells to transition to a new, dissociative, MLP cell state in only 7-14 days. (c1-c3) and (d1-d3) show the original HCT-8 cells cultured under same condition on PA gel and polystyrene substrates. Note on 8th day, dissociation of colonies happened and cells show MLP.

Figure S1:

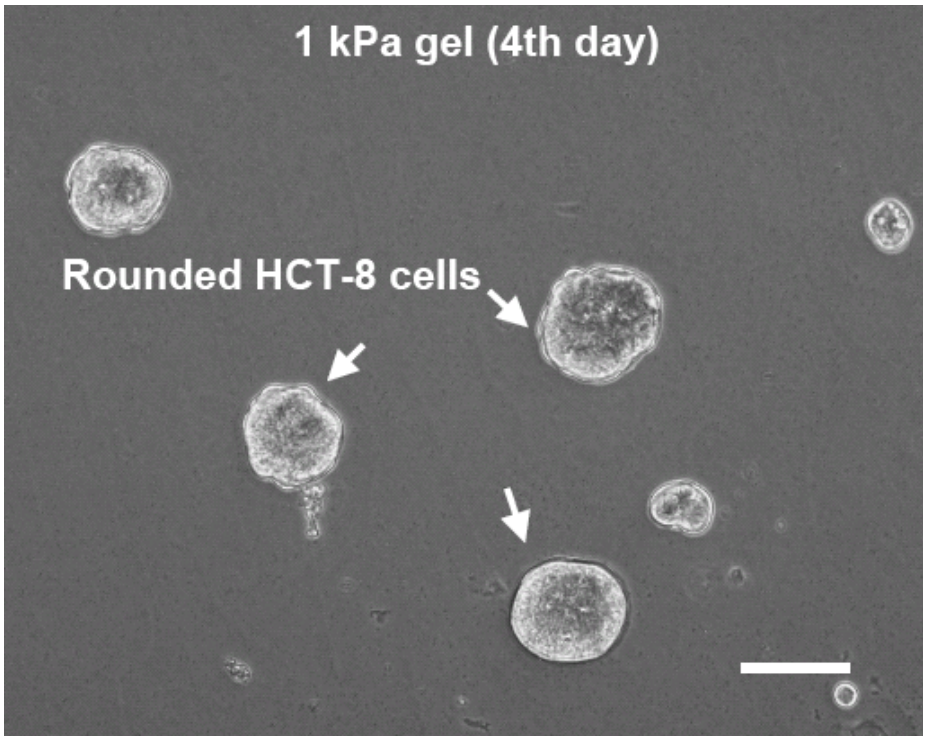

Soft gels
Intermediate stiff gels
Stiff gels
Living HCT-8 cell monolayer
48.92 kPa
21.57 kPa
5.24 kPa
1.20 kPa
Force F = kd (nN)
Indentation δ (μm)

(a)

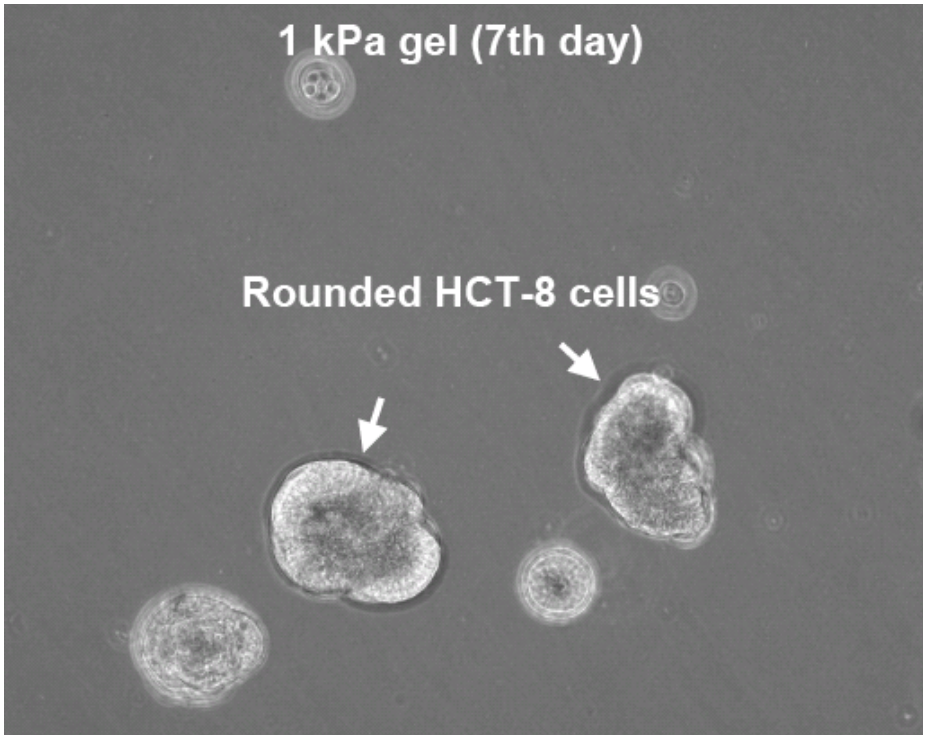

Soft gels
Intermediate stiff gels
Stiff gels
Living HCT-8 cell monolayer
Occurance (%)
Microenvironment modulus (kPa)

(b)

Figure S2:

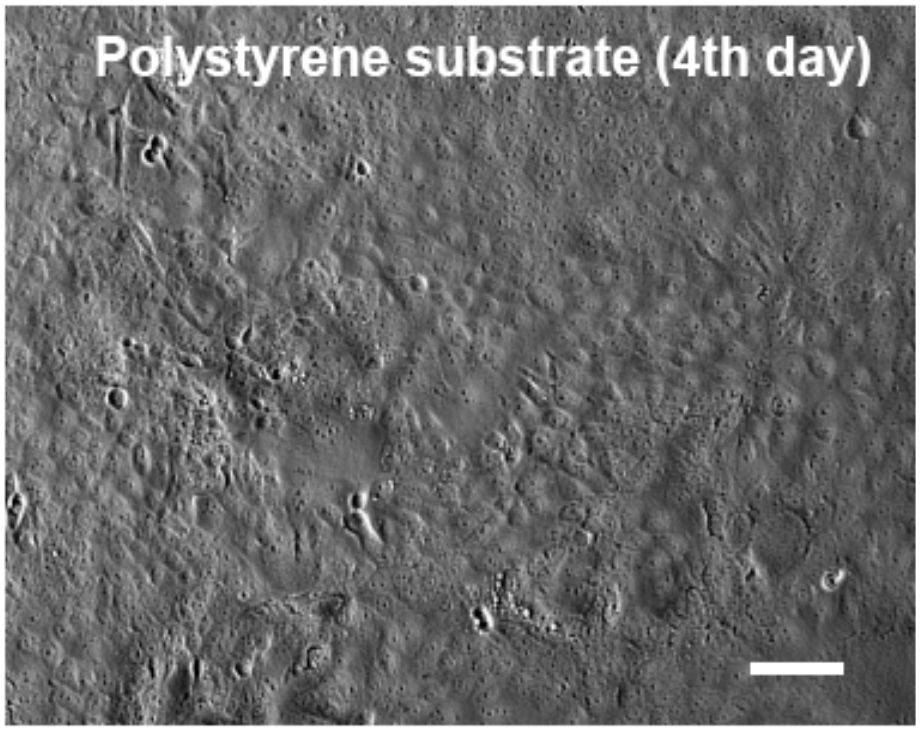

1 kPa gel (4th day)
Rounded HCT-8 cells

(a)

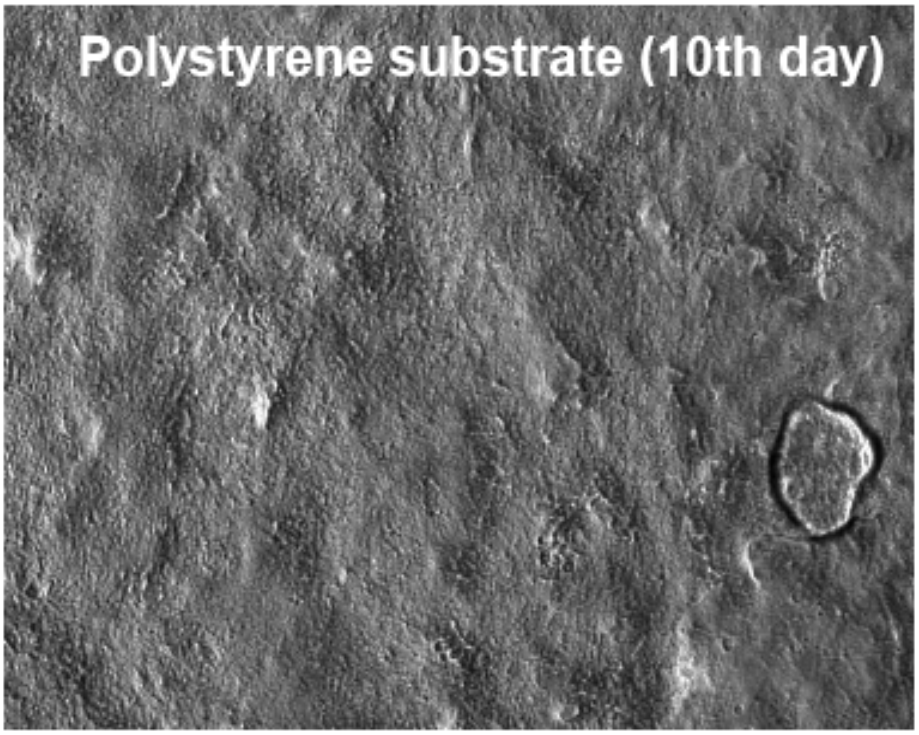

1 kPa gel (7th day)
Rounded HCT-8 cells

(b)

Figure S3:


Polystyrene substrate (4th day)

(a)


Polystyrene substrate (10th day)

(b)

Figure S4:

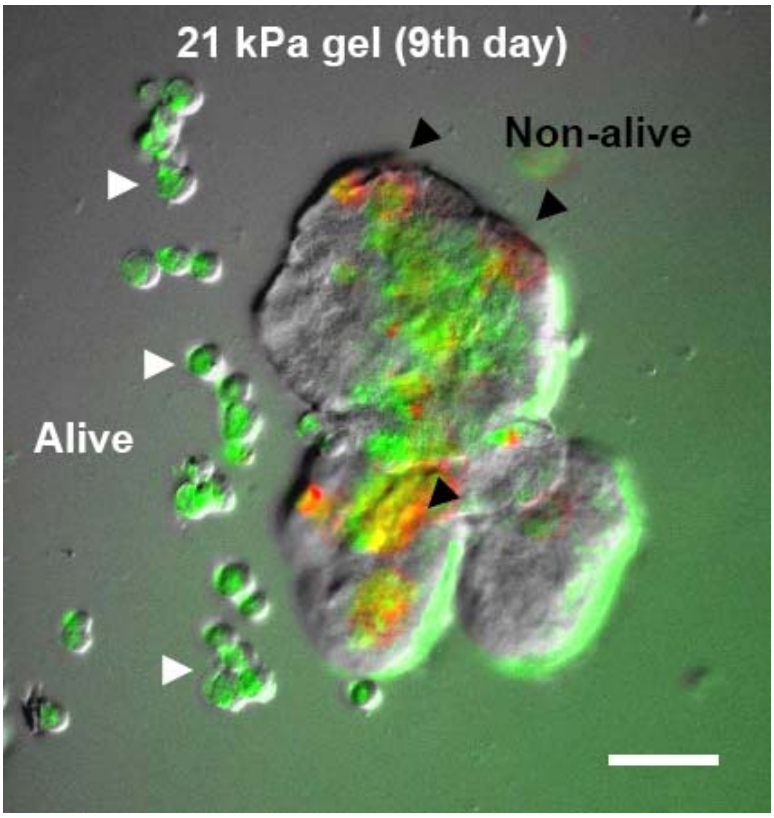
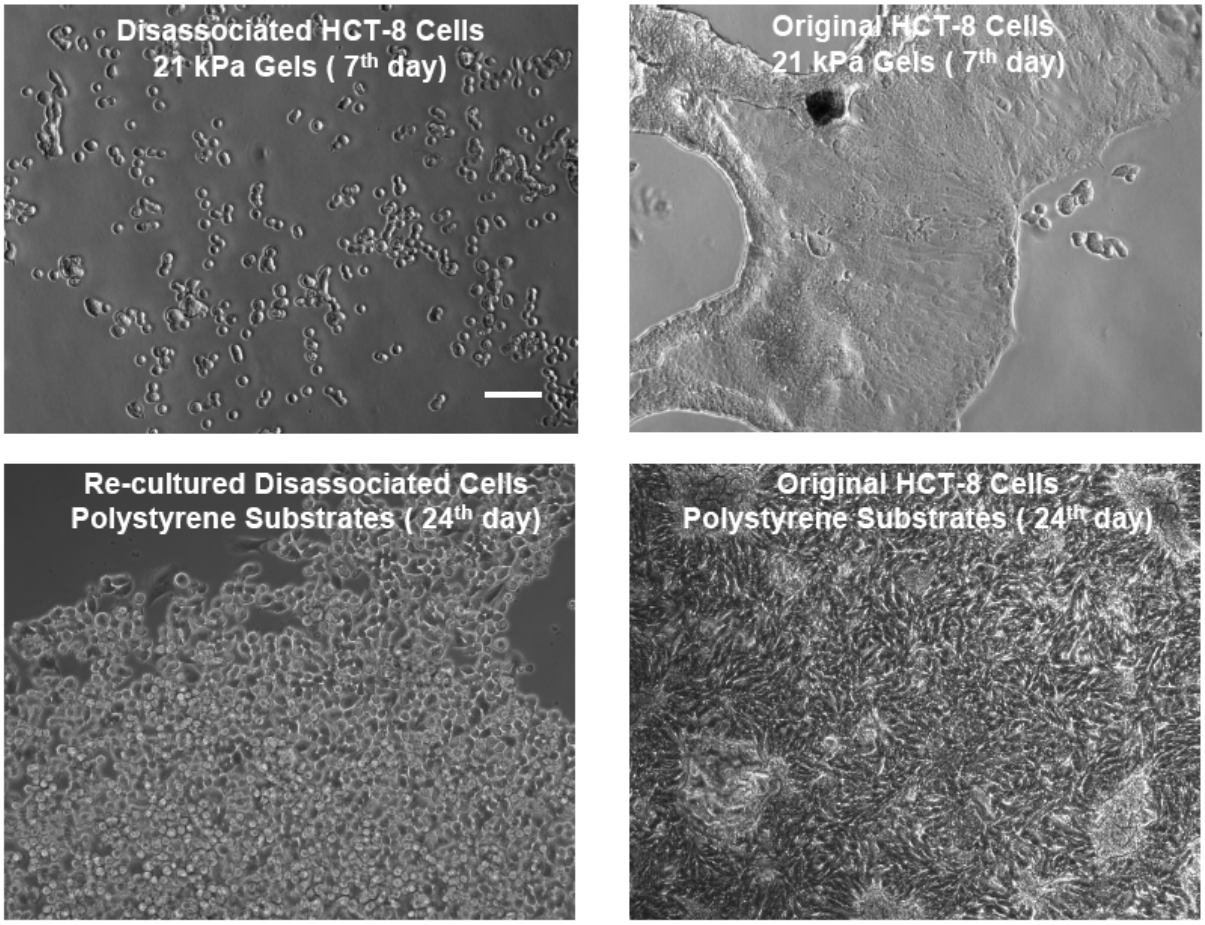
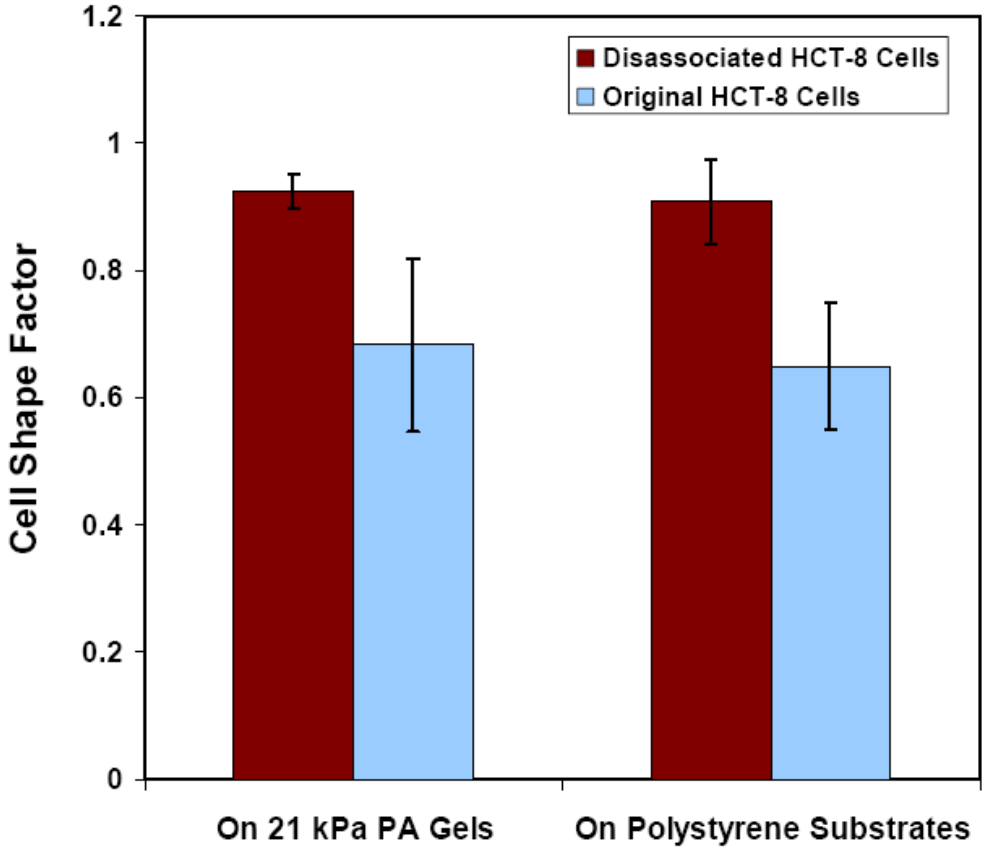


Figure S5:

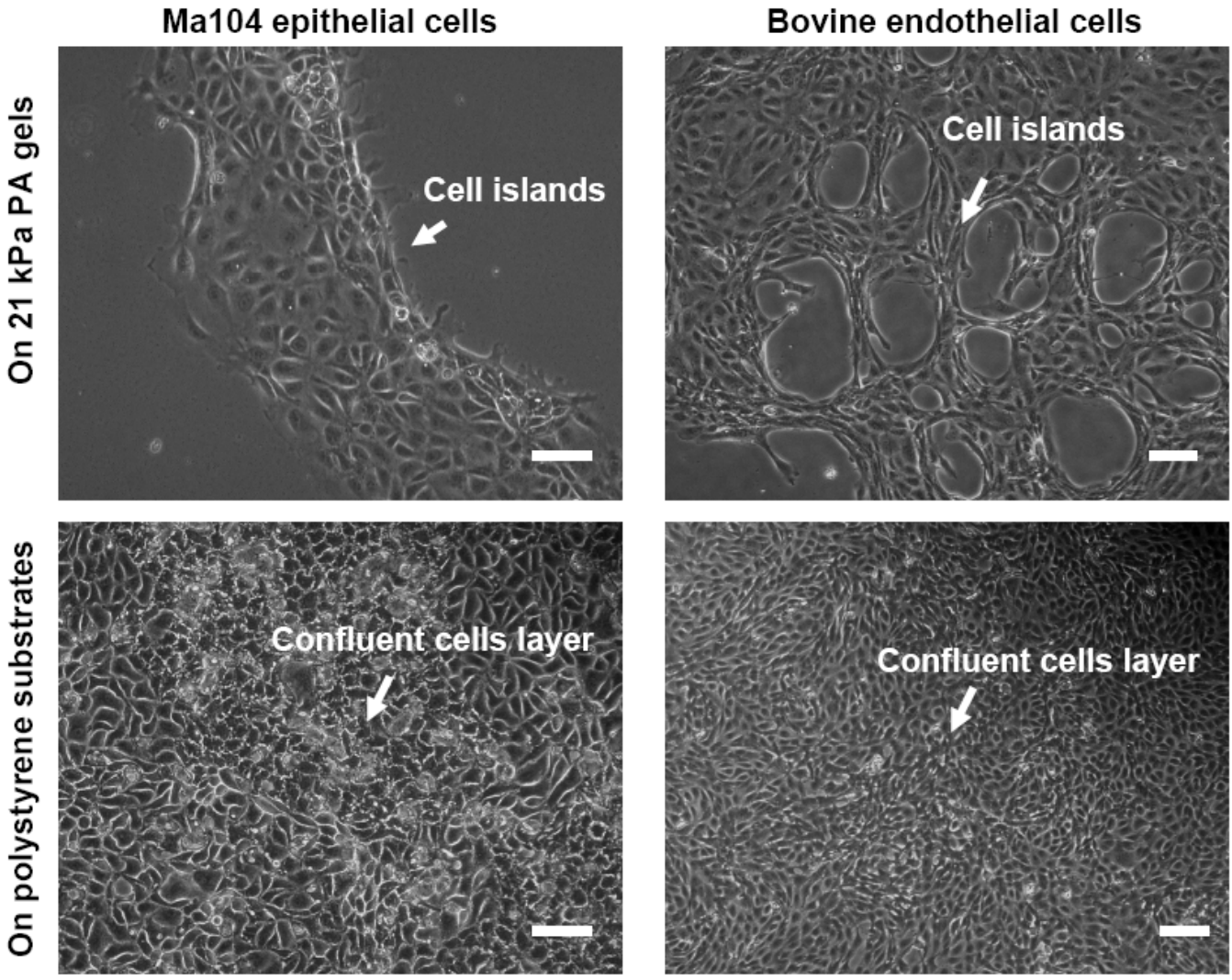

Disassociated HCT-8 Cells
21 kPa Gels (7th day)
Original HCT-8 Cells
21 kPa Gels (7th day)
Re-cultured Disassociated Cells
Polystyrene Substrates (24th day)
Original HCT-8 Cells
Polystyrene Substrates (24th day)

(a)


Disassociated HCT-8 Cells
Original HCT-8 Cells
Cell Shape Factor
1.2
1
0.8
0.6
0.4
0.2
0
On 21 kPa PA Gels
On Polystyrene Substrates

(b)

Figure S6:


Ma104 epithelial cells
Bovine endothelial cells
On 21 kPa PA gels
On polystyrene substrates
Cell islands
Cell islands
Confluent cells layer
Confluent cells layer

(a) **(b)**

Figure S7:

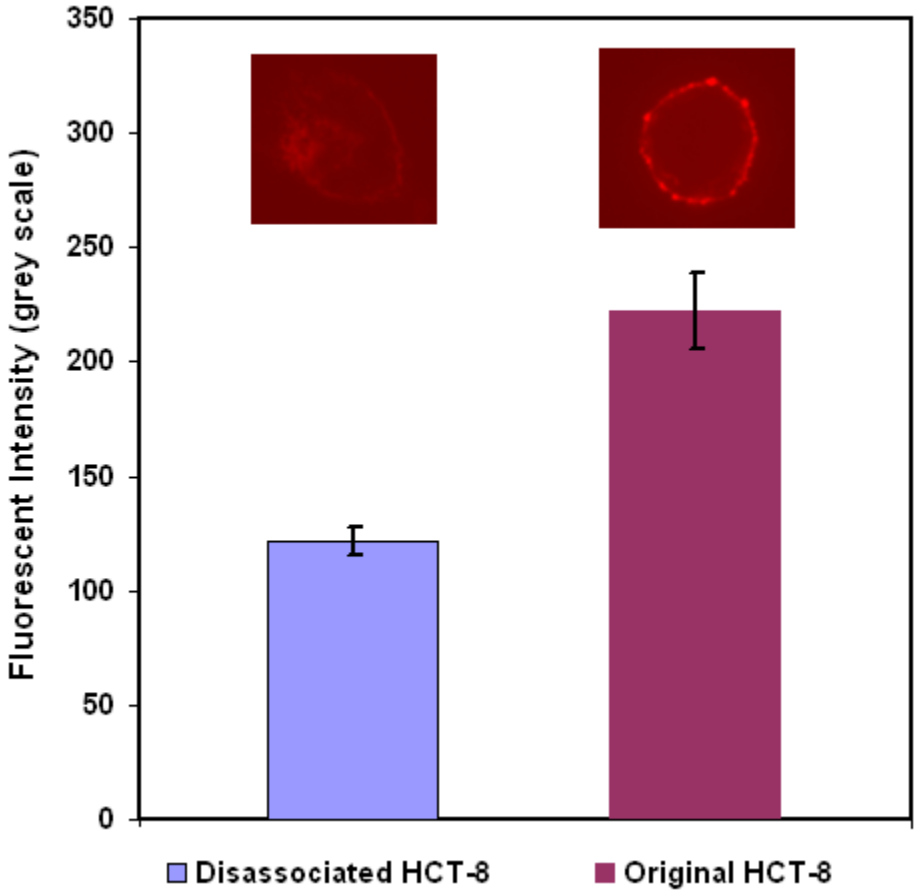

350
300
250
200
150
100
50
0
Fluorescent Intensity (grey scale)
Disassociated HCT-8
Original HCT-8

Figure S8:

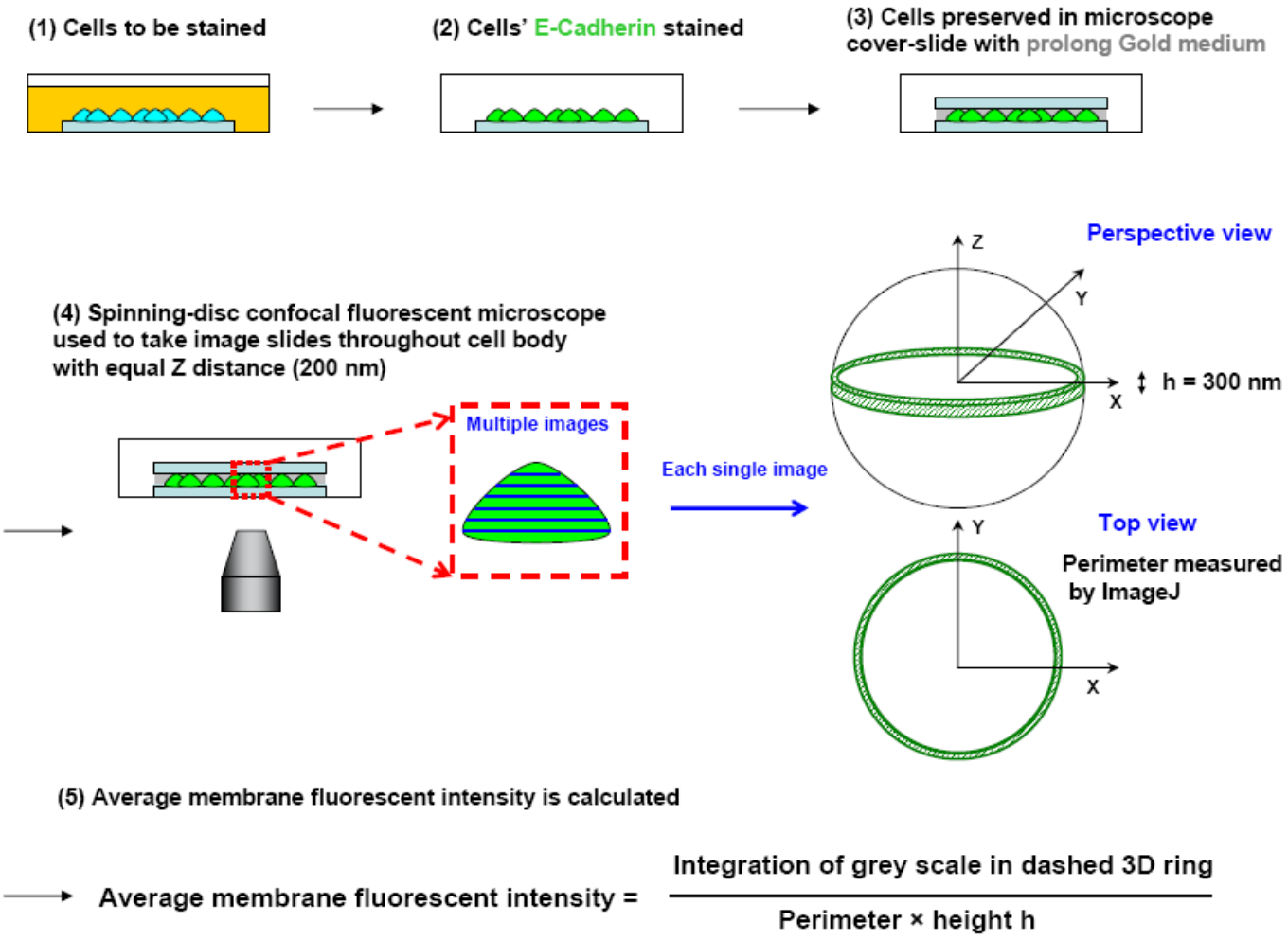

(1) Cells to be stained
(2) Cells' E-Cadherin stained
(3) Cells preserved in microscope cover-slide with prolong Gold medium
(4) Spinning-disc confocal fluorescent microscope used to take image slides throughout cell body with equal Z distance (200 nm)
Multiple images
Each single image
Perspective view
Z
Y
X
h = 300 nm
Top view
Y
X
Perimeter measured by ImageJ
(5) Average membrane fluorescent intensity is calculated
Average membrane fluorescent intensity = Integration of grey scale in dashed 3D ring / Perimeter × height h

Figure S9:

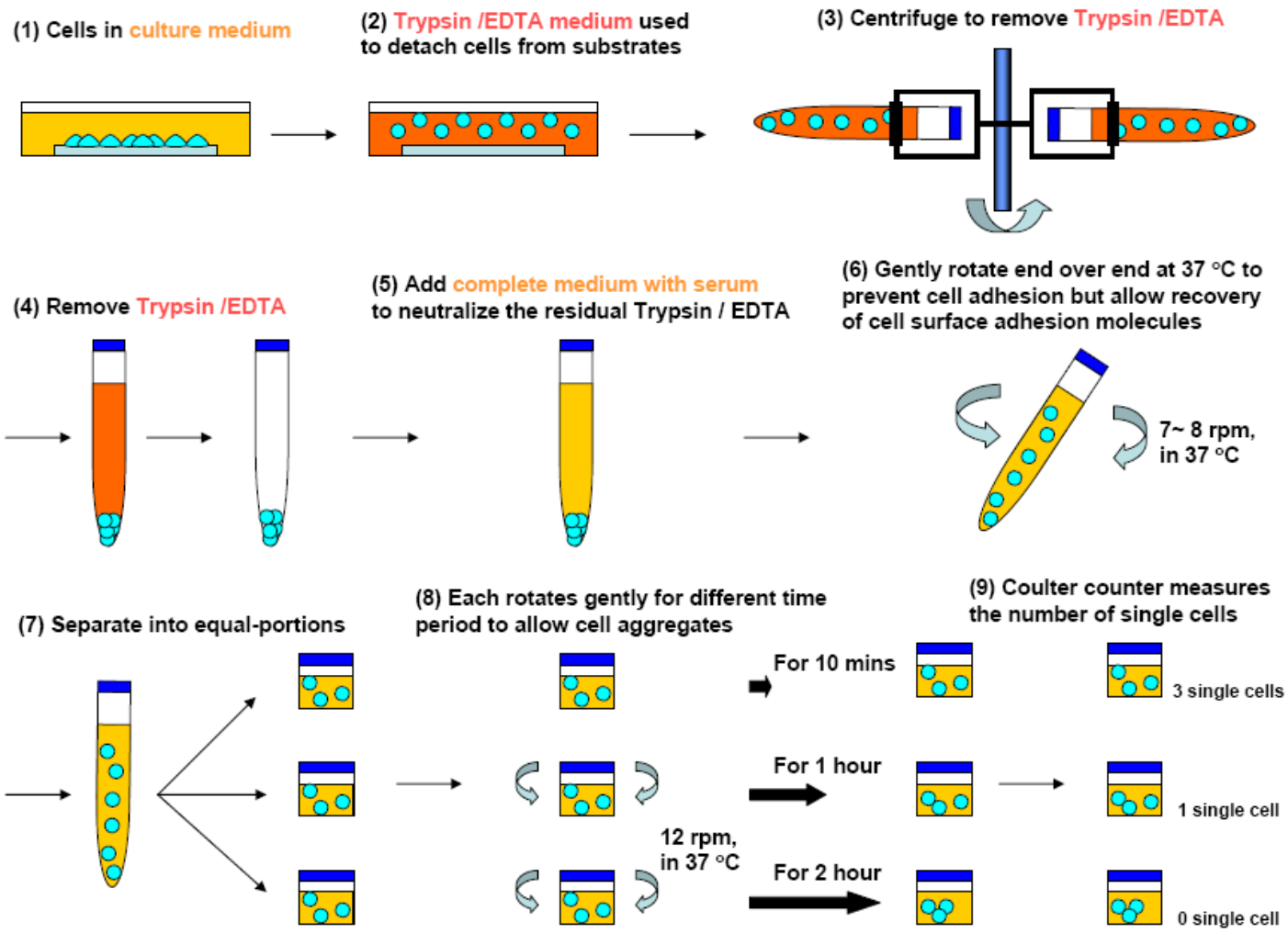
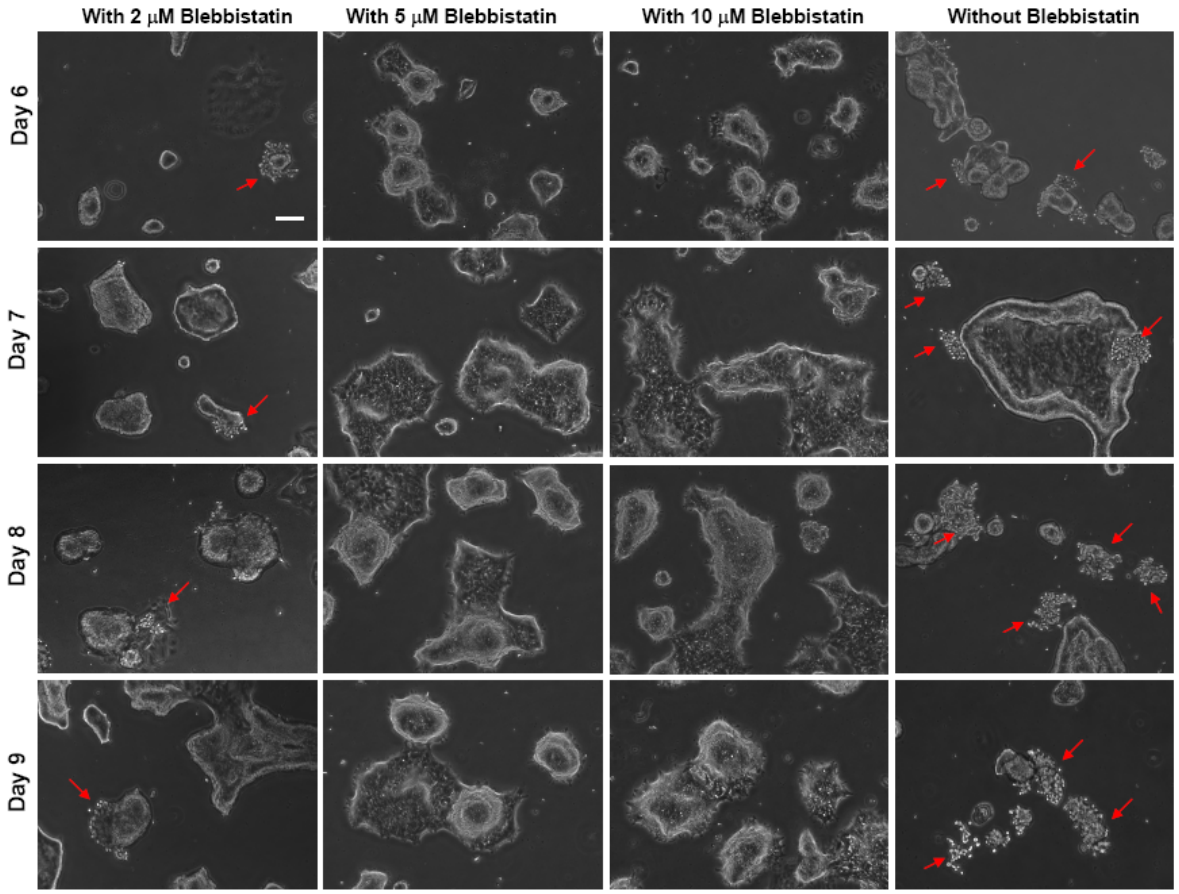
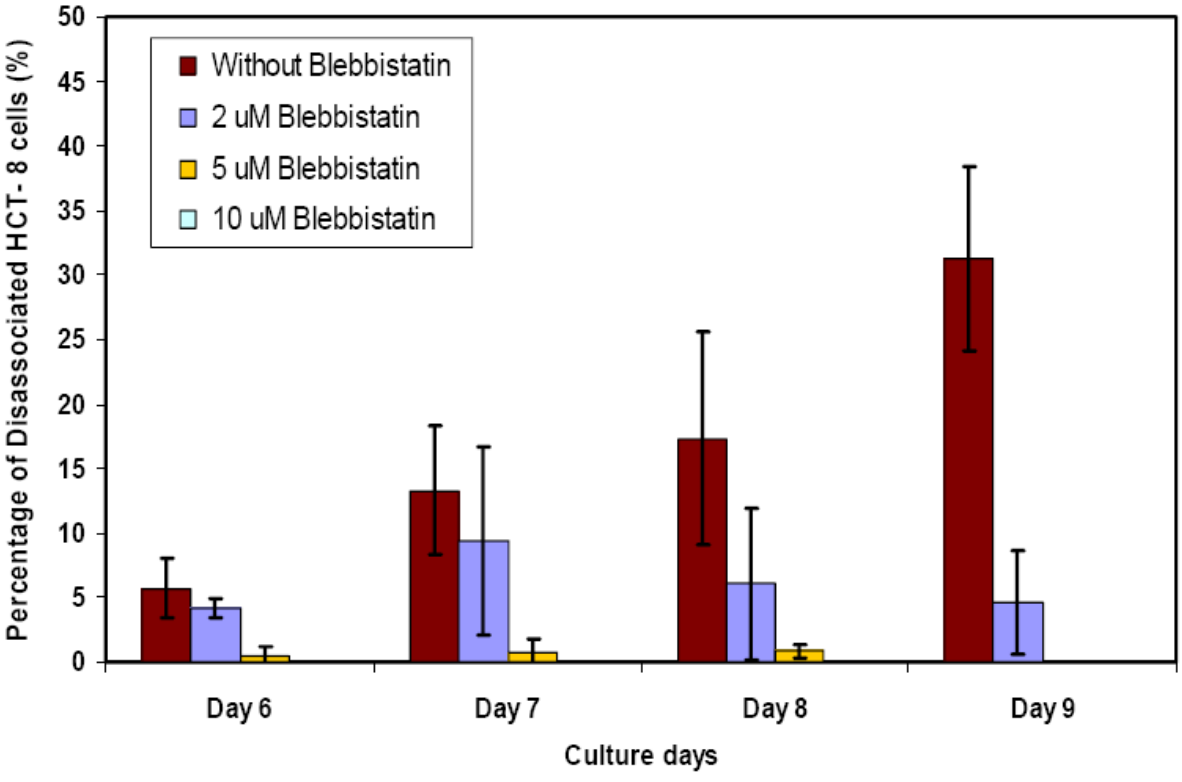

(1) Cells in culture medium
(2) Trypsin /EDTA medium used to detach cells from substrates
(3) Centrifuge to remove Trypsin /EDTA
(4) Remove Trypsin /EDTA
(5) Add complete medium with serum to neutralize the residual Trypsin / EDTA
(6) Gently rotate end over end at 37 °C to prevent cell adhesion but allow recovery of cell surface adhesion molecules
7~ 8 rpm, in 37 °C
(7) Separate into equal-portions
(8) Each rotates gently for different time period to allow cell aggregates
12 rpm, in 37 °C
For 10 mins
For 1 hour
For 2 hour
(9) Coulter counter measures the number of single cells
3 single cells
1 single cell
0 single cell

Figure S10:

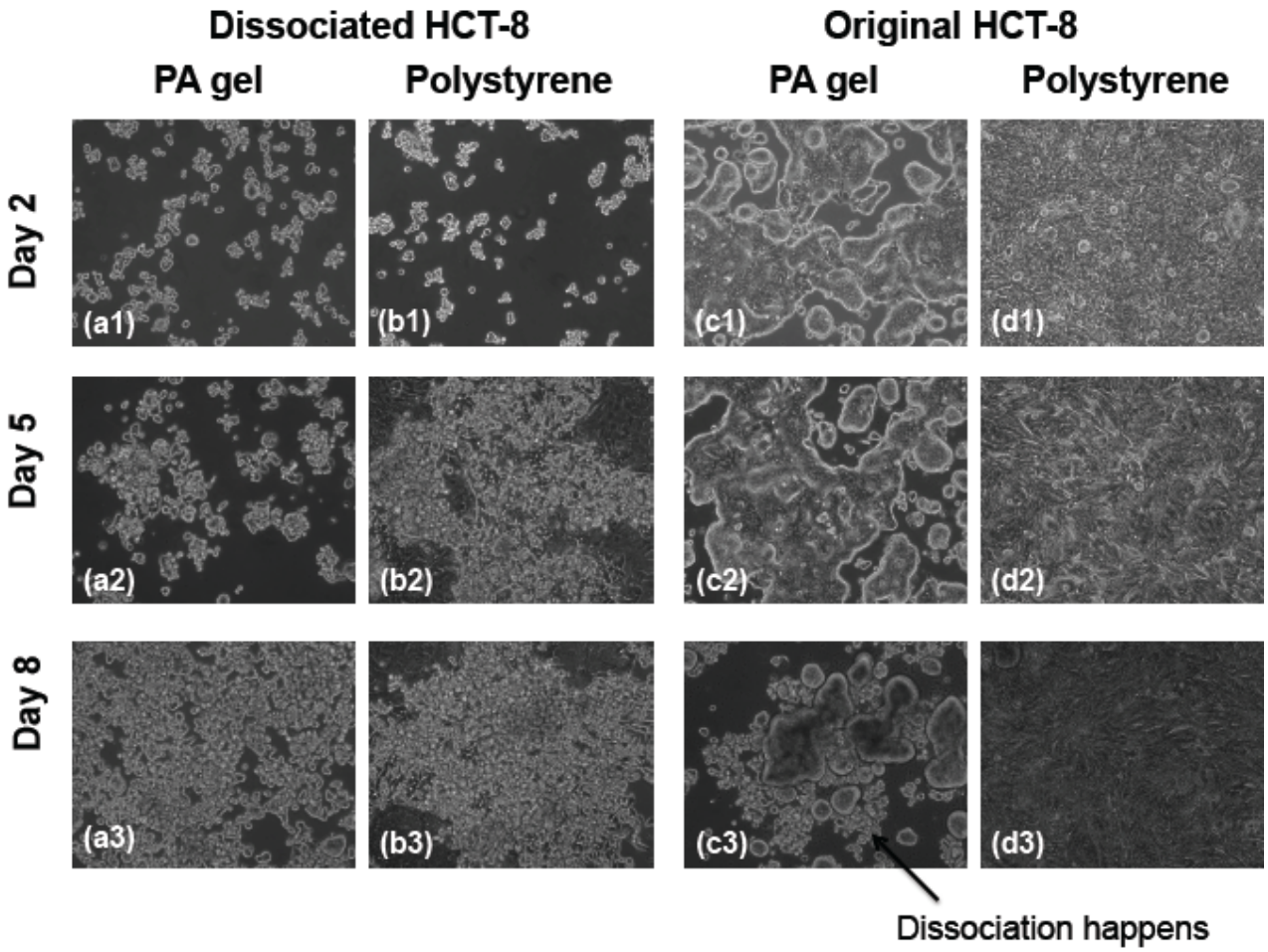


(a)



(b)

Figure S11:


Dissociated HCT-8
Original HCT-8
PA gel
Polystyrene
PA gel
Polystyrene
Day 2
Day 5
Day 8
(a1)
(b1)
(c1)
(d1)
(a2)
(b2)
(c2)
(d2)
(a3)
(b3)
(c3)
(d3)
Dissociation happens

4. Supporting Tables:

Table S1| The relative concentration of acrylamide and N, N'- Methylene bis-acrylamide solutions used in preparation of different stiffness polyacrylamide gels and the resulting Elastic modulus determined by Atomic Force Microscopy (AFM)(9, 10).

Table S1:

	Soft gels	**Intermediate-stiff gels**	**Stiff gels**
Concentration of Acrylamide (mol./v)	8%	8%	8%
Concentration of Bis-acrylamide (mol./v)	0.01%	0.13%	0.48%
Stiffness calibrated by AFM (kPa)	1.05 ± 0.17	20.73 ± 1.03	47.05 ± 1.86

5. Supporting Videos:

Video S1| Real-time video recorded the cell disassociation cascade from a cell cluster. The disassociation process is finished within 5 hours and disassociated cells extrude filopodia and migrate away from the parent island. The time scale shown is Hours: Minutes.

Video S2| The video scan of the entire 21 kPa gel surface on which cells have dissociated after 14 days of culture. It shows that the majority (70 - 90%) of HCT-8 cell colonies are dissociated to single cells (shiny single dots). The microscope objective used is 4×.

6. Supporting References:

1. Kuhlenschmidt, M. S., E. Schmell, C. W. Slife, T. B. Kuhlenschmidt, F. Sieber, Y. C. Lee, and S. Roseman. 1982. Studies on the intercellular adhesio of rat and chicken hepatocytes, conditions affecting cell-cell specificity. The Journal of Biological Chemistry 257:3157-3164.
2. Zhou, J., P. Su, L. Wang, J. Chen, M. Zimmermann, O. Genbacev, O. Afonja, M. C. Horne, T. Tanaka, E. Duan, S. J. Fisher, J. Liao, J. Chen, and F. Wang. 2009. mTOR supports long-term self-renewal and suppresses mesoderm and endoderm activities of human embryonic stem cells. Proceedings of the National Academy of Sciences 106:7840-7845.
3. Yang, S., and T. A. Saif. 2005. Micromachined force sensors for the study of cell mechanics. Review of Scientific Instruments 76:044301.
4. Yang, S., and T. A. Saif. 2007. Force response and actin remodeling (agglomeration) in fibroblasts due to lateral indentation. Acta Biomaterialia 3:77-87.
5. Straight, A. F., A. Cheung, J. Limouze, I. Chen, N. J. Westwood, J. R. Sellers, and T. J. Mitchison. 2003. Dissecting Temporal and Spatial Control of Cytokinesis with a Myosin II Inhibitor. Science 229:1743-1747.
6. Kovacs, M., J. Toth, C. Hetenyi, A. Malnasi-Csizmadia, and J. R. Sellers. 2004. Mechanism of Blebbistatin Inhibition of Myosin II. The Journal of Biological Chemistry 279:35557-35563.
7. Guo, W.-H., M. T. Frey, N. A. Burnham, and Y.-l. Wang. 2006. Substrate Rigidity Regulates the Formation and Maintenance of Tissues. Biophysical Journal 90:2213-2220.
8. Farman, G. P., K. Tachampa, R. Mateja, O. Cazorla, A. Lacampagne, and P. P. d. Tombe. 2008. Blebbistatin: use as inhibitor of muscle contraction. European Journal of Physiology 455:995-1005.
9. Wang, Y.-L., and R. J. Pelham, Jr. 1998. Preparation of a flexible, porous polyacrylamide substrate for mechanical studies of cultured cells. Methods Enzymol 298:489–496.
10. Engler, A. J., F. Rehfeldt, S. Sen, and D. E. Disher. 2007. Microtissue elasticity: measurements by Atomic Force Microscopy and its influence on cell differentiation. Methods in cell biology 83:521-545.