Electronic Supplementary Material (ESI) for Biomaterials Science.

SUPPORTING INFORMATION

Contents

METHODS

ECM Coating Hydrogels

Gradient hydrogels were sterilized under 302nm UV light for 10min. Hydrogels were rinsed with pH 8.5 HEPES buffer (Life Technologies) before being covered with 2ml of 1 mg/mL N-sulphosuccinimidyl-6-(4'- azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH; Thermo Scientific) in pH 8.5 HEPES. Sulfo-SANPAH was activated under 365nm UV light for 10 min before being removed. Hydrogels were rinsed 3 times with HEPES buffer and covered with 2ml of protein solutions, consisting of either collagen I (Col) (0.1g/ml), fibronectin (Fn) (25μg/ml), or laminin (Ln) (25μg/ml) in pH 8.5 HEPES buffer. Hydrogels were incubated overnight in protein solutions at 4˚C for hydrogels in collagen solution and at 37.5˚C for hydrogels in fibronectin and laminin solutions. Hydrogels were rinsed with PBS the next day and before use to remove excess ECM protein.

Immunofluorescent Staining and Imaging

At the end of the culture time, all cells were fixed for 15 min at 24˚C with 4% paraformaldehyde (Santa-Cruz) and permeabilized for 15 min at 24˚C with 1% Triton X-100 (Sigma-Aldrich) before being stained with antibodies. Cells were incubated with primary antibodies overnight at 4˚C and with secondary antibodies for 1 hour at 37˚C. Primary and secondary antibodies were diluted with bovine serum albumin (Merk). The primary antibodies used were YAP (1:100) (sc-101199), MRTF-A (1:100) (sc-390324) and α-actinin (1:100) (A7811). The secondary antibodies used were Alexa Fluor 488 (1:200) and Alexa Fluor 594 (1:200). The cell stains Lamin-A (1:500) (ab205769), rhodamine phalloidin (1:200) and Alexa Fluor 647 phalloidin (1:200) were incubated at the same time as secondary antibodies. Nuclei were stained with DAPI (1:500) for 15min at 24˚C before hydrogels were mounted to glass coverslips for imaging. Samples were rinsed 3 times with PBS for at least 3 min in between each staining step and before mounting.

Image Analysis

Images were captured using a Nikon C2+ confocal microscope using NIS-Elements Advanced Software (Nikon). For H9C2 cells, a Z-stack of 3 images at 2μm steps was captured and combined as a maximum projection image. For the NRVCMs, maximum projection images captured several layers of cells that could not be distinguished from one another. Instead, a single image focused on the layer of cells closest to the hydrogel substrate was analyzed. Images were taken along the axis of the stiffness gradient and the location of each image relative to the edge of the hydrogel was recorded so that the stiffness at that point could be estimated.

Images were prepared for analysis in Fiji, where images were split into individual channels and the brightness and contrast of the F-actin stand and DAPI stain were adjusted to enhance their visibility. At this step, any cells that were out of focus were excluded. Images were then analyzed in Cell Profiler using a custom pipeline that analyzed cell morphology and fluorescent intensity local to the cytoplasm and to the nucleus. Using the major and minor axis measurements from Cell Profiler, the aspect ratio of the cells and the nuclei were calculated through the following equation:

$$\frac{Major\ Axis\ Length}{Minor\ Axis\ Length} = Aspect\ Ratio$$

Intensity values were then normalized to the area of the cells to express fluorescent intensity as a function of area. For YAP and MRTF-A, the degree of nuclear localization was estimated by calculating the Nuclear to Cytoplasmic ratio (Nuc:Cyt) using the following equation:

$$\frac{Nuclear\ Intensity}{Cytoplasmic\ Intensity} = Nuc{:}Cyt$$

Statistical Analysis

Statistical analysis was performed in GraphPad Prism. Based on the location on the hydrogel where the data was acquired, data from the images analyzed in cell profiler was graphed against stiffness. Pearson's correlation coefficient was calculated to determine if there was a relationship between stiffness and the dependent variables and whether any trends identified represented a positive or negative

relationship. To compare stiffness-matched measurements from cells cultured on different ECM coatings, a Two-way ANOVA was performed on each measure, followed by either Tukey's or Dunnett's multiple comparisons where significant differences were detected. All data is presented as mean ± SEM.

Figure S1

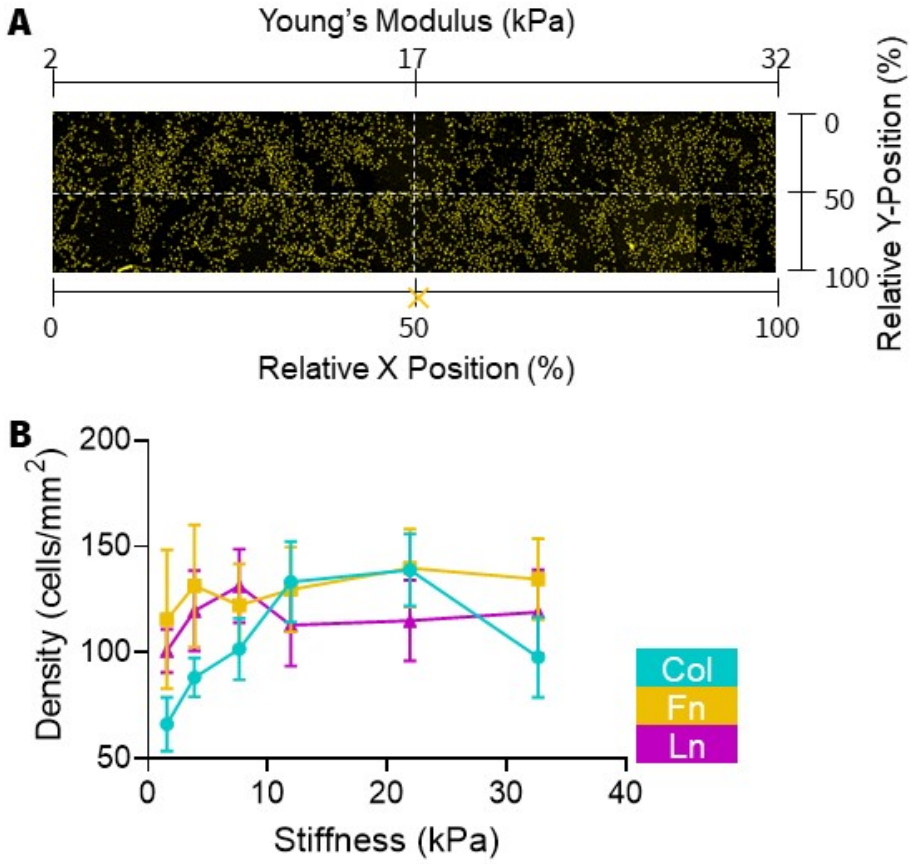

Figure S1. Distribution of H9C2 Cells. H9C2 cells were evenly distributed across the substrate surface. **A)** The representative distribution of H9C2 cells across the substrate was constructed from 27 adjacent images taken across a 9x4mm^2 section of the hydrogel. The average position of cells on the X and Y axis is indicated by the yellow cross. **B)** Based on the DAPI stain, the cell density at each stiffness was calculated. Across all stiffnesses and ECM, no significant differences in cell density were detected (Two-way ANOVA, P>0.05, n=7), however on collagen coated hydrogels, cells tended to be more densely packed between 12 and 22kPa.

Figure S2

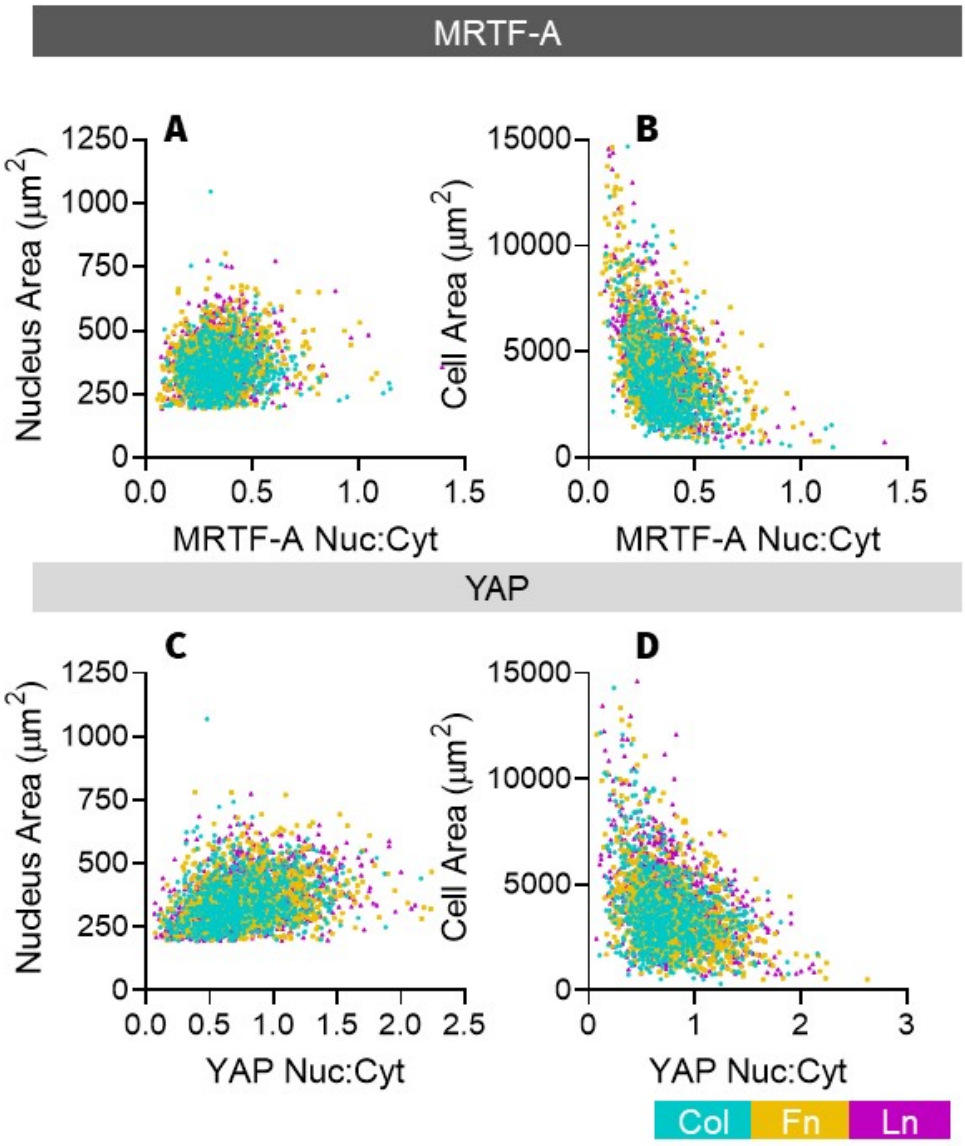

Figure S2. Relationship between mechanomarker localization and cell morphology. MRTF-A and YAP localization was compared to nuclear area and cell area. A) No discernable trends were observed between nuclear area and MRTF-A localization, but a weak correlation can be detected between nuclear-MRTF-A localization and increasing nuclear area in Fn and Ln coated hydrogels, but not Col coated hydrogels (Pearson's correlation, Col-R^2=0.002, P>0.05, n= 4; Fn- R^2=0.01, P<0.05, n=4; Ln- R^2=0.14, P<0.05, n=4). **B)** Conversely, a weak negative relationship can be observed between the nuclear localization of MRTF-A and cell area (Pearson's correlation, Col-R^2=0.002, P>0.05, n= 4; Fn- R^2=0.01, P<0.05, n=4; Ln- R^2=0.14, P<0.05, n=4). The same trends can be seen between YAP and **C)** nuclear size (Pearson's correlation, Col-R^2=0.03, P<0.05, n= 4; Fn- R^2=0.04, P<0.05, n=4; Ln- R^2=0.08, P<0.05, n=4) and **D)** cell size (Pearson's correlation, Col-R^2=0.10, P<0.05, n= 3; Fn- R^2=0.13, P<0.05, n=3; Ln- R^2=0.17, P<0.05, n=4), whereby YAP becomes increasingly nuclear localized with increasing nucleus size and decreasing cell size.