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Cell force measurements in 3D microfabricated environments based on compliant cantilevers

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Fabrication of µ-flowers

SiO_2 growth and thinning (Fig. 2 A-B). µ-Flowers were fabricated by means of surface microtechnology. First a wet oxidation was performed to grow a 200 nm thick layer of SiO2 on a Si (100) 100 mm wafer, in a furnace (Centrotherm, Blaubeuren, DE). After oxidation, the top surface of the wafer was protected with 2 µm of spin coated photoresist AZ® 1512 HS (MicroChemicals GmbH, Ulm, DE) and the oxide on the back side of the wafer was completely removed in a buffered hydrofluoric acid solution (BHF) made by a 7:1 mix of NH_4F (concentration 40% in water) and HF (concentration 49% in water). Photoresist was removed from the top of the wafer with remover SVC-14 (Micro Resist Technology GmbH, Berlin, DE), followed by rinsing with deionized (DI) water. The SiO_2 film on the front side was thinned by etching in BHF, and its thickness was measured to be 104 ± 1 nm (49 measurements across the whole wafer surface). The measurements were performed with a spectroscopic reflectometer Nanospec 6100 (Nanometric Inc., Milpitas, CA).

Ti deposition (Fig. 2C). A layer of Ti was subsequently evaporated on top of SiO_2 in a LAB 600 H e-beam evaporator (Leybold Optics GmbH, Alzenau, DE). A small area of the wafer was masked during the evaporation, to keep it free from Ti and use it as a baseline for the measurement of Ti thickness. The measurement was performed with an Alpha-Step 500 mechanical profilometer (Tencor, Milpitas, CA), and resulted in a thickness of 59 ± 2 nm.

Au patterning via lift-off (Fig. 2D). Au patterns were fabricated and functionalized with a lift-off process. First, the wafer was dehydrated on a hot plate at 150° C for 3 min 30 s. A sacrificial layer of LOR resist (MicroChem Corp., Newton, MA) was spun onto the wafer for 30 s at a speed of 6500 rpm, followed by baking on a hotplate at 190° C for 4 min 10 s. The nominal LOR thickness was 400 nm. Photoresist AZ® 1512 HS (MicroChemicals GmbH, Ulm, DE) was spun onto LOR at a speed of 2800 rpm for 1 min 30 s, to reach a nominal thickness of 1.6 µm. All the steps just detailed (dehydration, spin coating, baking) were performed with an EVG® 150 automated resist processing system (EVG, Sankt Florian am Inn, AT). The resists were exposed to UV light on a MJB4 mask aligner (Süss MicroTec AG, Garching, DE), using an I-line filter and a dose of 42 mJ/cm^2. A chrome mask previously written on a DWL 200 laser lithography system (Heidelberg Instruments Mikrotechnik GmbH, Heidelberg, DE) was used during wafer exposure, to transfer circular rings onto the resist (Fig. 6D). After exposure the wafer was manually developed in a beaker containing 100 ml of MF-CD 26 developer (Micro Resist Technology GmbH, Berlin, DE). The beaker was gently rotated and shaken to refresh the exhausted developer at the photoresist interface. Development was stopped after 1 min 30 s by dipping the wafer in DI water, and followed by rinsing with DI water and drying with a nitrogen gun. The wafer was thus protected by photoresist, except for concentric ring patterns that were exposed and removed with developer. The diameter of the central circle was 9 µm, while the width of the external rings was 7 µm. A Ti/Au (5/40 nm) layer was deposited onto the wafer with a LAB 600 H e-beam evaporator (Leybold Optics GmbH, Alzenau, DE), with Ti acting as an adhesion layer for Au. The sacrificial photoresist was removed by overnight immersion in Microposit 1165 remover (Shipley Co., Inc., Malborough, MA), followed by rinsing in isopropyl alcohol, rinsing in DI water and spin drying. In this way Au rings were fabricated on top of the Ti/SiO_2 bilayer. Their thickness has been measured to be 44 ± 2 nm with an Alpha-Step 500 mechanical profilometer (Tencor, Milpitas, CA).

2nd photolithographic mask (Fig. 2E). A new photoresist layer was patterned to shape the cantilevers of µ-flowers. A film of AZ® 1512 HS (MicroChemicals GmbH, Ulm, DE) was spin coated at a speed of 3000 rpm for 1 min 30 s, to achieve a thickness of 1.5 µm. A chrome mask containing the patterns to define the cantilevers was aligned on the wafer, to center the µ-flowers with respect to the Au rings (Fig. 6E). The alignment was done manually with optical microscopes on a MJB4 mask aligner (Süss MicroTec AG, Garching, DE), with a typical alignment error of about 1 µm. The photoresist was then exposed with UV light, using an I-line filter and a dose of 42 mJ/cm^2. After exposure, the wafer was manually developed in a beaker containing 100 ml of MF-CD 26 developer (Micro Resist Technology GmbH, Berlin, DE), followed by rinsing as described before. The wafer was thus selectively protected by photoresist in the shape of µ-flowers. 4 different geometries of µ-flowers were fabricated, by changing the number of cantilevers (3, 4, 6, and 8 cantilevers per µ-flower, as shown in Fig. 2). Each of these geometries was then reproduced by varying the length of the cantilevers to 4 different values (20, 30, 40, and 50 µm). In this way every chip (dimension 1x1.2 cm^2) contained 16 versions of µ-flowers, and each of these versions was replicated 600 times, for a total of about 10000 µ-flowers per chip. µ-Flowers dimensions are shown in Fig. 2. The pitch between subsequent µ-flowers was 60, 80, 100, and 120 µm, scaling accordingly to the length of cantilevers.

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Cantilever shaping by etching (Fig. 2F). The part of Au rings exceeding the photoresist was etched in a water solution containing KI (25 g/l) and I_2 (12 g/l) for 30 s, followed by rinsing in DI water. In this way Au spots were left only on the cantilevers. By using this process, Au spots were perfectly centered with respect to the long axis of the cantilevers, independently of the alignment error of the 2nd mask onto the Au rings. This error affected only the position of Au spots along the cantilever length, but since this error (~ 1 μm) is much smaller than the length of cantilevers, it is tolerable. It is now clear why Au rings were deposited, instead of depositing directly Au spots. In the latter case, a misalignment between Au spots and cantilevers would have caused (i) Au spots not to be centered with respect to the long axis of some cantilevers, and (ii) Au spots to be cut and reduced in size on some cantilevers more than on others, in an uneven way (they would have been cut by Au etching on cantilevers perpendicular to the misalignment direction, but not on cantilevers parallel to the misalignment direction). The non-protected area of Ti was etched as well, by means of an inductively coupled plasma generated in a mix of Cl_2 and BCl_3 gases. For this etching an STS Multiplex Inductively Coupled Plasma (ICP) etcher was used (Surface Technology Systems, Newport, UK). The wafer was then diced into 1x1.2 cm^2 chip.

Cantilever release and cleaning (Fig. 2, G-H). Chips were rinsed in DI water, and glued with QuickStick^{TM} 135 (Electron Microscopy Sciences, Hatfield, PA) on a blank dummy Si (100) wafer, in order to continue the process with machines compatible with 100 mm wafers. Dummy and chips where loaded in an Alcatel 601E deep reactive ion etcher (Alcatel, Paris, FR) and SiO_2 was etched from the chips areas not protected by photoresist, using a plasma from C_2F_6 gas (ICP coil power: 1800 W, power of substrate bias: 400 W). In this way the bilayered Ti/SiO_2 cantilevers were shaped on the Si substrate (Fig. 2F). Cantilevers were then released from the substrate by an isotropic Si etching performed with plasma generated from SF_6 gas. To avoid excessive heating of cantilevers, a low power was used during Si etching (ICP coil power 800 W, power of substrate bias = 0 W). Chips were detached from the dummy wafer by melting QuickStick^{TM} 135 on a hotplate at 135° C. Residues of glue were carefully removed from the chip underside with acetone, using a swab to prevent any wetting of the topside (which would be harmful for released cantilevers, due to capillary forces). The photoresist was stripped from cantilevers by means of oxygen plasma followed by immersion in Microposit 1165 remover for 30 min at 70° C (Shipley Co., Inc., Malborough, MA). The oxygen plasma stripping was performed in a TePla 300 reactor (PVA TePla AG, Kirchheim, DE) and segmented in three short steps of 10 s each, to minimize cantilever heating. For the same reason the power of the machine was kept low (100 W, 200 ml/min oxygen flux).

Spring constants calculation

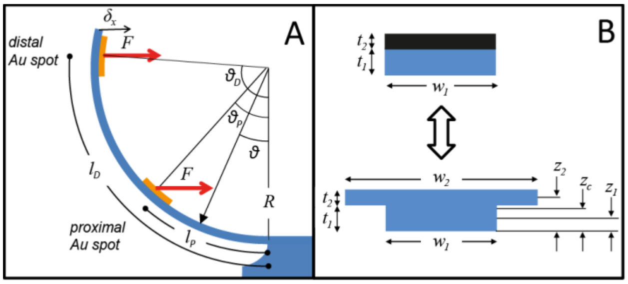

Figure S1. (A) Schematic of a bent cantilever used for the calculation of the spring constants experienced by forces applied at the distal Au spot and at the proximal Au spot. (B) Scheme showing the mechanical equivalence between a bilayered cantilever with rectangular section and a T-shaped cantilever made out of one single material.

The following calculations are derived from the analysis of the deflection of curved beams[1]. Let's consider the bent cantilever shown in Fig. S1 A. By using Castigliano's second theorem it is possible to calculate the deflection δx produced by a horizontal force F applied either at the proximal Au spot or at the distal one. The theorem states that $\delta x = \partial U/\partial F$, being U the strain energy of the cantilever expressed as a function of the applied load F. Since the length of the cantilever is much bigger than its thickness, the strain energy can be approximated with the energy of flexure U_f of a straight beam:

$$U \cong U_f = \int_0^{l_i} [M(l)]^2 /(2EI) dl$$

where $l_i = l_P$ or l_D, E is the Young's modulus of the cantilever, I the second moment of area and $M(l)$ is the bending moment generated by the load F at any point l along the cantilever length. Denoting the angular position of any section of the cantilever by ϑ, the moment is $M = FR(\cos\vartheta - \cos\vartheta_i)$, with $\vartheta_i = l_i/R = \vartheta_P$ or ϑ_D respectively. Substituting dl with $Rd\vartheta$ we have

$$U_f = \int_0^{\vartheta_i} [FR(\cos\vartheta - \cos\vartheta_i)]^2 R/(2EI) d\vartheta .$$

We can now calculate the deflection δx as

$$\begin{aligned} \delta x &= \partial U / \partial F \cong \partial U_f / \partial F = \\ &= (FR^3 / EI)\left[\vartheta_i / 2 - (3/2)(\cos\vartheta_i \sin\vartheta_i) + \vartheta_i \cos^2\vartheta\right], \end{aligned}$$

and hence the spring constant for an applied horizontal force F

$$k = F / \delta x = EI \Big/ \left[R^3 \left(\frac{\vartheta_i}{2} - \frac{3}{2}\cos\vartheta_i \sin\vartheta_i + \vartheta_i \cos^2\vartheta_i \right) \right].$$

To compute k we need to calculate the second moment of area I for the bilayer cantilever. For this purpose an equivalent T-shaped cross section is developed (see Fig. S1 B), made out of a single material (SiO_2), with thickness t_1 and t_2 equal to the original bilayer thicknesses, and with the width of the second layer increased by the ratio between the Young's moduli, i.e. $w_2 / w_1 = E_{Ti} / E_{SiO2}$ (1). Being z_1 and z_2 the positions of the neutral axis of the two elements, t_1 and t_2 the thicknesses of these same elements, the neutral axis z_c of the equivalent T-shaped beam is

$$z_c = (z_1 w_1 t_1 + z_2 w_2 t_2)/(w_1 t_1 + w_2 t_2)$$

Having z_c and knowing that $I_i = w_i t_i^3/12$ we can calculate the second moment of area of the equivalent T-shaped beam

$$I = I_1 + w_1 t_1 (z_1 - z_c)^2 + I_2 + w_2 t_2 (z_2 - z_c)^2 .$$

For the fabricated cantilevers we have a radius $R = 28$ μm, $l_P = 20$ μm, $l_D = 50$ μm, $w_1 = 5$ μm, $t_1 = 104$ nm and $t_2 = 59$ nm. Taking

$E_{SiO_2} = 66$ GPa(2) and $E_{Ti} = 116$ GPa (value for bulk Ti) and carrying out the computation for k we obtain

$$k_P = 313\ \text{nN/µm};$$
$$k_D = 5.5\ \text{nN/µm}.$$

When computing the spring constant, the main sources of uncertainty are the values of the Young's moduli and the thickness of the cantilever. Young's moduli of very thin metal films, as the one fabricated in the context of this work, are known to be dependent on the particular process used during the fabrication, on the substrate on which the films are deposited, as well as on the thickness of the films themselves, with variations of E_{Ti} that can be in the order of 30%[3, 4]. For these reasons care has to be paid when using data from the literature or from tabulated values for bulk materials. The other most significant source of uncertainty is the thickness of the SiO_2 film composing the cantilevers. We have precisely measured it with a spectroscopic reflectometer, but the subsequent etching process, performed to release the cantilevers, could have modified its initial thickness. In order to quantify the uncertainty on k, we can compare two extreme cases. Case (i): a maximum value of 128 GPa for E_{Ti} (4) and a completely unaffected SiO_2 layer; case (ii): a minimum value of 90 GPa for E_{Ti} (3) associated with a 10% reduction of the SiO_2 layer. We obtain the following asymmetric uncertainties for k:

$$k_P = 313^{+13}_{-92}\ \text{nN/µm};$$
$$k_D = 5.5^{+0.2}_{-1.6}\ \text{nN/µm}.$$

To quantify forces more precisely it would be required to overcome this uncertainty, by a direct calibration of the spring constant. It would be also necessary to determine the direction of the force vector, since the effective k constant depends on it. However the ratio k_P / k_D is independent on these uncertainties, since they would change the values of both k_P and k_D by the same factor. Hence the considerations done about the division of the data in two sets, one for proximal and one for distal adhesions, are not undermined.

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