Supplementary Material (ESI) for Lab on a Chip

Fibronectin isolation and fluorescent labeling

Human plasma Fn was isolated from fresh human plasma (Swiss Red Cross) using gelatin-sepharose chromatography[1] and labeled with Alexa 488 or Alexa 633 (Molecular Probes) using established protocols[2].

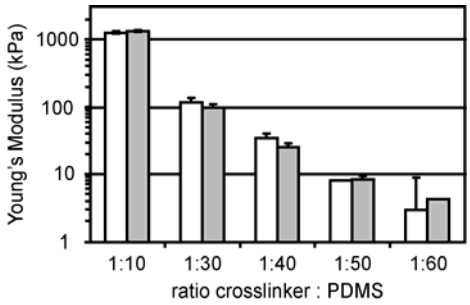
Scanning electron microscopy

The etching and replication of the microstructures were observed with a scanning electron microscope (LEO 1530, in-lens detector, thin coating of Pt for conductivity).

PDMS can be produced with a wide range of stiffnesses

Polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, US) is a crosslinked polymer, and stiffness can be controlled by varying the crosslinking density. Mixtures of 1:10 (weight ratio, w/w curing agent to prepolymer), 1:30, 1:40, 1:50, 1:60, 1:70 and 1:100 ratios were investigated. To create thick films of PDMS the different mixtures were cast inside a 14 cm TCPS Petri dish and cured for 4 hrs at 80°C. The resulting thickness was approximately 1 mm. For the mechanical tests standard dumbbell-shaped specimens were punched out (12 mm gauge length, 2 mm width) and the thickness of each sample was measured. Tensile tests were carried out at room temperature with an Instron tensile tester (model 4411, 1 N load cell, 12 mm/min cross-head speed, corresponding to initial strain rate of 1 min^{-1}). The slope at the start of deformation was used to calculate the Young's modulus (E). For each crosslinker concentration, three samples were measured

on the same day after curing and 16 days later to test if the mechanical properties change after storage. By changing the crosslinker concentrations of the PDMS precursor mixture, the Young's modulus of microwell structures was varied over several orders of magnitude (Suppl. Fig. 1). For soft PDMS mixtures from 1:60 or 1:50 ratios of crosslinker to PDMS, Young's modulus measurements of 3 or 8 kPa, respectively, could be reached and no further crosslinking after 16 days was observed. Variability in the Young's modulus increased in very soft samples (1:60) and may have resulted from crosslinking heterogeneity or insufficient mixing. At lower concentrations of 1:70 or above, the material did not crosslink but remained a highly viscous liquid. These data suggest PDMS is not suitable as a model substrate below ~3 kPa, in contrast to other reports[3, 4].



Suppl. Figure 1: Varying the crosslinking density of PDMS allows one to tailor the mechanical properties of the substrate. The measured Young's modulus ranged between 3 kPa and 1.3 MPa using 1:60 or 1:10 ratios of crosslinker to polymer, respectively (E).

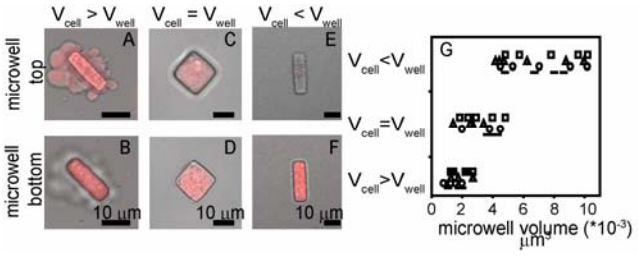
Cell culture

Primary human umbilical vein endothelial cells (HUVECs) (PromoCell, Heidelberg, Germany) were maintained for less than 6 passages in Endothelial Cell Growth Medium plus Supplement (2% serum; PromoCell). Fibronectin coated microwells were seeded with $50*10^3$ cells/cm^2. Cells were allowed to adhere within the microwells for 30 minutes and afterward rinsed by gentle pipetting. Cells were then cultured for 24 hours in either low (2%) or high (10%) serum prior to cell staining and imaging.

Cell volume determines the efficacy of shape control in 3D

The microwell culture concept relies on a cell-adhesive surface inside 3D microstructures with a fixed volume and shape. Cell shape is only completely controlled by the microwell when cell and microwell volumes are similar, and this fit is only rarely achieved using a surface with only one type of microwell. However, the use of a library of different dimensions on the same culture surface (Fig. 2G) allows the selection of cells which are fully constrained in 3D but not protruding from the top of the microwell. The range of microwell volumes adequate for 3D shape control of HUVECs was determined by analyzing confocal z-stacks of calcein positive cells to determine whether cells were too large for the microwell (Suppl. Fig. 2A, B), equal in volume to that of the microwell (Suppl. Fig. 2C, D), or too small to completely fill the microwell (Suppl. Fig. 2E, F). An analysis of 115 cells revealed that for a primary HUVEC culture, well volumes of ~$2*10^3$ to $4.5*10^3$ μm^3 were sufficient to adequately control 3D cell shape (Suppl. Fig. 2G). Cell shape did not appear to affect the ability of HUVECs to completely fill the microwells.

For instance, cells were as efficient at filling spindle-shaped wells with sharp corners as they were at filling circles or squares (data not shown). Although it is difficult to determine whether one or two cells is filling a well with calcein staining, nuclear staining with ethidium homodimer-1 in combination with actin staining using phalloidin confirmed that microwells within this volume range were generally filled with only one cell (>90%; data not shown).



Suppl. Figure 2: Microwells which covered a wide range of projected surface areas and volumes were produced, and the fidelity of cell shape control within microwells depended upon the ratio of cell volume to well volume. Images are shown of an endothelial cell in a smaller well (A, B), a cell in a well of approximately equal volume (C, D), and a cell in a larger well (E, F). Confocal slices were taken at the upper (A, C, E) and lower planes of microwells (B, D, F) which were 10 μm deep. Images shown are confocal overlays of calcein AM-labeled living cells (red) on the DIC transmitted images (grayscale). The range of well volumes which were approximately smaller than, equal to, or larger than cell volumes were quantified (G). Quantification was based on confocal z-stacks taken with 3 μm increments, and each triangular (triangles), circular (circles), square (squares), or rectangular data point (dashed line) represents at least 2 cell measurements in that size category. All scale bars 10 μm.

Visualization of actin, nuclei, and fibronectin

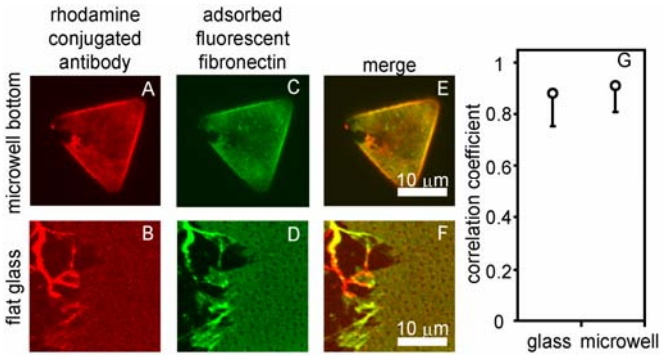
For actin stress fiber imaging, cells were permeabilized with 0.1% Triton X-100 plus 1.5% formalin in PBS for 10 minutes and then fixed in 3% formalin in PBS for 10 minutes. Samples were next blocked in 4% bovine serum albumin (Sigma-Aldrich) in PBS for one hour. Finally, samples were simultaneously incubated with Alexa Fluor 488 Phalloidin (1:400 dilution; Molecular Probes) and ethidium homodimer-1 (1.5 μM; Molecular Probes) for 20 minutes.

For testing the efficacy of immunohistochemistry (IHC) within microwells, microwell samples were coated with Fn labeled with either Alexa 488 or Alexa 633 prior to cell seeding. After 24 hours, samples were fixed and blocked as described above. Microwells were next incubated with a sheep anti-human Fn polyclonal antibody (1:200; Serotec AHP08) for one hour, rinsed thoroughly, and finally incubated with a rhodamine conjugated donkey anti-sheep IgG secondary antibody (1:200; Chemicon AP184R) for one hour. Samples were rinsed thoroughly prior to imaging.

In order to compare confocal images of fluorescently labeled Fn which was adsorbed to microwell surfaces with images of Fn visualized through IHC, glass surfaces or microwells were first coated with Alexa fluorophore labeled Fn. After culturing HUVECs for 24 hrs, the cells were fixed and the Fn was stained with a rhodamine-conjugated secondary antibody as described above. Finally, 27 by 27 μm square confocal images (256 by 256 pixels) of fluorescently- or IHC-labeled Fn were only acquired underneath HUVECs on flat glass surfaces or on the bottom surface of microwells that were filled by a cell, and the 2D correlation coefficient of the two raw 16-bit images was computed using Matlab (The Mathworks; Natick, Massachusetts). Both Alexa 488- and

633-labeled Fn were compared against the rhodamine-conjugated secondary antibody to ensure fluorescent bleed did not complicate the analysis. Acquisition parameters were held constant between glass and microwell samples.

Although microwells were fabricated into thin films to permit high resolution microscopy of cellular features, the microwell culture platform might limit the ability to visualize intracellular proteins with IHC due to diffusive limitations. Therefore, we tested the efficacy of IHC staining (Suppl. Fig. 3A, B) of Fn (Suppl. Fig. 3C, D) which was bound to the bottom surface of microwells containing a HUVEC (Suppl. Fig. 3A, C, E) or bound to glass surfaces underneath an adherent HUVEC (Suppl. Fig. 3B, D, F). Correlation coefficients between images of adsorbed Fn which was previously labeled with Alexa 488 or Alexa 633 and IHC-stained Fn visualized with a rhodamine-conjugated secondary antibody were calculated, since diffusion limited IHC staining in microwells should lead to a lower correlation coefficient between images of microwell bottoms relative to correlation coefficients of images on flat glass surfaces. However, Suppl. Fig. 3G demonstrates that Fn on the bottom surface of microwells containing cells could be stained and imaged using IHC as effectively as Fn underneath cells on 2D glass. In addition, similar results were found when Alexa 488- or Alexa 633-labeled Fn were compared against the rhodamine-conjugated secondary antibody, thus excluding a contribution from fluorescent bleed (data not shown).

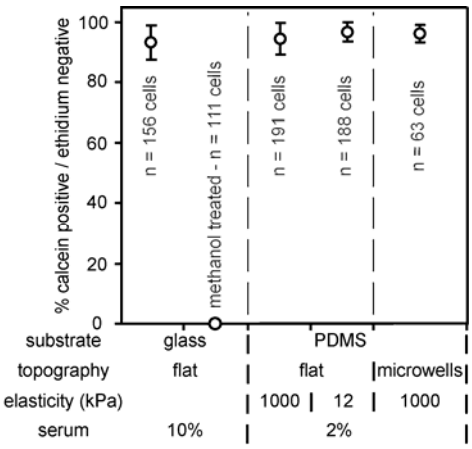


Suppl. Figure 3: Confocal images are shown of IHC-stained Fn using a rhodamine-conjugated secondary antibody (A, B). The adsorbed Fn was previously labeled with either Alexa 488 or 633 (C, D). Merged images are shown for a 20 μm edge triangular microwell filled with a single HUVEC (E) and Fn underneath a small portion of a well spread HUVEC on flat glass (F). Correlation coefficients were calculated (G) to quantify the efficacy of IHC underneath cells in microwells (A, C, E) relative to cells adherent to flat glass substrates (B, D, F) to determine whether increased diffusive barriers are present using microwell samples. All fields of view were ~28 by 28 μm, confocal settings such as laser transmissivity and photomultiplier tube voltage were kept constant, and raw data images were used for correlation coefficient calculations.

HUVECs are viable in microwells

Living cells were distinguished from apoptotic or necrotic cells by the enzymatic conversion of calcein AM to fluorescent calcein and the exclusion of ethidium homodimer-1. Microwell samples containing HUVECs cultured for 24 hours were simultaneously incubated with 5 μM calcein AM and 1.5 μM ethidium homodimer-1 in phosphate buffered saline (PBS) for 20 minutes.

Cell confinement on patterned 2D surfaces has been shown to induce apoptosis, maintain cells in a quiescent state, or permit progression through the cell cycle restriction point in G1 as cell spreading is increased[5-7]. Positive staining for calcein and exclusion of ethidium homodimer-1 from cell nuclei was used to determine if cell confinement in 3D microwells initiated cell death. In control studies, 93% of HUVECs in 10% serum on glass at ~75% confluence were viable, and viability was not inhibited when cells were cultured in low serum on stiff (1 MPa; 94% viable) or soft (12 kPa; 96.5% viable) flat PDMS (Suppl. Fig. 4). Thus, PDMS was not itself toxic for cells, similar to previously published data[3]. Cell confinement in 3D also did not lead to a significant increase in cell death. Approximately 95% of cells which were cultured in microwells with circular, square, and triangular shapes where 3D cell shape was completely controlled were viable. For those microwells which were completely filled by HUVECs and thus dictated their 3D cell shape, well bottom surface area, or projected 2D surface area, was on average 255±155 μm^2. However, the total well surface area, or total surface area in contact with cells, was 808±226 μm^2. For microwells of different shapes, the relationship between 2D projected area and total microwell surface area changes since different 3D shapes have different surface area to volume ratios for a constant volume. On 2D surfaces, HUVECs on micro-contact printed islands could spread up to 4000 μm^2 due to the extra degree of freedom imposed by the lack of volume restriction[6]. Microwells with shallower depths would more closely approximate 2D surfaces as the projected 2D surface area increases for a given constant cell volume. Finally, these results indicate that the decreased exposure to serum for cells in microwells relative to on flat surfaces does not lead to a loss of cell viability.



Suppl. Figure 4: Cell viability was verified for HUVECs whose 3D shape was controlled by positive staining for calcein AM, which is fluorescent inside living cells, and negative staining of ethidium homodimer, a membrane impermeable dye which becomes fluorescent when bound to nucleic acids of apoptotic or necrotic cells. The percentage of calcein positive/ethidium negative cells are shown for cells on glass in high serum, cells on glass which were treated with methanol, cells on flat hard or soft PDMS, and single cells in hard microwells which were binned according to 2D projected surface area. Data in each group was acquired from at least 3 separate experiments, and the percentage of viable cells was computed from all cells.

References

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