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CYCLE XXXIII**

Investigation of cell-microenvironment interactions by Atomic Force Microscopy techniques

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Chapter 1

Introduction

1.1 Cell-microenvironment interactions

Tissues are composed by two main components: cells and the extracellular matrix (ECM). The ECM is the non-cellular component of all tissues, forming the physical microenvironment surrounding cells, and playing, both, a structural and a signalling role.

It has been known for many years that tissues grow and remodel in response to changes in mechanical forces, it is the cells in the tissues that are responsible for this remodelling. A typical example is the bone, which changes its shape, density, and stiffness when its mechanical loading conditions are altered¹.

The ECM is responsible of the external force transmission to the single cells, though also the single cells are capable of exerting force on the microenvironment².

The precise composition of an ECM is tissue specific, but it is usually composed of a 3D network of fibrous and crosslinked proteins (e.g., collagen, laminin and fibronectin), proteoglycans, matricellular proteins³. Its structure and composition can be dynamically modulated by cells and determines its unique biophysical and structural properties, such as the topography and the stiffness, distinguished by specific pore sizes, fibre sizes, fibre orientation and ligand density. ECM proteins can serve as scaffold for the anchoring of cells, they can guide or restrain cell and tissue movements (Figure 1).

Far from being just an inert physical scaffold, the biochemical and biophysical properties of the ECM provide cues to the cells. The cells sense those features and undergo conformational changes in their adhesion sites and cytoskeletal structure that in turn activate signalling to influences cell behaviour. This coordinated interaction between cells and their physical microenvironment creates a complex cross-talk that is fundamental in the guidance of tissue morphogenesis^{4,5}. It is now well recognised that mechanical forces play a fundamental role in the regulation of basically all cell functions, including gene expression, protein synthesis, cell growth, death, proliferation and differentiation^{6,7}.

The mechanism through which cells can perceive the physical properties of the surrounding microenvironment and convert this information in a biochemical signalling is called *mechanotransduction*^{6,7}.

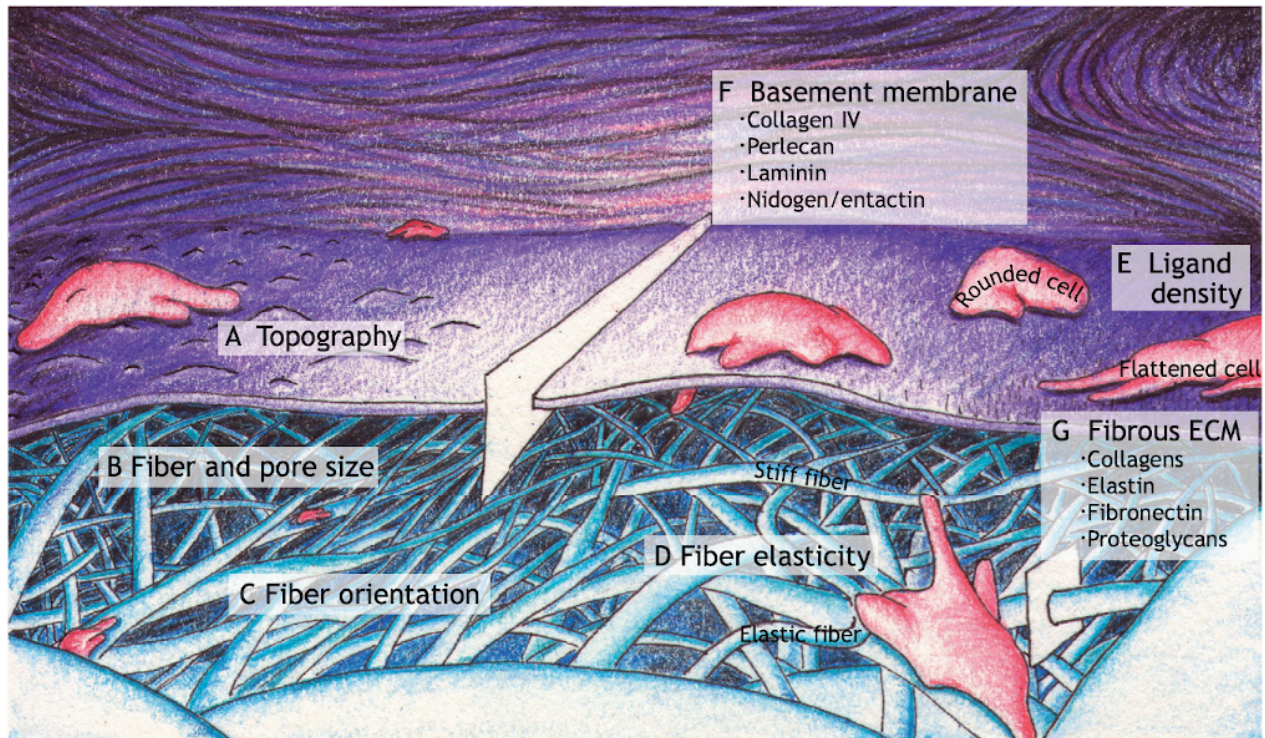


Figure 1. Examples of physical properties of the extracellular matrix. (A) Topography. (B) Varying fibre diameters and sizes of pores between ECM fibres. (C) Differences in fibres orientation, oriented and non-oriented fibres. (D) Fibres elasticity. (E) Differences in ligand density affecting cells shape. (F-G) Different types of ECMs, basement membrane and fibrous ECM. Image adapted from Walma et al⁶.

1.2 Mechanosensing and transduction

At the base of mechanotransductive processes are the events taking place at the interface between the cell and the ECM where the microenvironmental biophysical cues are translated into intracellular signals. The involved (extra)cellular structures in this cell/microenvironment interface, such as the ECM, the glycocalyx, the cell membrane, the integrin adhesion complexes (IAC) and the cytoskeleton, are highly complex and interlaced in an intricate crosstalk. IAC are the major relay station of the transduced signals between the cellular out- and inside, mostly by modulating the cytoskeletal organisation and mechanics and the location of mechanosensitive signalling proteins.

The IAC represent the principal interfacial mechano-sensors that perceive the information of the cellular outside by the binding of the integrin receptors to ligands in the ECM^{9,10} (see Figure 2). Integrins can assume different conformations ranging from the inactive closed-bent (with low ligand affinity) to the active open-extended conformation. Integrin activation can take place in different ways: from the outside through the formation of the bond between the integrin and the ECM-ligand, through internal pathways, or by the modulation of membrane or by the actin cytoskeletal tension.

Inside of the cell's body, once activated, the integrins are then linked via adaptor proteins (such as talin and vinculin) to the filaments of the actin cytoskeleton, engaging in this way the so-called **molecular clutches**.

These ECM/integrin/talin/actin filaments linkages enable the transmission of force that derives from the **actin retrograde flow**, which again is generated by both the actin polymerisation and myosin-driven contraction (see Figure 2).

However, whether this initial structure disassembles immediately or instead is reinforced and matures by recruitment of further proteins and integrin clustering depends on the extent of the force loading of the molecular clutch. At sufficient force loading the integrins are maintained in their activated conformation (which can happen very quickly, in less than a second¹¹) and their ECM/ligand binding is strengthened increasing the lifetime of the bonds¹². This force-dependent reinforcement and the consequential increase in lifetime allows the recruitment of further essential IAC components and eventually generates Focal Adhesion complexes FAs, that's represent the final stabilized adhesion spot.

These processes regulate the sequential reorganization of the actin filaments (in terms of tension, spatial organization and density) in relation to the **external** cues, and they are responsible for the determination of the effective cell shape and mechanical properties of cells at physiological strain level.

Exploiting this overall mechanism, the cellular physical sensing is capable of "interpreting" mechanical and morphological properties of the ECM, through the spatial organisation and dimensionality of adhesion sites, with precision at the nanoscale, manifesting it in the extent to which IAC mature from nascent adhesions into FAs and then in the internal cytoskeletal architecture.

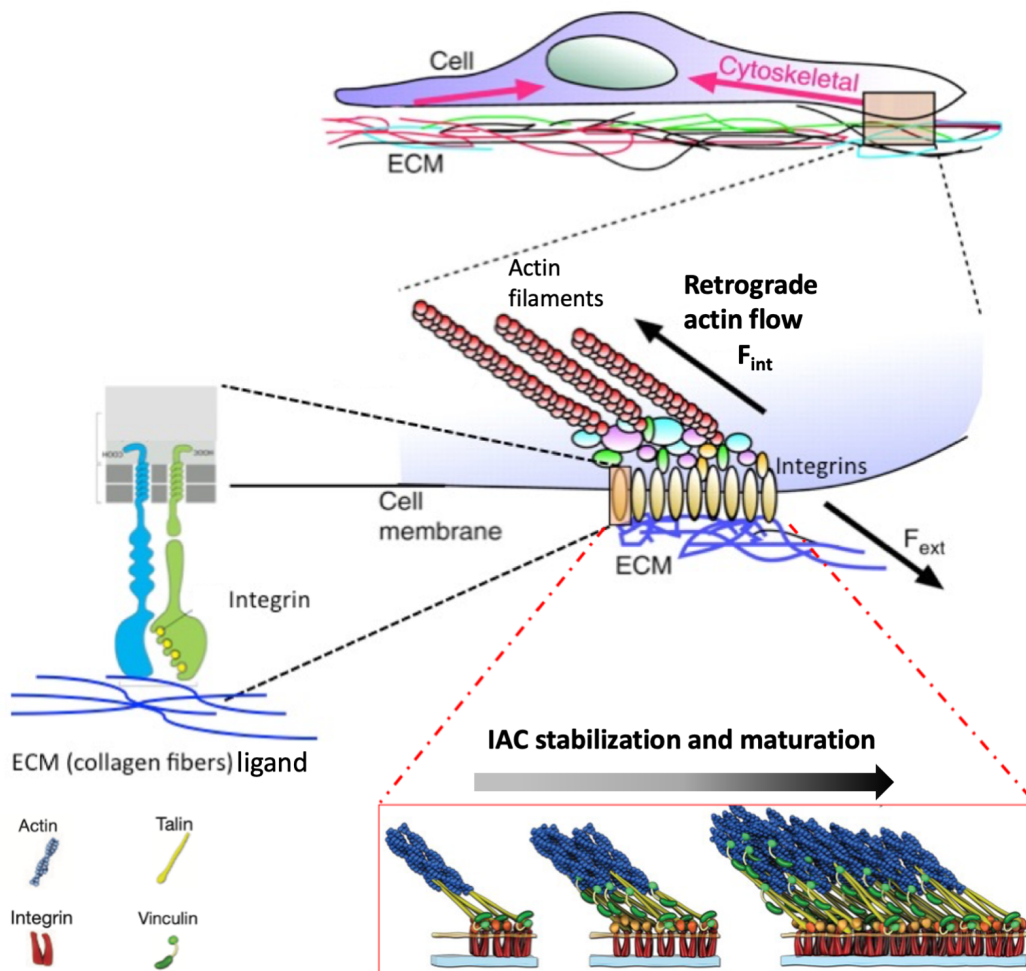


Figure 2. integrin-mediated mechanosensing and mechanotransductive sequence after the binding with the extracellular matrix. The insets illustrate the initial ECM-integrin-talin-vinculin-actin linkage in the nascent adhesion. The Figure contains adapted elements of images from Case and Waterman⁹ and from Chen¹³.

1.3 Mechanical properties of tissue components in cancer diseases and other pathologies

The biochemical and mechanical properties of the ECM tune the growth, survival, migration, invasion and differentiation of cells within the tissue to influence their fate and thus regulate tissue development, phenotype and homeostasis. Not surprisingly, diseases such as cancer, that are defined by disrupted tissue homeostasis and loss of differentiated phenotype, or chronic inflammation diseases are accompanied by alterations in the composition, organization and mechanical properties of the ECM that contribute not only to malignant transformation but also to tumour progression and metastases^{14–16}. Tumours are frequently accompanied by a condition (desmoplasia) that is characterized by chronic inflammation and increased levels of remodelled¹⁵ and cross-linked ECM proteins that stiffen the tissue¹⁷ (see Figure 3). Cancers develop a desmoplastic

response in which the tissue contains higher amount of ECM proteins such as fibronectin, tenascin, and hyaluronic acid (HA) and other proteoglycans and matricellular proteins as well as greater quantities of crosslinked collagen I type¹⁸. A modified ECM has also been implicated in metastases^{14,19}, which is the invasion and dissemination of tumour cells from primary tumours to distant tissues, and is the leading cause of mortality in most cancer patients.

The so-called “invasion-metastases cascade” begins with the extravasation of cancer cells to lymphatic and blood vessels, and the cell ability to invade is accompanied by various molecular changes²⁰ leading, among others, to alteration of organization of actin filaments²¹. Therefore, the cell stiffness play roles in transmigration of cancer cells through the ECM in order to reach the vessels (i.e. the softer is the cell, the easier is to migrate through the tissue). Indeed, while cancer ECM has been found to be generally stiffer than normal one, seminal studies have shown that cancer cell themselves are more compliant than normal cells^{22,23}.

Interestingly, even if the chronic inflammation is usually associated as a marker for the possible following cancer development¹⁷, the existence of a silent inflammatory tissue state (that precedes the clinical symptoms) has been recently demonstrated which is characterized by structural damage and perforation of the basement membrane that eventually leads to a softening of the ECM¹⁶.

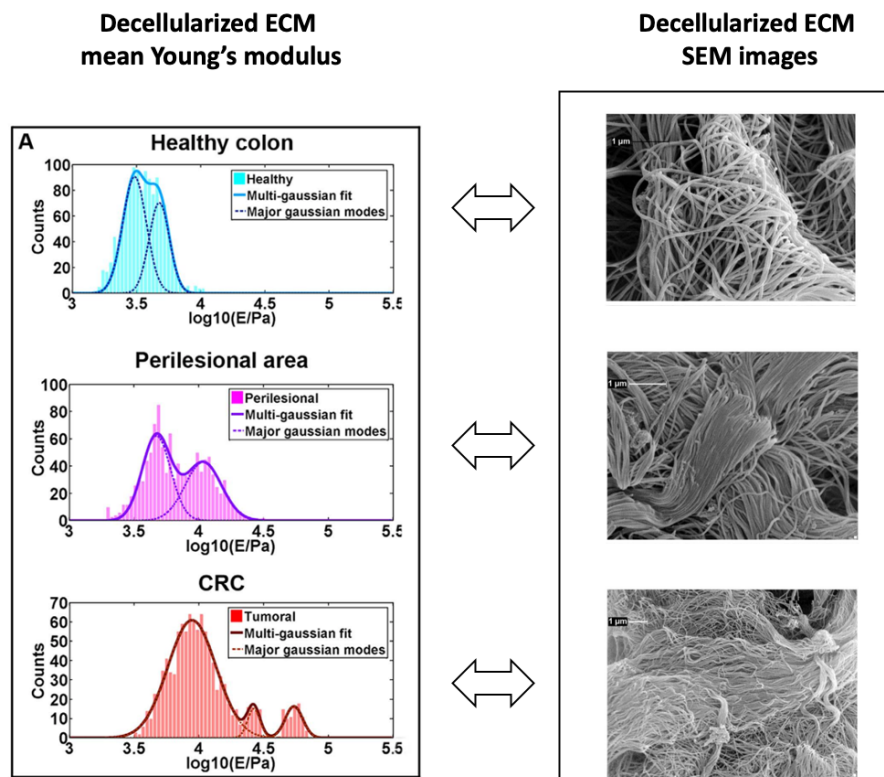


Figure 3. Example of desmoplasia: ECM remodelling (increase of fibre organization) and corresponding increasing in stiffness, from healthy tissue to neoplastic (CRC). Image adapted from Nebuloni et al¹⁵.

1.4 Mimicking the ECM

The integrin-mediated cellular mechanosensing is capable of “interpreting” mechanical and structural properties of the microenvironment with high precision at the nanoscale.

Dissecting and understanding comprehensively how exactly the environmental physical and structural properties influence mechanotransduction remains partially understood due to the complexity of the involved biological structures of the ECM. This limits also the attempts to artificially reproduce the complex nanoscale morphology and stiffness of the ECM. A realistic model of the ECM complexity should be based on the 3D organization of biochemical and biophysical cues as an essential aspect regulating cellular functions. However, the full control over the properties of artificial 3D structures mimicking ECMs is very hard to achieve and the fabrication is still in its infancy^{24,25}.

On the other hand, the mimicking of few selected physical properties (see Figure 4) like the ligand spacing^{26,27}, or the tissue stiffness²⁸ has been widely used to dissect the properties that trigger specific cellular responses like cellular adhesive capacity, proliferation and differentiation. However, the ECM configuration can be rather disordered at the local nanoscale level, often showing irregularities, anisotropies and density gradients²⁹, setting an upper limit in the capability of micro/nano-fabricated regularly patterned substrates to mimic the ECM *in vitro*.

An important achievement was the discovery of the importance of such *disordered* structure in tuning cell behaviour. Indeed, it has been demonstrated how also the nano-disorder is a crucial factor both for the recreation of ECM-like microenvironment^{30–32} and in the triggering of mesenchymal cell differentiation³³. Eventually, the identification of the ECM parameters relevant for cellular behaviour and evolution is crucial to improve conventional *in-vitro* culture systems and to move towards a new generation of engineered *active* biomaterials, able to determine the cell fate^{31,32}.

This can provide a significant breakthrough in the biomedical field, from the point of view of both, basic and applied research, allowing to develop specific culturing devices or to greatly improve the potency and functionality of implants and interfaces in general^{34,35}.

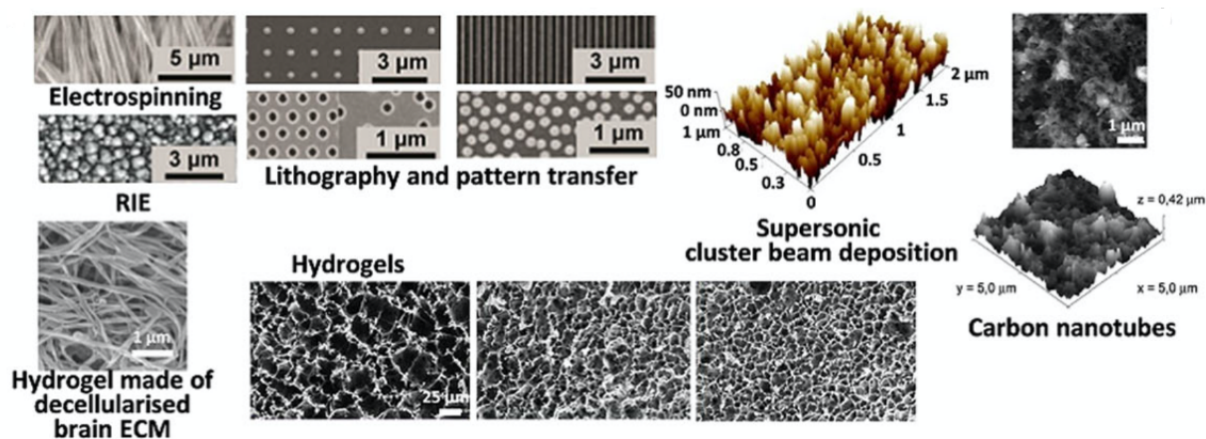


Figure 4. Examples of biomaterials and approaches to reproduce the peculiar morphology and mechanical properties of the ECM. Image adapted from Chighizola et al.³⁶.

1.5 Atomic force microscopy to study biophysical cues in mechanotransductive interactions

The purpose of mechanobiology is to dissect the biophysical markers of the cells and the surrounding microenvironment and understand how these structural properties are intertwined and influence each other to regulate tissues survival. As a consequence, it is fundamental to get precise characterization of the structural and mechanical properties of the microenvironment, the cells or the whole tissue. The investigation of such processes relies furthermore on the ability to apply accurately controlled physical stimuli to the samples and to measure the reactions of their mechanobiological alterations³⁷.

The study of biophysical aspects of cells and their microenvironment or the nature of their interaction requires specific force-sensing instrumentation, like optical/magnetic tweezers³⁸, traction force microscopy³⁹ and atomic force microscopy⁴⁰ (AFM).

AFM belongs to the branch of scanning probe microscopy^{40,41} (SPM), the AFM probes consist in an interacting tip attached to an elastic lever (cantilever) that deflects under the interaction forces between the probe and the surface of the sample (see Figure 5). The corresponding cantilever deflections are measured by a quadrant photodiode that detects the displacement of a laser reflecting from the backside of the cantilever, while the nanometric displacement of the probe/sample is controlled through piezoelectric actuators.

Given the nature of the interaction between the probe and the sample, the AFM can be run in fluid, enabling the investigation of cells (and other biological samples in general) in their natural environment and eventually characterize (quantify) their natural response to specific stimuli⁴².

In recent years it has gained increasing importance in the mechanobiology field as an instrument with versatile application modes that allow high resolution morphological imaging (i.e., surface topography) and characterisation of

mechanical properties (e.g., stiffness or viscoelasticity) of biological samples, also simultaneously^{43–45}.

This system can be operated in different modes depending on which structural and/or mechanical parameters of the measured samples are to be characterised. In the scanning mode, the cantilever deflection is kept constant through a feedback circuit while it is moved over the sample surface, recording the pointwise z-piezo displacement Z_p , the surface morphology of the sample can be reconstructed as $Z_p = Z_p(x,y)$. This standard imaging application is e.g. widely used to image the nanotopography of cells⁴⁶, biomaterials and native ECMs⁴⁷.

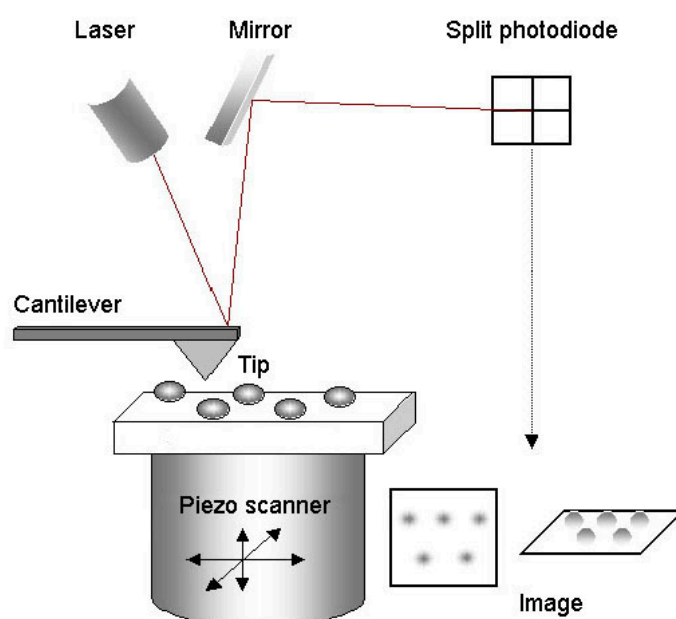


Figure 5. Schematic representation of the AFM scanning principles.

Keeping fixed the sample position it is also possible to apply forces by pushing the (colloidal or pyramidal) probe against the sample (Figure 6). Measuring the deflection of the cantilever through the extent of laser dislocation, the upward force acting on the tip can be calculated, the sample deformation can be measured and then mechanical properties of the sample can be determined. The nanomechanical analysis of soft biological samples (such as cells and many tissues, or extracellular matrices) with AFM is still to-date not straightforward. Attention must be drawn to the choice of the right experimental conditions (like the probe shape⁴⁵), for the application of contact mechanics models^{48,49} and also in the data analysis^{45,50}.

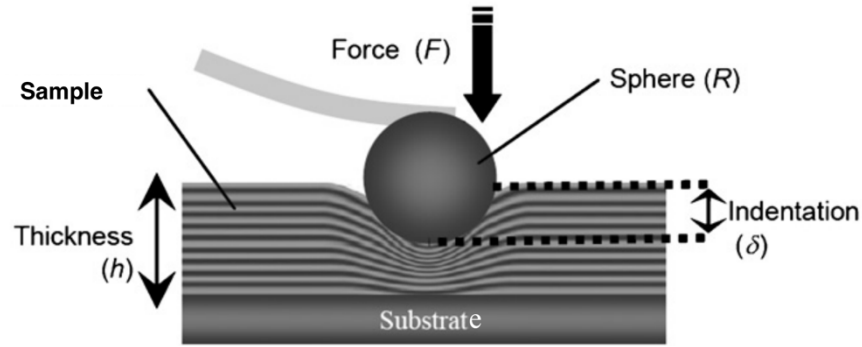


Figure 6. Schematic of the indentation of a sample by colloidal probe tip for mechanical measurement. Image adapted from Schneider et al⁵¹.

Instead of applying forces to the cell/sample with the AFM probe to deform it in order to analyse the mechanical properties, the AFM can also be used to measure cellular adhesion forces¹¹ (e.g., integrin/ligand binding). The common approach is to use a single cell, attached to tipless cantilever (Figure 7), as a probe, or to use a suitable functionalized probe, and bring it gently in contact with the substrate/cell of interest^{52,53}. In this case, after a suitable time of contact to enable the desired interaction, the probe is then retracted in order to break the generated bonds and the downward deflection (representing the force needed to break those bonds) is recorded.

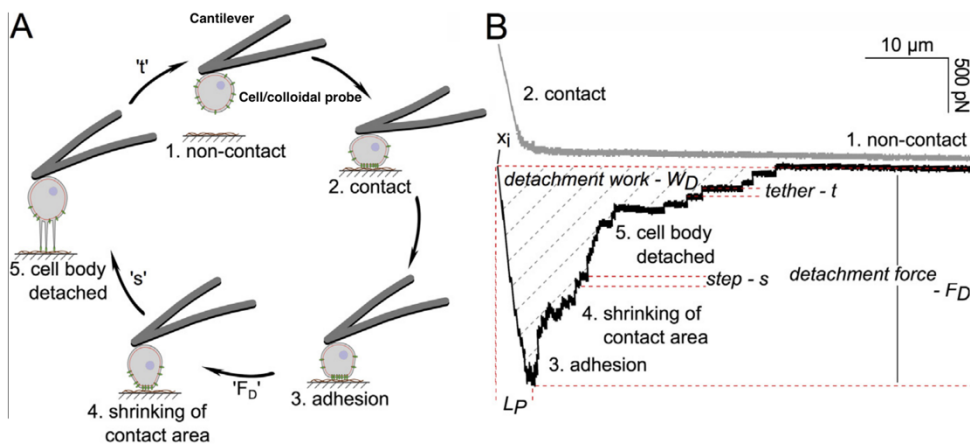


Figure 7. Schematic illustration of a single cell force spectroscopy experiment (A) and of the adhesion events detected (B). Image adapted from Friedrichs et al⁵⁴.

1.6 PhD project and objectives

Given the complex relation between cells and the microenvironment and its importance for the tissue's development and survival, the focus of my PhD project has been the characterization of cell adhesion capability in dependency specific environmental properties by means of Atomic Force Microscopy (AFM) techniques. Starting from the results obtained during the timespan of my Master Thesis, in my PhD I have completed the development of the protocol for the fabrication and calibration of very large atomic force microscopy colloidal probes (CPs)⁵⁵, in order to have a reliable scaffold for both, the functionalization of the large contact surface in the field of AFM force spectroscopy, and for testing the biomechanical markers in cells and ECMs that cover dimensions also on the micrometre scales (Chapter 3). Moreover, large CPs allow averaging over the length scale typical of few μm^2 , providing a more reliable readout.

The developed protocol was used to create a novel nanotopographical colloidal probes (nt-CPs) with the purpose to characterize, through adhesive force spectroscopy, the early mechanotransductive events of cells in relation to different morphologies⁵⁶ of the microenvironment. The created CPs have been morphologically functionalized with a nanostructured cluster-assembled thin film made of biocompatible Zirconium-Oxide (Zirconia, ns-ZrO_2) nanoparticles.

The thin film was fabricated exploiting Supersonic Cluster Beam Deposition (SCBD) apparatus^{57–59}, which allows the production of nanostructured surfaces (see Figure 12,15) characterized by a disordered topography that resembles that of the ECM. This disordered nanotopography is accurately controllable and reproducible in terms of *roughness, cluster size distribution and porosity*⁶⁰.

This work has proved to be efficient, providing on the one side a robust and reliable protocol which gave us the opportunity to characterize the impact of subtle nanoscale differences, and on the other side a solid base for the analysis of the adhesive behaviour of single cells with piconewton sensitivity (see Chapter 4).

Along with a continuous improvement and optimization of the procedure, the aforementioned protocol has been applied also for the investigation of the involvement of the pericellular brush layer (the glycocalyx⁶¹) in the cellular ability to perceive and react to different nanotopographies (see Chapter 5). Indeed, it has been recently proposed that the glycocalyx has an active role in driving the integrin clustering⁶² and membrane bending⁶³, impacting the adhesive capability of cells.

The great versatility of the AFM, combined with the developed protocols involving the use of colloidal probes, allowed to study the adhesive response on the nanoscale of the cells cultured on peculiar substrates. This paved the way for a better understanding of the biophysical cues that trigger the origin of mechanotransductive events, which in turn is essential to achieve the real reproducibility of the phenomenon and its exploitation in practical applications.

In parallel with this activity, I have contributed to scientific collaborations with national (Istituto dei Tumori, Milan) and international (Department of Biophysical Microstructures at the Institute of Nuclear Physics, Polish Academy of Science (IFJ PAN, Kraków, Poland)) groups.

The collaboration with IFJ PAN was focused on the investigation of the mechanical modification of prostate cancer cells in reaction of specific anti-cancer microtubule-targeting drugs. The purpose of this study was to exploit the mechanical modifications of these cells as a fingerprint for their ability to survive in different chemical microenvironment, and characterizing their drug resistance⁶⁴.

The collaboration with Istituto dei Tumori aimed to study the influence of cellular microenvironment on the progression of peritoneal metastases (PM) derived from colon-rectal carcinoma (CRC). Specifically, I performed mechanical measurements at the microscale of ECMs derived from human healthy peritoneal tissues and affected by CRC tumour. The aim of this research was to exploit the knowledge of the reciprocal influence between cells and ECM and develop a new scaffold for the *in vitro* culturing of PM, providing a new model for the investigation of tumour invasiveness and for test of new anti-cancer drugs.

1.7 Thesis outline

Section 1. Protocol development and characterization of the early adhesion events at the cell-microenvironment interface

- In Chapter 3, the AFM colloidal probes are presented as suitable and versatile tools for the investigation of the cell-microenvironment interactions: from nano/micro-mechanics to force spectroscopy, together with all the criticisms and possible issues in the fabrication-calibration procedure of such probes⁵⁵.
- In Chapter 4, a brand new nanotopographical colloidal probes are used to characterize the early mechanotransductive events at the cell-membrane level resulting from different morphological stimuli⁵⁶.
- In Chapter 5, the structure of the glycocalyx, a pericellular sugar brush, and its involvement in the cellular ability to perceive and react to different nanotopographical features is dissected.

Section 2. Study of mechanobiological implication in cancer diseases

- In Chapter 6, the cytoskeletal rearrangement, and consequent modification in mechanical properties, as a cellular ability to react to micro-environmental changes is discussed. In particular, the biomechanical modification of prostate cancer cells as mechanism to develop resistance against microtubule-targeting drugs is presented⁶⁴.
- In Chapter 7, the importance of the ECM properties in the development of peritoneal metastasis (PM) is discussed. In particular, it is shown how the fine characterization of the physico-chemical properties of the ECM could be exploited to build a new scaffold to engineer *in vitro* PM.
- Conclusion and perspective, the obtained main results are briefly summarized, along with a discussion about potential improvement.

Appendix.

- In Appendix A, the problem of the finite-thickness effect in the study of the cell mechanics is introduced⁶⁵, in particular the need to characterize accurately the cell culture substrate and others related issues. Then some possible optimization procedures are presented.
- In Appendix B, the limits of the contact-mechanics Hertz Model⁴⁸ are discussed and a possible overcoming of these limits through Sneddon approximated corrections⁶⁶ is presented.
- In appendix C, a list of values of the effective correction parameter β (see Eq of the thermal noise, section 2.4) is presented as function of the reduced mass and reduced gyration radius (for information see Chapter 3).

Chapter 2

Materials and Methods

2.1 Atomic Force microscopy set-up.

All the AFM experiments, from scanning to force spectroscopy measurements, has been performed using a Bioscope Catalyst (Bruker). High sensitivity of the system, even in “noisy” conditions like working in fluid a fixed temperature, was obtained by means of both an active anti-vibration base (DVIA-T45, Daeil Systems) and an acoustic enclosure for the AFM (Schaefer Italy). During the AFM measurements, the temperature of the medium was maintained at 37°C using a perfusion stage incubator (PSI, Bruker) and a temperature controller (Lakeshore 331, Ohio, USA).

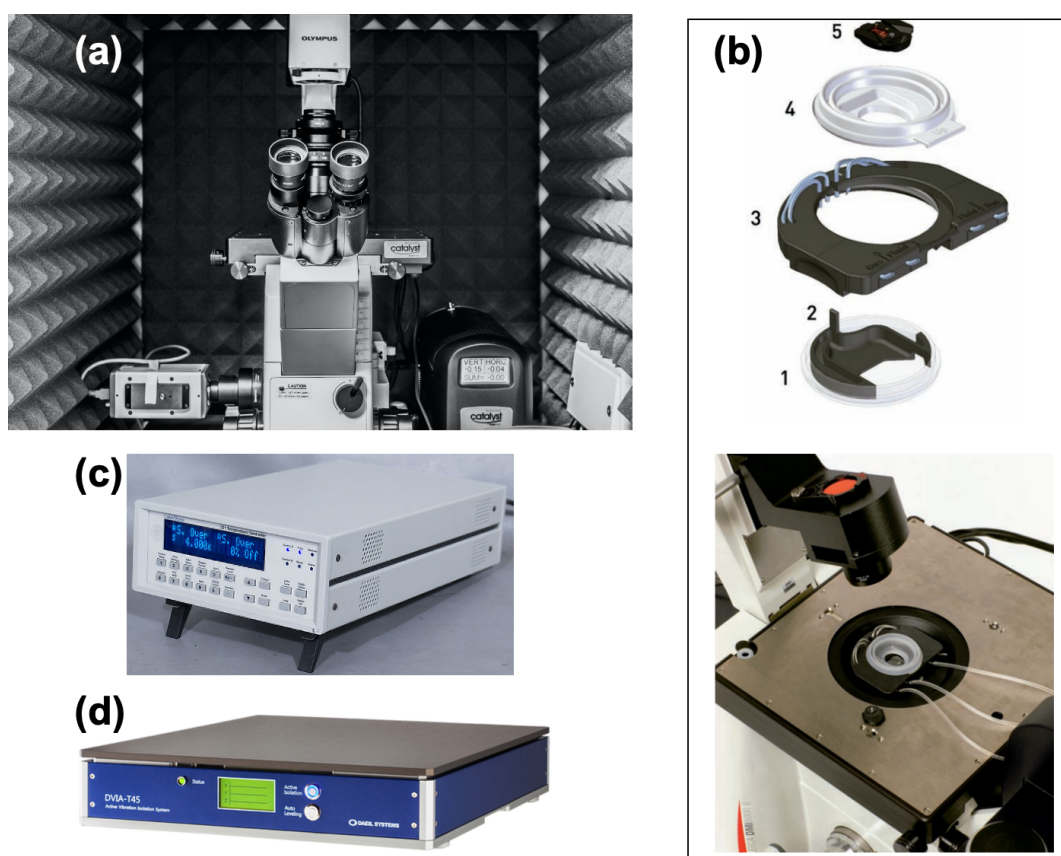


Figure 8. Picture of the single instruments used for the experimental activities. (a) AFM Catalyst inside of the acoustic enclosure (www.marcoferrari.studio/dip-fisica-unimi). (b) Scheme of the PSI components and its allocation inside of the AFM (Bruker, Application Note #131). (c) Temperature Controller 331 (www.lakeshore.com). (d) Active isolation system DVIA-T45 (www.daeilsys.com).

2.2 Miro (Microscope Registration and Overlay).

The software integrates AFM imaging with optical microscopy, automatically importing and rescaling AFM images into the optical microscope ones. This software can be used to direct the location of the AFM imaging and force measurements.

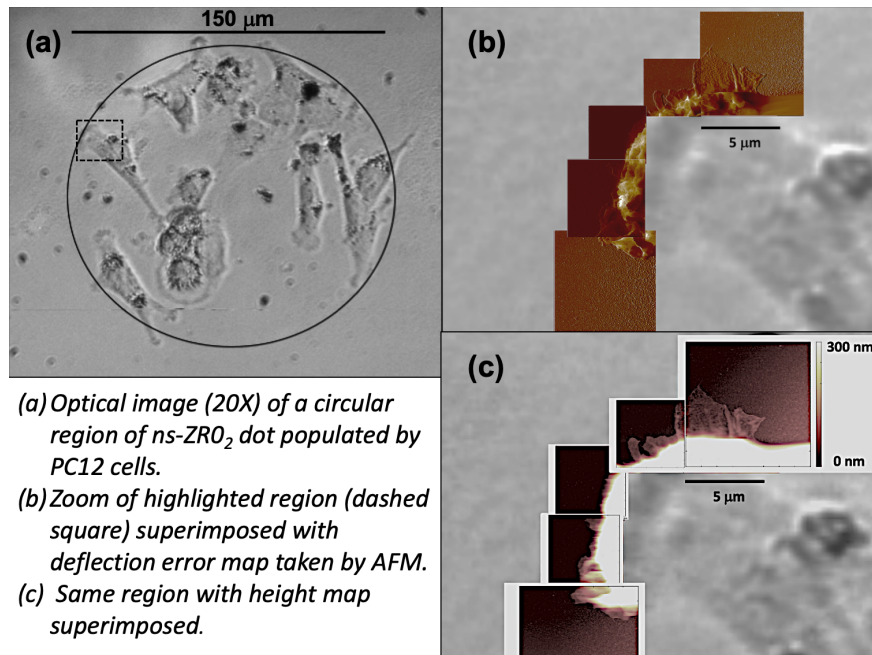


Figure 9. Examples of the overlapping images between optical and AFM images. Image adapted from Schulte et al³¹.

2.3 Fabrication of the CPs

The CPs are fabricated using a custom established protocol described in Refs^{55,67}. After mounting the probe into the AFM tip-holder, the beads were captured using the XY motorized stage of the AFM. The AFM is integrated in an optical inverted microscope (Olympus X71), which help locating and capturing the beads. Depending on the size of the desired microspheres, environmental capillary adhesion or Vaseline is exploited to temporarily attach the sphere to the tipless cantilever. Then the probe is transferred into a high-temperature oven (PXZ series, Fuji Electronics) already at the target temperature and heated for two hours. The temperature is chosen slightly below the softening point of the sphere materials, which is qualitatively defined as the temperature at which a solid object begins to collapse under its own weight. The temperature used were T= 780 °C for borosilicate spheres and T=700 °C for soda-lime spheres.

2.4 Probe Calibration (force constant and radius)

The probes are calibrated using the Thermal Noise method^{68,69} (see Chapter 3 for details). The standard formula for the evaluation of the cantilever stiffness is

$$k = \frac{\hat{\alpha}}{\chi^2} \frac{k_B T}{(\hat{s} S_z)^2 P_V} = \beta_0 \frac{k_B T}{(\hat{s} S_z)^2 P_V}$$

Where k_B is the Boltzmann's constant, T is temperature, S_z is the deflection sensitivity and P_V is the area below the first resonant peak in the power spectral density (PSD) of the raw deflection signal (in units of V^2/Hz). The factor $\hat{\alpha}$ takes into account the vibrational energy stored in the first normal mode of the cantilever, χ compensate the difference between the static and dynamic deflection sensitivity, and $\hat{s}$ corrects the torque effects, emerging from the tip height and the probe tilt, on the measured deflection sensitivity. Usually, with standard sharp probes, with rectangular cantilever and with the loading point at the very end of the cantilever, the parameters have fixed values $\hat{\alpha} = 0.971$ and $\chi = 1.09$. When a microsphere with a mass comparable to the one of the cantilever is attached, or the loading point is shifted (due to particular geometrical configurations) specific corrections to the thermal noise formula have to be applied^{55,70–72} (see Chapter 3).

We implemented a LabView software (Figure 10), connected to a Signal Access Module (SAM, Intermodulation Product) and an acquisition board (BNC-2110, National Instruments). The implemented set up makes possible to work directly to the raw deflection signal of the photodiode and implement the geometrical corrections needed for specific configurations of the probes created. To characterize the CP radius we used a custom protocols^{55,67} depending on the size of the attached sphere: for small spheres ($R < 10 \mu\text{m}$) the probe is used to scan a spiked calibration grating (TGT1, Tips Nano), the resulting image will be tens/hundreds of independent replicas of the sphere apical region. By fitting the spherical cap model to each of these replicas it is possible to determine the radius. For bigger spheres ($R \sim 20/30 \mu\text{m}$) we used calibrated images taken with the optical microscope.

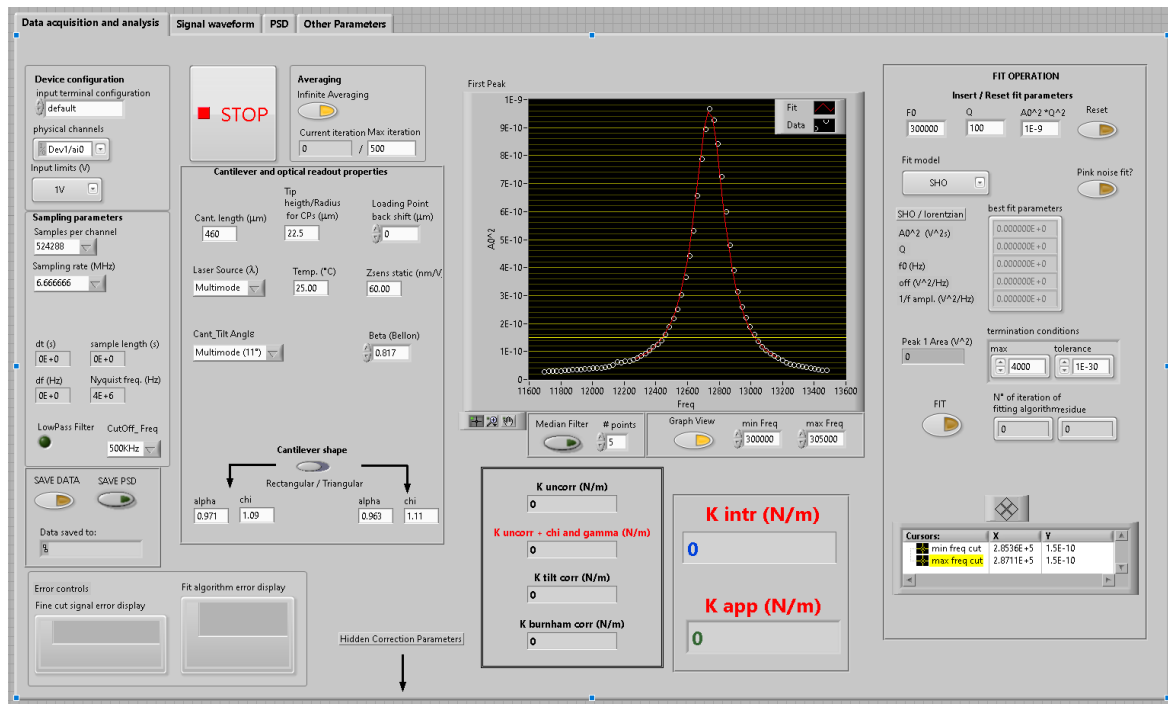


Figure 10. Front panel of the LabView software implemented for the application of the thermal noise method and the relative fine corrections.

2.5 Force spectroscopy measurements

Knowing the force constant of the cantilever is possible to measure the interaction forces between the sample and the probe. The typical output of a force measurement is the so-called Force Curve (FC) that couple the force felt/exerted by the tip of probe with the tip-sample distance. Figure 11 shows the movement (approaching and retracting) of the probe during the force spectroscopy experiment:

- The probe is approaching the surface of the sample and no interaction force is measured between the tip and the sample.
- The interaction forces (Van der Waals, capillary forces, DLVO, magnetic) causes the tip to jump into contact with the sample.
- The probe applies forces to the sample until a pre-set force threshold is reached and the (possible) deformation of the sample is recorded.
- The probe is then retracted from the sample and the adhesion force is recorded
- The cantilever is again away enough from the sample and no forces is measured.

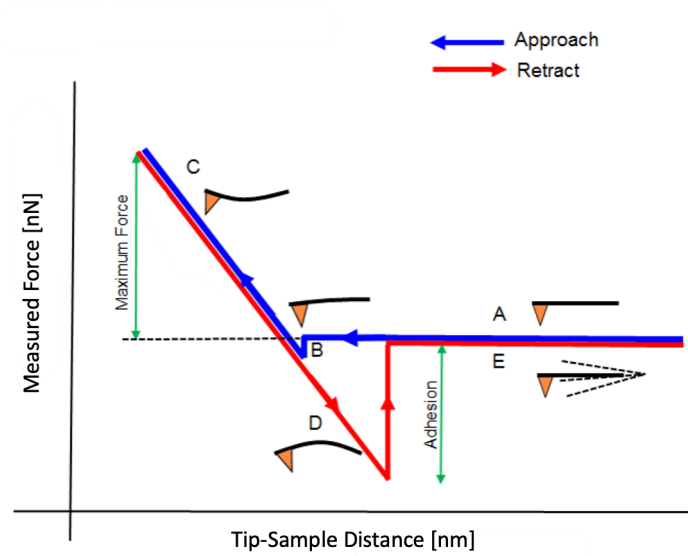


Figure 11. Plot of approach (blue) and retract (red) curve for a standard force spectroscopy measurement.

2.6 Adhesion force analysis

The values of several parameters were calculated for the analysis of the cell-probe detachment mechanisms (Figure 7B). In particular, we inferred the maximum cell-probe adhesion force F_a , defined as the maximum force needed to start the detachment of the probe from the cell, with respect to the zero level of the baseline. The maximum detachment force F_a depends on different properties of the cell, such as overall rigidity, cortex tension, cell shape, single binding strength and spacing. The work of detachment W required to separate the nt-CP from the cell, W describes the energy dissipated during the detachment and is calculated integrating the area between zero level and the profile of the force versus distance curve. Moreover, we extracted the number of $N_{j,t}$ and strength $F_{j,t}$ of the single unbinding events, defined as the difference in force measured before and after the detaching event, of every FC measured (for both, jumps j , and tethers t). The unbinding events must be identified in the retraction section of the FCs: we exploited the numerical derivative of the curves with respect to the tip-sample distance to detect the location of the events (see Figure S4 at pg 72).

2.7 Deposition of the ns-ZrO₂ film on the colloidal probes

The functionalization of the CP surface has been done exploiting an SCBD apparatus^{57,58} (see Figure below). Partially oxidised zirconia clusters are produced in the source trough an arc discharge and then extracted, together with a carrier gas (like Ar or He) into the vacuum of expansion chamber to form a seeded supersonic beam. Several aerodynamic lenses focused the beam that eventually enter inside of the deposition chamber where they directly intercept the target sample producing a cluster-assembled thin film⁶⁰. The roughness of the deposited film, defined as the standard deviation of the surface height values, evolves with the film thickness according to the power law $r_q \sim h^b$, where $b=0.35$ approximately (details about the roughness-thickness relation can be found in the suppl. Information pg 69-71).

Supersonic Cluster Beam Deposition (SCBD) Apparatus

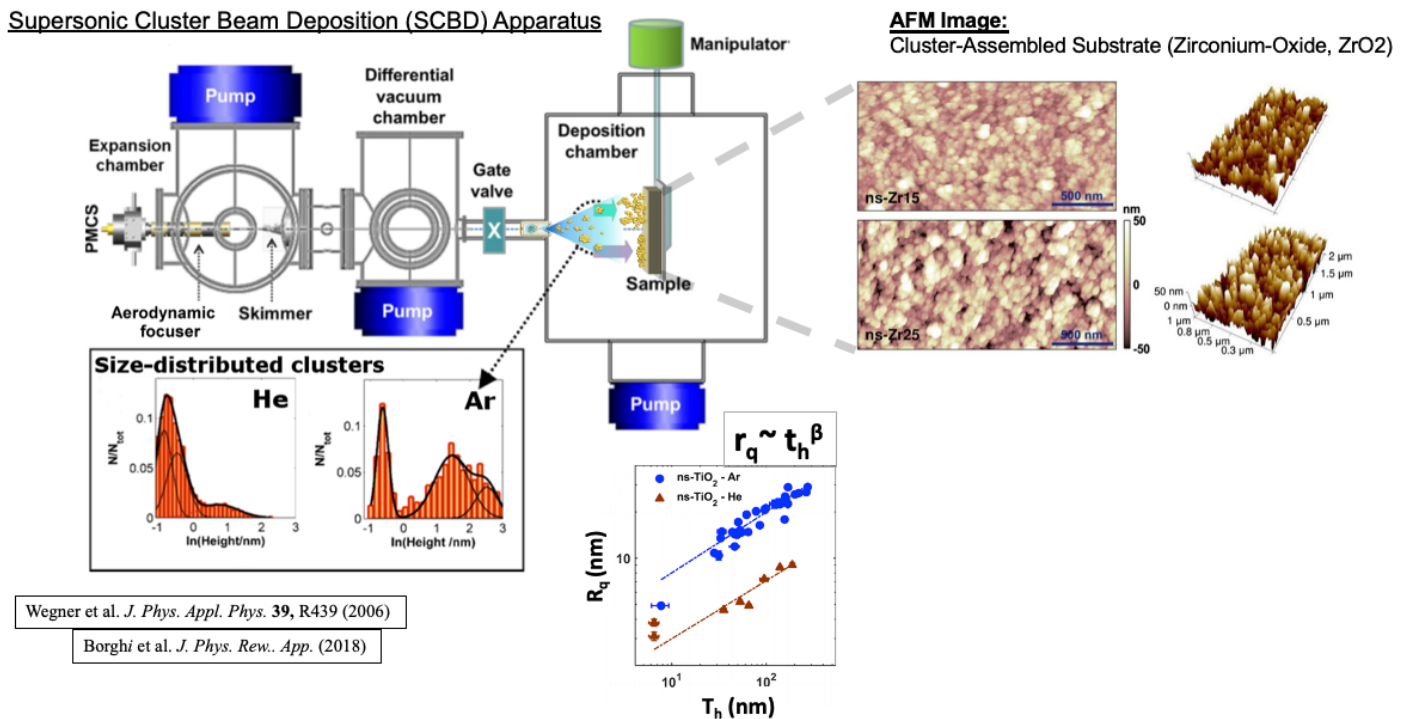


Figure 12. Supersonic cluster beam deposition (SCBD) apparatus. In the inset, two typical size distribution of Titania clusters produced using He and Ar as carrier gases are shown. Image adapted from Schulte et al.³². The graph demonstrates the precise roughness-thickness relation that correlates these two properties of the cluster-assembled thin film.

2.8 Cell culture

For routine cell culture (sub-culturing every 2-3 days), the cells were kept in an incubator (Galaxy S, RS Biotech) at 37°C and 5% CO₂, in RPMI-1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, 2 mM L-Glutamine, 10 mM HEPES, 100 units/mL penicillin, 1 mM pyruvic acid, and 100 µg/mL streptomycin (all reagents from Sigma Aldrich, if not stated otherwise). For the force AFM experiments, the cells were detached from the cell culture flasks with trypsin/EDTA solution, counted with an improved Neubauer chamber, and plated in low concentration of 4.000 cells/cm² (to guarantee the presence of single separated cells) on Ø 40 mm glass-bottom dishes for cell culture (Willco Wells) the day before the experiment. Phenol-red free solutions were used for the experiments, since this molecule was found to be harmful for the AFM tip holder. Directly before the cell plating, the Ø 40 mm glass-bottom dishes were coated with PLL (incubation with a 0.1% PLL solution for 30 min at RT, followed by several washing steps with PBS) and sterilised with UV light for 10 min. After cell plating, the cells were kept overnight in the incubator to guarantee good cell attachment before the AFM experiments.

Section 1

Chapter 3

Colloidal probes for mechanobiological investigation: protocol for the fabrication and calibration

The crosstalk between cells and ECM is essential for regulation of growth, development and maintenance of multicellular systems and tissues. Due the biophysical nature of some of these interactions between the single components (cells and ECM), like forces, stress, pressure and morphological interactions, a physical approach is essential to investigate those properties that characterize such complex phenomena.

Since AFM could reaches a spatial resolution down to nanometre scale and detect force down to few piconewtons, it has been widely exploited to investigate weak biological interactions^{73,74}, characterize 3-dimensional configuration of complex biomolecules^{75,76} or characterize the surface molecules of cell membranes^{77,78}.

However, several mechanobiological processes of crucial importance in the cell's life and tissues survival^{79–81} involve hundreds of molecules and proteins in their cascade reactions, and their effects are evident only on much larger scale, like cell differentiation, migration and proliferation⁸².

To have a physical readout of this changes, involving so many nanoscale players, micro-scale averaged observations (such as the Young's Modulus, the viscoelastic properties or mean cellular adhesion) needs to be acquired and, to this purpose, the standard pyramidal AFM tips may not be recommended⁴⁵: the nanometric curvature radius provides an interaction area usually too small and as a consequence it results in the application of high stresses. The not well characterized geometry provides furthermore results that are hard to interpret through the standard contact-mechanics models^{48,49}, and eventually data that potentially represents too localized properties.

Colloidal probes (CPs) mount a spherical and micrometric particle that is attached to the free end of the cantilever and replaces the sharp tip⁶⁷. This simple switching of geometry offers several advantages: the larger contact area allows detecting spatially averaged information of the collective modification the cellular architecture⁸³ or the ECM structure^{15,16}. The simple geometry provides an easier interpretation of data in terms of theoretical models and stresses are reduced limiting the damage of the samples.

CPs are purchased or fabricated in house with spheres of different dimensions, masses and materials. Large CPs are useful to study systems where the local inhomogeneities and variations could cover several tens of microns, offering the

chance to go beyond the micrometric characterization and moving towards the macroscopic properties of the specimens.

However, the protocols for fabrication, and more important the calibration of such probes, present practical criticisms and theoretical difficulties that must be taken into account. Here below my work concerning the large CPs fabrication and calibration issues is reported⁵⁵.



Large colloidal probes for atomic force microscopy: Fabrication and calibration issues

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Abstract

Atomic force microscopy (AFM) is a powerful tool to investigate interaction forces at the micro and nanoscale. Cantilever stiffness, dimensions and geometry of the tip can be chosen according to the requirements of the specific application, in terms of spatial resolution and force sensitivity. Colloidal probes (CPs), obtained by attaching a spherical particle to a tipless (TL) cantilever, offer several advantages for accurate force measurements: tunable and well-characterisable radius; higher averaging capabilities (at the expense of spatial resolution) and sensitivity to weak interactions; a well-defined interaction geometry (sphere on flat), which allows accurate and reliable data fitting by means of analytical models. The dynamics of standard AFM probes has been widely investigated, and protocols have been developed for the calibration of the cantilever spring constant. Nevertheless, the dynamics of CPs, and in particular of large CPs, with radius well above 10 μm and mass comparable, or larger, than the cantilever mass, is at present still poorly characterized. Here we describe the fabrication and calibration of (large) CPs. We describe and discuss the peculiar dynamical behaviour of CPs, and present an alternative protocol for the accurate calibration of the spring constant.

KEYWORDS

atomic force microscopy, calibration, colloidal probes, deflection sensitivity (invOLS), spring constant

1 | INTRODUCTION

Atomic force microscopy (AFM) is a powerful tool to investigate molecular interactions at bio-interfaces, as well as their mechanical properties, with nanometric spatial resolution and 1 to 10 pN force resolution.^{1,2}

The force sensing capability of AFM is provided by the use of probes consisting of an elastic microlever (cantilever) with an interacting tip at his apex. A deflection Δz of the cantilever as small as 1/10 of a nanometre can be detected by means of an optical beam deflection,³ and translated into a tip-sample force F according to Hook's law: $F = k_{\text{app}}\Delta z$. The value of the spring constant of the cantilever can be selected in the range 0.01 to 100 N/m. Here an apparent spring constant k_{app} is used because due to the cantilever tilt θ (usually $\theta = 10^\circ$ – 15° , see Figure 1l) both the force F and the measured

deflection Δz are perpendicular to the sample surface, rather than to the cantilever axis. The intrinsic spring constant k in turn relates the deflection and the force normal to the cantilever axis.

While sharp AFM tips are mandatory when high spatial resolution is necessary, large spherical probes, also known as colloidal probes (CPs),^{4–7} can be advantageous when one or more of the following requirements are important: a well-defined interaction geometry (sphere on flat) and reduced stress and strain in mechanical tests; a good spatial averaging; a broader and smoother surface for probe functionalization. The well-defined interaction geometry makes CPs a suitable tools for the investigation of mechanical properties of soft or biological samples^{8–11}; the larger contact area allows to detect weak interaction forces^{12,13} and generally CPs provide an easier interpretation of data in terms of theoretical models.^{8,11,14,15}

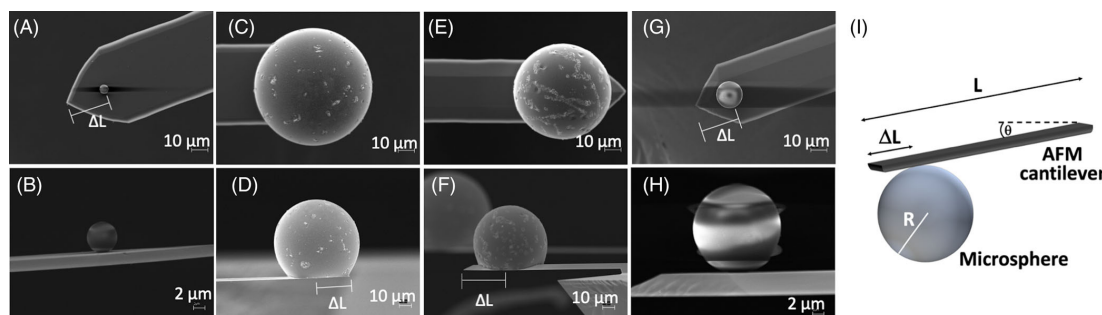


FIGURE 1 SEM images of some CPs fabricated (see Table 1). A, CM1, borosilicate; B, CM1 at higher magnification; C and D, CM4, soda-lime; E and F, TM4, soda-lime; G and H, TM3, borosilicate. I, Schematics of a CP, with the relevant parameters highlighted

The obvious price to pay when using CPs is the dramatic reduction of the spatial resolution, although the optimal spatial resolution clearly depends on the typical length scale of the physical phenomena under investigation. For example, as long as one is interested in characterizing the average rigidity of mesoscopic cellular regions (cell body vs periphery), tissues or extracellular matrices, CPs provide enough spatial resolution despite their micrometric radii.^{9,16–18}

Large CPs, where the radius R can be far larger than $10\ \mu\text{m}$, are very useful to study extremely weak interaction forces, or the mechanics of samples over a wide range of contact area, averaging nanoscale inhomogeneities and local variations of the sample (eg, in the case of the mechanical response of tissues). Large CPs allow to better compare the results obtained by AFM to those obtained by other indenters^{19–22} and in principle to bridge nano- and microscale mechanics to macroscale mechanics.^{23,24}

Despite the many advantages of CPs, some issues related to the calibration of these probes must be addressed in order to exploit their full potential, especially in the case of large CPs.²⁵ In particular, calibration of AFM probes require the determination of the intrinsic and apparent spring constants k and k_{app} and of the static deflection sensitivity S_z (in nm/V), also known as inverse optical lever sensitivity (invOLS), which converts the raw output ΔV of the photodetector (in Volts) into a deflection Δz (in nm) normal to the sample surface: $\Delta z = S_z \Delta V$.²⁶ The force F is then calculated as:

$$F = k_{\text{app}} S_z \Delta V \quad (1)$$

The calibration of both k (k_{app}) and S_z for CPs presents issues, which become more important as the probe radius increases.

The issues related to the calibration of CPs arise when the radius and mass of the attached sphere are comparable to the length and mass of the cantilever, respectively. In order to describe the effect of the attached sphere on the cantilever dynamics, two dimensionless parameters can be introduced (see also Section 2)²⁷: the reduced mass $\tilde{m}$ is the ratio of the mass of the sphere m_s to the mass of the cantilever m_c ; the reduced gyration radius of the sphere $\tilde{r}$ is proportional to the ratio of the radius R of the sphere to the length L of the cantilever.

The basic cantilever calibration parameters, such as the resonant frequency ν_0 , the quality factor Q , the deflection sensitivity S_z , the spring constant k , depend on $\tilde{m}$ and $\tilde{r}$.

In this report, we discuss some critical aspects of CP production and calibration, and present methods to minimize their impact on the accuracy of AFM measurements.

2 | METHODS

2.1 | Fabrication of the CPs

We have produced eight monolithic glass probes following an established custom protocol²⁸ (see Table 1).

We used borosilicate glass spheres (Thermo Fisher Scientific, series 9000 glass particles) with radius $R = 2, 5, 10\ \mu\text{m}$ (probes CM1–3 and TM1–3), and soda-lime spheres (Polyscience, glass beads Amine, N° 23 584–10) with nominal radius $R = 30\ \mu\text{m}$ (probes CM4 and TM4); we could not find on the market borosilicate glass spheres with radius above $10\ \mu\text{m}$. These latter spheres are of far better quality than soda-lime glass spheres, in terms of surface roughness and morphology. Soda-lime glass spheres present extended surface defects (including craters, protrusions; see Figure 1C–F).

In order to span a wide range of reduced masses and gyration radii, we used two different kinds of silicon tipless (TL) cantilevers: tapping mode (TM; TL-NCH-50, Nanosensor) and contact mode (CM; TL-CONT, Nanosensor).

After mounting the cantilever into the AFM (Bioscope Catalyst, Bruker) tip holder, the beads were captured using the XY motorized stage of the AFM. The AFM is integrated in an optical inverted microscope (Olympus X71), which helps locating and capturing the beads. Depending on the size of the spheres, environmental capillary adhesion or Vaseline is exploited to attach the sphere to the tipless cantilever. The cantilevers with the attached beads are then transferred into a high-temperature oven (PXZ series, Fuji Electrics) already at the target temperature, and heated for 2 hours. We used different temperatures for the different materials of the sphere: 780°C for borosilicate

TABLE 1 Properties of the CPs fabricated for this study

Probe	Bead radius (nm)	Cantilever length (μm)	Cantilever width (μm)	Cantilever thickness (μm)	$\tilde{m}$	$\tilde{r}$	$\Delta L/L$	β
CM1	2189	450	50	2	9.76×10^{-4}	5.7×10^{-3}	0.048	0.817
CM2	4462	450	50	2	8.18×10^{-3}	1.16×10^{-2}	0.053	0.826
CM3	7696	450	50	2	4.24×10^{-2}	2.02×10^{-2}	0	0.849
CM4	36 130	125	30	2	7.02	9.5×10^{-2}	0.088	1.018
TM1	1767	125	30	4	1.54×10^{-3}	1.67×10^{-2}	0	0.817
TM2	4292	125	30	4	2.21×10^{-2}	4.06×10^{-2}	0.112	0.836
TM3	7690	125	30	4	1.27×10^{-1}	7.2×10^{-2}	0.175	0.8768
TM4	31 752	125	30	4	9.62	3×10^{-1}	0.501	1.111

Note: These probes were fabricated using two different kind of tipless cantilevers: Tapping mode (TM) and contact mode (CM).

and 700°C for soda-lime glass, respectively. These temperatures are slightly below the softening point of the materials, which is qualitatively defined as the temperature at which a solid object begins to collapse under its own weight. After 2 hours, the microsphere is covalently attached to the cantilever; the glass locally melts at the sphere-cantilever interface, forming a neck, which is more or less extended depending on curing time and temperature.

Besides being much smoother than the soda-lime spheres and less dispersed in radius, borosilicate glass spheres present typically a lower deformation after the heating procedure (compare Figure 1B,H and Figure 1D,F). Moreover, the radius of soda-lime glass spheres can differ significantly from the nominal one.

2.2 | Calibration of CPs

2.2.1 | Radius

1. $R \leq 10 \mu\text{m}$. The characterization of the radius of the CP is obtained by an AFM reverse imaging of the probe,^{5,29,30} as described in Reference 28. The CP is used to scan a spiked calibration grating³¹ (TGT1, Tips Nano). Spikes are separated by approximately 3 μm , therefore a 20 $\mu\text{m} \times 20 \mu\text{m}$ image typically contains a hundred independent replicas of the probe apical region. Upon collecting several such images, the volume, projected area and height of each replica are measured. By fitting a spherical cap model to the experimental data, in the form, for example, of a volume vs height curve: $V = \frac{\pi}{3}h^2(3R-h)$, it is possible to determine the value of the sphere radius R , with an accuracy as good as 1%. While the spherical cap model adopted here allows using all data points from the three-dimensional reverse AFM image, deconvolution methods³² are limited by the fact that only so-called certainty regions can be determined, corresponding to those points where the tip apex is effectively in contact with the surface of the calibration sample, and analytical formulas are limited to the study of single profiles.³³ Since the spikes are relatively close, the sphere can penetrate through them by only a few hundred nanometres, at best. For this reason, as

long as gratings with sparser, and taller spikes are not available, the application of this method is limited to relatively small spheres.

2. $R = 30 \mu\text{m}$. CPs with $R > 10 \mu\text{m}$ are too large for the calibration by reverse AFM imaging. The radius of these probes is measured optically, using a calibrated metallographic optical microscope equipped with a calibrated 50 $\times$ objective (AXIO, Zeiss). The error associated to the optical characterization of the probe radius is 1 μm (standard deviation). Since the nominal radius of the soda-lime spheres attached to the tipless cantilevers is $R = 30 \mu\text{m}$, the relative error of its characterization is approximately 3%. Alternatively, it is possible to follow an AFM-based reverse imaging approach based on single profiles, by scanning the large CP across a calibration grating with well separated ($>10 \mu\text{m}$) and tall ($>1.5 \mu\text{m}$) sharp steps. The radius R can be obtained by fitting an arc of circumference to the AFM profile. For a better characterization of R , we suggest to rotate the calibration grating in order to obtain different nominally equivalent profiles of the sphere, and average the different values.

2.2.2 | Nominal and reduced masses

We calculated the mass of the spheres m_s using the nominal densities of borosilicate glass $\rho_{\text{BG}} = 2.23 \text{ g/cm}^3$ and soda-lime glass $\rho_{\text{SLG}} = 2.52 \text{ g/cm}^3$ and the calibrated radius R as: $m_s = \rho_{\text{BG/SLG}} \frac{4}{3} \pi R^3$.

The mass of the cantilever m_c was evaluated assuming a rectangular section, using the nominal dimensions (length L , width w , thickness t) and the silicon density $\rho_{\text{Si}} = 2.33 \text{ g/cm}^3$ as: $m_c = \rho_{\text{Si}} Lwt$. We ignored the fact that some cantilevers have a trapezoidal cross section.

The total mass m_p of the probe was evaluated by summing the cantilever and the sphere masses: $m_p = m_c + m_s$.

The reduced mass $\tilde{m}$ and the reduced gyration radius $\tilde{r}$ of the sphere are defined as²⁷:

$$\tilde{m} = \frac{m_s}{m_c}, \tilde{r} = \sqrt{\frac{7}{5} \frac{R}{L}} \quad (2)$$

We evaluated the effective mass $\tilde{m}^*$ of the cantilever, before and after the attachment of the sphere, as the effective mass of a SHO,

using the standard formula: $m^* = k/\omega_0^2$, where k is the spring constant and ω_0 is the angular frequency of the oscillator.

2.2.3 | Deflection sensitivity

The calibration of S_z is performed by engaging the AFM tip on a very rigid surface and collecting a series of raw deflection vs z -piezo-displacement curves. Assuming that neither the tip nor the surface is deformed, the deflection sensitivity S_z is calculated as the inverse of the slope of the curves.²⁶ This sensitivity is referred to as the static deflection sensitivity, and differs from the dynamic deflection sensitivity $S_{z,dyn}$ ^{34,35}; the latter sensitivity must be used when the cantilever oscillates instead of being statically bent due to an end loading, as in the application of the thermal noise method for the calibration of the cantilever spring constant.

It must be noted that S_z strongly depends on the loading configuration.^{36–38} In particular, the static sensitivity of a tilted tipless cantilever differs from that of a standard tilted cantilever, with a sharp tip, or a sphere, attached to its end.

In the case of a standard probe, the force is not applied at the cantilever end, but rather at the tip apex, which is some distance apart, therefore a torque is generated. Moreover, because of the tilt angle θ , during the standard operation of an AFM the interaction force is perpendicular to the sample surface, instead of the cantilever axis, while during the thermally driven oscillation of the cantilever the effective driving force is always perpendicular to the cantilever axis. The corresponding cantilever displacement is therefore different from that obtained by the application of the same force at the cantilever end.

Corrections for the deflection sensitivity S_z are necessary, to account for the differences in the loading configuration of tipless and tipped cantilevers in the different experimental conditions. Correction factors are therefore used to determine both the intrinsic spring constant k using the thermal noise method, and the apparent spring constant k_{app} used to rescale the raw deflection signal into a force (Equation 1). While the latter correction factor is discussed in Section 2.2.5, the first factor is introduced in the following paragraphs.

As mentioned, during the thermal noise calibration, the cantilever is driven by a distributed thermal force, which can be considered as effectively located at the cantilever end, where it acts perpendicularly to the cantilever axis. Assuming that the deflection sensitivity depends on the parameters θ , R , L , ΔL introduced in Figure 11, the correction factor is therefore $\hat{s} = S_z^{0,0,L,0}/S_z^{\theta,R,L,\Delta L}$, where $S_z^{\theta,R,L,\Delta L}$ represents the deflection sensitivity measured in a particular loading configuration and $S_z^{0,0,L,0}$ is the sensitivity in the thermal loading configuration. According to Reference 37 (Equation 3 and other equations therein):

$$1/\hat{s}^2 = \left(1 - \frac{\Delta L}{L}\right)^2 \left[\frac{1 - \frac{3}{2} \frac{R/L}{(1 - \frac{\Delta L}{L})} \tan \theta}{1 - 2 \frac{R/L}{(1 - \frac{\Delta L}{L})} \tan \theta} \right]^2 \cos^2(\theta) \quad (3)$$

The $\cos^2(\theta)$ term in Equation (3) accounts for the tilt θ of the cantilever³⁹; the term in square brackets accounts for the torque applied to the cantilever because of the finite height of the tip; the first term $(1 - \Delta L/L)^2$

accounts for the fact that when the static deflection sensitivity is calibrated, the deflection at the loading point, rather than at the end of the cantilever, is measured.^{37,38} A special case of Equation (3) was first proposed by Hutter³⁸ under the hypothesis $\Delta L = 0$; in this case, Equation (3) simplifies to:

$$1/\hat{s}^2 = \left[\frac{1 - \frac{3}{2} \frac{R}{L} \tan(\theta)}{1 - 2 \frac{R}{L} \tan(\theta)} \right]^2 \cos^2(\theta) \quad (4)$$

For standard AFM tips, the radius R of the sphere in Equations (3) and (4) must be replaced by the tip height H .³⁷

2.2.4 | Thermal noise and CPs

Several approaches have been developed to characterize the spring constant of a cantilever,^{6,40–45} among which, the thermal noise method^{40,41,46,47} is based on the equipartition theorem.

Under the assumption that the mass m_t of the tip is negligible with respect to the mass m_c of the cantilever, the probe (cantilever + tip) can be modelled as a point body with effective mass m^* , bound to an ideal spring with stiffness k , in thermal equilibrium with its environment.

Assuming that the tip oscillates only along the direction perpendicular to the cantilever, and being $z_\perp$ its displacement relative to the equilibrium position, its average potential energy is $U = \frac{1}{2}k \langle z_\perp^2 \rangle$, where $\sqrt{\langle z_\perp^2 \rangle}$ is the thermally driven root mean square oscillation amplitude of the free end of the cantilever, where the tip is located, ranging from approximately 5 to 500 pm. According to the equipartition theorem, $U = \frac{1}{2}k_B T$, where k_B is the Boltzmann constant and T is the local absolute temperature. It then follows:

$$1/2 k \langle z_\perp^2 \rangle = 1/2 k_B T \quad (5)$$

If $\langle z_\perp^2 \rangle$ is measured, the cantilever spring constant k is then calculated from Equation (5) as:

$$k = \frac{k_B T}{\langle z_\perp^2 \rangle} \quad (6)$$

When the optical beam deflection method is used to measure the cantilever deflection, with the laser aligned at the end of the cantilever, Equation (6) is replaced by Equation (7)^{37,38,48}:

$$k = \frac{\hat{\alpha} k_B T}{\chi^2 (\hat{S}S_z)^2 P_V} \quad (7)$$

In Equation (7), $P_V = \langle \Delta V^2 \rangle$ is the area below the first resonant peak in the power spectral density (PSD) of the raw deflection signal (in units of V^2/Hz).

The factor $\hat{\alpha}$ in Equation (7) takes into account the fraction of the total oscillation amplitude stored in the first normal mode of the cantilever, when one goes beyond the single harmonic oscillator (SHO)

approximation.⁴⁶ For the first normal mode of a tipless rectangular cantilever, $\hat{\alpha} \equiv \hat{\alpha}_1 = 0.971$.

The factor $\chi^{34,35,48}$ corrects for the differences between the static (which is typically measured) and dynamic deflection sensitivities: $\chi = S_{z,\text{dyn}}/S_z$.^{46,49} Indeed, as pointed out by Butt and Jaschke in their seminal work,⁴⁶ the cantilever inclination, rather than its vertical displacement, is measured using the optical beam deflection apparatus; the slope of the free end of the statically loaded cantilever differs from those of the modal shapes of the oscillating cantilever.^{46,48,50} For the first normal mode of a tipless rectangular cantilever, assuming a negligibly small laser spot aligned at the end of the cantilever, $\chi \equiv \chi_1 = 1.09$. For the general case, the reader is referred to References 37 and 50 for the calculation of χ .

The factor $\hat{s}$ represents the correction to the deflection sensitivity due to the change in the loading condition of the cantilever (Equations 3 and 4).

In principle, the thermal noise method could be applied to higher normal modes, in which case Equation (7) should be rewritten in terms of the parameters $P_{V,i}$, $\hat{\alpha}_i$, χ_i , where the index $i = 1, 2, 3, \dots$ refers to the i -th normal mode. In practice, due to the finite laser spot size, which causes an averaging of different cantilever inclinations across a finite area, the first normal mode has to be preferred³⁵; moreover, the first normal mode typically provides the largest oscillation amplitude, that is, the largest signal to noise ratio.

The factors $\hat{\alpha}$ and $1/\chi^2$ can be merged in a single parameter $\beta = \hat{\alpha}/\chi^2$. For the first normal mode of an ideal rectangular cantilevers, $\beta = \beta_0 = 0.817$.

The implementation of the thermal noise model for AFM cantilevers assumes that the mass of the cantilever is uniformly distributed. For this statement to be valid, the mass of the tip must be negligible compared to the mass of the cantilever. When large spheres are attached at the end of a cantilever, this hypothesis is not always satisfied. Since the mass of the microsphere scales up with the cube of radius R , it can easily reach values comparable to the mass of the cantilever. This typically happens for a radius $R > 10 \mu\text{m}$, for a $200 \mu\text{m}$ long, silicon nitride cantilever, and a glass sphere.

2.2.5 | Loading point and spring constant

For tipless cantilevers, the loading point is located at the free end of the lever. For sharp tips, the loading point corresponds to the location of the tip apex along the cantilever. For CPs, the loading point is located at the sphere apex, that is, approximately a distance ΔL from the cantilever end (Figure 1).

The attachment of the sphere to the tipless cantilever is not expected to change the intrinsic spring constant of the probe. Since the determination of the deflection sensitivity S_z can be critical for CPs (see discussion in Section 3.6), it can be convenient to calibrate the intrinsic spring constant k_{TL} of the tipless cantilever, before the attachment of the sphere, and then correct its value to determine the intrinsic spring constant $k_{\text{TL}}^{\text{LP}}$ of the tipless cantilever at the loading point

of the CP; the latter spring constant can be supposed to be equal to the spring constant of the CP at the loading point, that is, $k_{\text{TL}}^{\text{LP}} = k_{\text{CP}}^{\text{LP}}$.

The spring constant $k_{\text{TL}}^{\text{LP}}$ of the tipless cantilever corresponding to the loading point of the CP is calculated as^{51,52}:

$$k_{\text{TL}}^{\text{LP}} = \left(\frac{L}{L - \Delta L} \right)^3 k_{\text{TL}} \quad (8)$$

In Equation (8), ΔL is the distance of the loading point of the CP from the cantilever free end. In the case of rectangular, axisymmetric and relatively long cantilevers, Equation (8) is valid for arbitrary offset ΔL , not only in the limit $\Delta L/L \ll 1$.⁵²

Alternatively, the thermal noise method can be applied directly to the CP, provided the measured spring constant k_{CP} is corrected first by Equation (8) (to obtain $k_{\text{CP}}^{\text{LP}}$), and then by Equation (11), to take into account the peculiar dynamics of the CP, as described in the Results.

When the optical beam deflection method is used to measure the cantilever deflection, in order to rescale deflections into forces perpendicular to the sample surface, an apparent spring constant k_{app} must be used, instead of the intrinsic spring constant k .³⁷ The relation between the apparent and the intrinsic spring constants, assuming the sphere is located at the cantilever end, is:

$$k_{\text{app}} = \left\{ \left[1 - \frac{3R}{2L} \tan \theta \right] \cos^2(\theta) \right\}^{-1} k \quad (9)$$

where the correction factor accounts for the tilt of the cantilever θ and the torque applied on the cantilever because of the finite tip height; it was proposed by Heim et al.,⁴⁰ and later corrected by Hutter³⁸ and generalized by Edwards et al.³⁷ and Wang.⁵³ For standard AFM tips, the radius R of the sphere in Equation (9) must be replaced by the tip height (and not by half that value), as reported in Reference 37.

Following the approach of Edwards et al.,³⁷ for CPs obtained from tipless cantilevers, assuming a sphere on cantilever configuration and considering a back-shift ΔL of the loading point, Equations (8) and (9) can be combined as:

$$\begin{aligned} k_{\text{CP,app}}^{\text{LP}} &= \left\{ \left[1 - \frac{3R/L}{2(1 - \Delta L/L)} \tan \theta \right] \cos^2(\theta) \right\}^{-1} \left(\frac{L}{L - \Delta L} \right)^3 k_{\text{TL}} \\ &= \left\{ \left[1 - \frac{3R/L}{2(1 - \Delta L/L)} \tan \theta \right] \cos^2(\theta) \right\}^{-1} k_{\text{CP}}^{\text{LP}} \end{aligned} \quad (10)$$

Equation (10) allows to calculate the apparent spring constant, at the loading point, of the CP from the intrinsic spring constant of the previously calibrated tipless cantilever.

The intrinsic spring constants are calibrated using the thermal noise method.^{41,46} Some details of the implementation of the thermal noise method for CPs are discussed in Section 3. The cantilever deflection signal is extracted from the AFM using a signal access module (intermodulation products) and sampled using an external ADC board (NI-DAQ PCI-6115). Signal acquisition and processing is

TABLE 2 Descriptions and abbreviations of the spring constants considered in this study

Spring constant name	Description
k_{TL}	Measured in the tipless configuration and related to the free end of the cantilever
k_{TL}^{LP}	Measured in the tipless configuration and related to the selected loading point of the probe
k_{CP}^{LP}	Measured with the attached sphere and related to the loading point of the probe corresponding to the position of the centre of the sphere
k_{app}	Apparent spring constant due to the tilt and torque of the cantilever in the standard experimental configuration

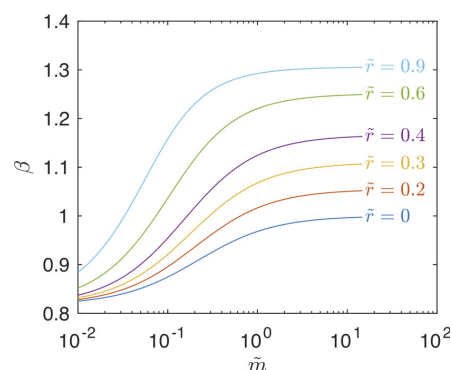
performed using custom software (LabView, National Instruments; see Reference 48 for practical indications). Alternatively, the Sader method can be used to calibrate the intrinsic spring constant of the cantilevers.^{45,54} The spring constant of a probe can be measured in different configurations (tipless cantilever vs CP) and it is always related to the loading point of the probe. For CPs, the loading point (LP) is located at the point where the tip/probe is attached to the cantilever; for TL cantilever, the LP is located at the cantilever free end. In Table 2 we summarize all the possible spring constant evaluated in this work, with the corresponding abbreviations.

3 | RESULTS

3.1 | Impact of the added mass on the dynamics of CPs

The laser doppler vibrometry (LDV) and in general interferometric techniques^{47,55-57} permit to measure with great accuracy the cantilever deflection instead of inclination, also in the case of oscillating cantilevers, including for the calibration of the spring constant by the thermal noise method. Interferometric techniques offer the advantage of not requiring the calibration of deflection sensitivity.

Using interferometric approaches it was recently demonstrated that the cantilever oscillation dynamics changes if a mass is attached to the free end.^{27,58} In particular Laurent et al²⁷ have measured the shape of first five eigenmodes along the profile of a rectangular cantilever, as a function of the reduced mass of the sphere $\tilde{m}$, and of the reduced gyration radius of the sphere $\tilde{r}$, introduced in Section 2. Apart from causing a decrease of the resonant frequency, the large attached mass of the sphere induces a shift of the normal mode nodes towards the free end of the cantilever, with the progressive shift of the centre of mass towards the cantilever free end and the increase of the contribution of the first normal mode to the total oscillation amplitude. As a consequence, both factors $\tilde{\alpha}$ and $\tilde{\chi}$ are expected to be influenced by the presence of a large sphere at the end of the cantilever. It turned out indeed that the factor β_0 calculated for an ideal tipless cantilever

**FIGURE 2** Scaling of the factor β (see Section 2.2.4) as function of $\tilde{m}$, for different $\tilde{r}$, calculated according to the procedure described in Reference 27 based on the modified modal shape functions for a sphere-loaded cantilever

is no longer accurate, and leads to an underestimation of the spring constant measured by the thermal noise method.²⁷

Figure 2 shows the values of the factor β calculated according to the procedure described in Reference 27 based on the modified modal shape functions for a sphere-loaded cantilever, for both $\tilde{m}$ and $\tilde{r}$ varying over a broad range, considerably extending the previously reported results.

Once the spring constant $k_{CP,0}$ of the CP has been calibrated by the thermal noise method using the standard prefactor β_0 , the correct spring constant k_{CP} is calculated according to the formula:

$$k_{CP} = \frac{\beta}{\beta_0} k_{CP,0} \quad (11)$$

Representative curves $\beta \equiv \beta(\tilde{m})$ for different values of $\tilde{r}$ are reported in Figure 2. In order to facilitate the application of Equation (11), Table 3 reports the values of the correction factor β for typical values of $\tilde{m}$ and $\tilde{r}$.

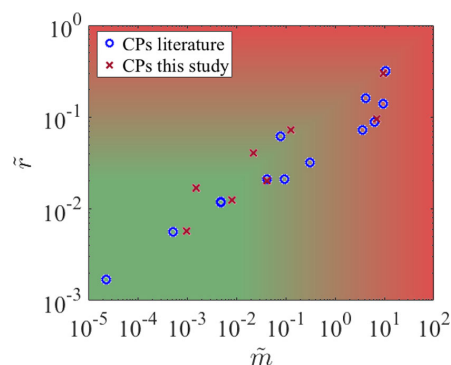
3.2 | A survey of CPs from the literature and the present study

CPs are fabricated with different cantilever and microsphere-relative dimensions and masses. Figure 3 reports the results of a literature survey about the values of $\tilde{m}$ and $\tilde{r}$ of CPs used in the experiments. Most of the CPs have relatively small spheres attached to the cantilevers, resulting in a reduced mass $\tilde{m}$ on the order of 0.01 or even below.^{10,59-61} Nevertheless, very large CPs are also used,^{10,62-65} with $\tilde{m}$ values as large as 5, up to 10.

In order to study how $\tilde{m}$ and $\tilde{r}$ of CPs affect the cantilever dynamics, we fabricated several CPs with different dimensions in order to cover the whole range shown in Figure 3. In particular, we produced eight probes, using both shorter and rigid tapping mode (TM), and longer and soft contact mode (CM) cantilevers. The properties of the CPs

TABLE 3 Values of the correction factor β (Equation 11) for different values of $\tilde{m}$ and $\tilde{r}$

		$\tilde{r}$										
		0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
$\tilde{m}$	0	0.817	0.817	0.817	0.817	0.817	0.817	0.817	0.817	0.817	0.817	0.817
	0.001	0.818	0.818	0.818	0.819	0.819	0.820	0.821	0.822	0.823	0.824	0.826
	0.002	0.819	0.819	0.820	0.820	0.821	0.823	0.825	0.827	0.829	0.832	0.834
	0.005	0.821	0.822	0.823	0.825	0.827	0.831	0.835	0.840	0.846	0.852	0.859
	0.01	0.825	0.826	0.828	0.832	0.837	0.844	0.852	0.861	0.872	0.884	0.897
	0.02	0.832	0.834	0.838	0.845	0.855	0.868	0.883	0.900	0.920	0.941	0.963
	0.05	0.851	0.854	0.864	0.879	0.901	0.927	0.957	0.990	1.024	1.059	1.093
	0.1	0.875	0.880	0.896	0.921	0.954	0.994	1.036	1.079	1.121	1.158	1.190
	0.2	0.905	0.913	0.936	0.972	1.018	1.068	1.117	1.161	1.199	1.230	1.254
	0.5	0.945	0.956	0.988	1.035	1.090	1.144	1.191	1.228	1.257	1.278	1.292
	1	0.968	0.981	1.016	1.068	1.124	1.177	1.220	1.253	1.276	1.292	1.304
$\tilde{m}$	2	0.983	0.996	1.034	1.087	1.144	1.195	1.236	1.265	1.285	1.299	1.309
	5	0.993	1.007	1.046	1.100	1.157	1.207	1.245	1.272	1.291	1.303	1.312
	10	0.996	1.011	1.050	1.105	1.162	1.211	1.248	1.275	1.293	1.305	1.313

**FIGURE 3** CPs used by the authors of publications as from a literature survey (blue circles) and CPs fabricated during this study (red crosses) represented according to their reduced mass $\tilde{m}$ and gyration radius $\tilde{r}$. The background colour highlights the region where corrections to the spring constant according to Equation (11) are negligible (green), and the region where the influence of the added mass becomes relevant (deviation larger than approximately 10%)

produced for this study are reported in Table 1 and also in Figure 3 (red crosses). We characterized the spring constant and the dynamics of our probes before and after the attachment of the microsphere.

3.3 | Shift of the resonant frequency of CPs

Figure 4 shows the thermal noise spectrum, that is, the raw PSD P_V in units of V^2/Hz , of the first resonant peak of the original tipless cantilevers, and of the CPs obtained upon attachment of the glass bead. Not surprisingly, as a consequence of the added mass, we observe a

decrease of the resonant frequency. These results confirm that a shift in the resonant frequency ν_0 is present even if the reduced mass is as small as $\tilde{m} = 9.76 \times 10^{-4}$ (Figure 5). This drop of the resonant frequency reaches a factor of 5 for largest masses (Figure 4D,H). These trends are in agreement with previous works.^{66,67}

Figure 4 shows that the increase of the full width at half maximum (FWHM) and of the oscillation amplitude are associated to the shift of the resonant peaks towards lower frequencies. For large CPs (Figure 4D,H), the peak is 30 times higher and 20 times narrower compared to the corresponding tipless cantilever case. These observations may be somewhat unexpected for probes whose mass is significantly increased. For instance, a reduction of the oscillation amplitude and a widening of the resonant peak is typically observed when the thermal noise spectrum is acquired in liquid, another case where the effective mass of the cantilever is increased significantly, due to the liquid viscosity that causes the cantilever to drag a finite volume of liquid during oscillation. As we will discuss later, this observation can be explained in terms of a combination of effects due to the increase of the inertial mass of probe⁶⁷ and the storage of a greater fraction of total oscillation in the first mode.²⁷

3.4 | Quality factor

The quality factor Q represents the ratio between the energy stored in the oscillation and the energy dissipated per cycle because of the viscous damping. For a single-mode oscillator, Q is defined as:

$$Q = \frac{m^* \omega_0}{\gamma} = 2\pi \frac{m^* \nu_0}{\gamma} \quad (12)$$

In Equation (12), γ is the damping coefficient, that is, the proportionality factor between the tip velocity and the viscous force. Q is related to

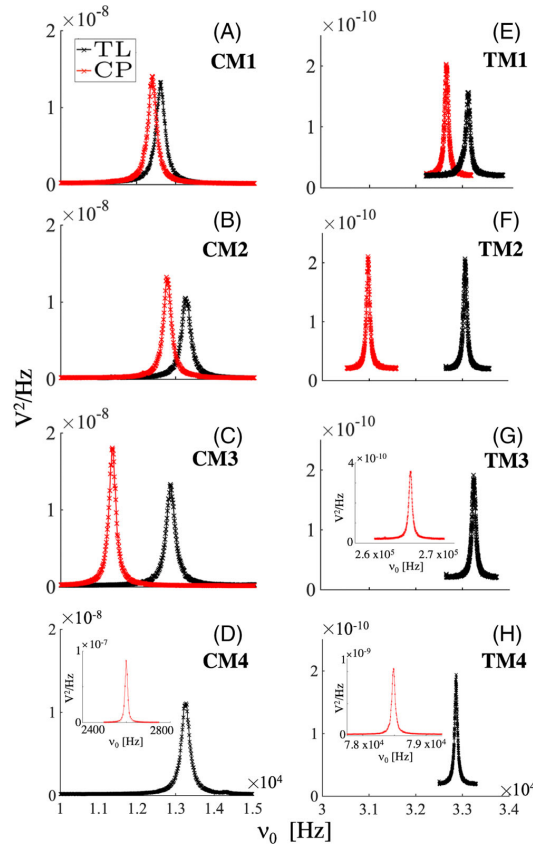


FIGURE 4 The measured PSD P_V at the first normal mode of the cantilever before (black) and after (red) the attachment of the microsphere for contact mode CPs. A–D, CMs; E–H, TMs. The mass of the attached sphere increases from top (CM1 (A)–TM1 (E)) to bottom (CM4 (D)–TM4 (H))

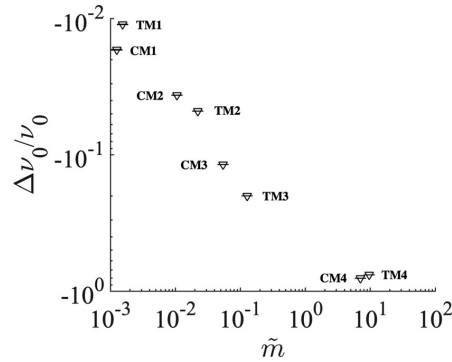


FIGURE 5 The relative shift of the measured resonant frequency ν_0 vs the reduced mass $\tilde{m}$. As shown in Figure 4, ν_0 decreases as $\tilde{m}$ increases

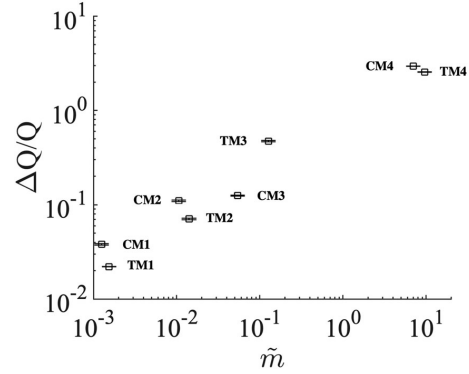


FIGURE 6 Relative shift of the measured quality factor Q vs the reduced mass $\tilde{m}$

the width of the resonant peak: at fixed mass m^* and frequency ν_0 , the higher is Q , the smaller is the FWHM, since $\text{FWHM} \approx \nu_0/Q$.

Figure 6 shows the relative increase of the quality factor Q of the CPs compared to the one measured in the tipless configuration. It is evident that the value of Q of CPs increases, rather than decreasing, with respect to the tipless cantilevers, and that the relative increase is larger for higher values of $\tilde{m}$.

To better understand the behaviour of Q upon the attachment of the sphere to the tipless cantilever, we calculated the effective mass $m^* = k/\omega_0^2$ for each probe, before and after the attachment of the bead. The ratio between the measured effective mass m^* and the nominal mass of the probe m_p (for both tipless probes and CPs) is shown in Figure 7A. The measured ratio m^*/m_p for the tipless cantilevers is 0.24 ± 0.02 (mean + standard deviation of the mean), in agreement with the theoretical value of 0.25 for the rectangular cantilever.⁴² A similar value is measured for those CPs with the smallest reduced mass, where the contribution of the cantilever mass is dominant (see Figure 7A). As $\tilde{m}$ increases, the effective mass m_c^* becomes larger than $0.25m_c$. For large CPs, m^* becomes similar to m_p , which is in turn very similar to m_s , the mass of the sphere; in this limit, the mass of the sphere is dominant.

Equation (13) represents a simple model for the effective mass m_{CP}^* of a CP; the concentrated mass of the sphere, located at the free end of the cantilever, is added to the effective mass of the tipless cantilever²⁶:

$$m_{CP}^* = m_c^* + m_s \quad (13)$$

Equation (13) clearly highlights that the cantilever and the sphere contribute differently to the inertia of the probe. The cantilever mass, which is uniformly distributed along the cantilever length, is strongly underestimated in the sum, being weighted by a factor of $1/4$; the mass of the sphere, instead, being truly concentrated at the free end of the cantilever, is fully represented with weight 1. Figure 7B shows the correlation between the measured m^* and the values of the effective

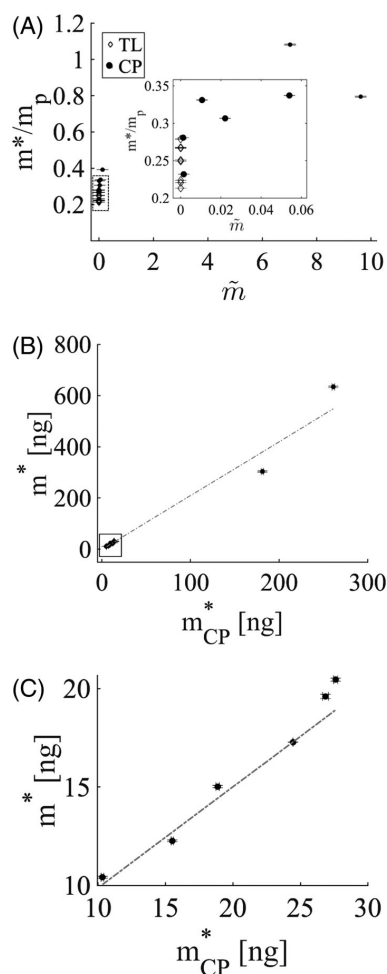


FIGURE 7 A Ratio of the effective mass m^* (calculated using Equation 12) to the nominal mass m_p of the fabricated probe as a function of the reduced mass $\tilde{m}$ for tipless cantilevers (diamonds) and CPs (filled circles). B, Effective mass m^* of the CPs (calculated as $m^* = k/\omega_0^2$) as a function of m_{CP}^* (defined in Equation 13), with linear fit ($y = mx + b$, $m = 1.052 \pm 0.005$, $b = -1.14 \times 10^{-12} \pm 1.27 \times 10^{-13}$). C, Zoom on the data presented in (B) for $m_{CP}^* < 30$ ng highlighted in the drawn box, with the linear fit ($y = mx + b$, $m = 1.027 \pm 0.008$, $b = -1.14 \times 10^{-12} \pm 5.37 \times 10^{-13}$)

mass predicted by Equation (13). The slope of the straight line fitting the experimental data in Figure 7B is 1.052 ± 0.005 . In the case of the first six probes (data highlighted in Figure 7C), a better correlation is found, the slope being 1.027 ± 0.008 .

Figure 7B shows that Equation (13) represents a good model for the effective mass of CPs, which in turn confirms the accuracy of the description of (large) CPs as effective SHOs with the total mass concentrated at the loading point. Indeed, as the radius R of the sphere

increases, the mass of the sphere increases rapidly, scaling as R^3 , and the CP becomes more and more equivalent to an SHO with a concentrated mass equal to the sphere mass. Deviations for the largest CPs can be attributed to uncertainties in the determination of the sphere mass, due both to the uncertainties in the sphere density or in the sphere volume. SEM images (Figure 1) show indeed that soda-lime glass spheres possess many defects, which could also be present in the inner volume, as voids, or segregated phases with different density. Moreover, upon softening in the oven, the spheres deform to some extent in the region close to the cantilever. This process is supposed to conserve the volume of the sphere, but could alter the measurement of the radius, which is inferred from the transversal section of the sphere; as a consequence, errors in the volume estimation are likely.

The increase of the quality factor is in turn explained by Equation (12). Q is proportional indeed to the resonant frequency ν_0 and the effective mass m^* . Since ν_0 drops by a factor of 5 (Figure 5), while m^* increases by a factor of 40 (it passes from $0.24 m_c$ to approximately $10 m_c$), the latter change dominates and Q increases (considerably).

Eventually, Figure 7B,C and Figure 6 explain why the amplitude of the resonant peak of the first normal mode is enhanced, as shown in Figure 4. Indeed, large CPs are well described by a single-mode harmonic oscillator model, because the higher-order modes provide a less and less important contribution to the tip motion, as their last node approaches the free cantilever end²⁷; as the quality factor Q increases significantly, the constraint of energy conservation causes the amplitude to increase.

In conclusion, Equations (12) and (13) and the observed trends of both resonant frequency and quality factor explain the peculiarity of the thermal noise spectra of CPs with a large microsphere attached to their free end, in particular the fact that these probes, despite a marked reduction of the resonant frequency, have extremely sharp resonant peaks, with large Q and amplitude. The apparent anomaly of CPs can be attributed to the fact that, in the AFM community, an increase of the effective mass and a drop in the resonant frequency of the cantilever are typically observed when operating a soft cantilever in liquid,^{68–70} and they are associated to a marked drop of the quality factor Q due to the viscous damping. With (large) CPs in air, however, the factor γ in Equation (12) does not change significantly, while the dramatic increase of the mass is not counterbalanced by the reduction of the resonant frequency. A recent study confirms that the added mass mechanism described in this work dominates the effects related to energy dissipation and viscous drag also in the case of large CPs in liquid, where the attachment of large beads to the cantilever is exploited to enhance the quality factor and the force sensitivity of the probes.⁶⁷

3.5 | Spring constant of CPs

Figure 8A shows a comparison of the spring constants k_{TL}^{LP} , calibrated for the tipless cantilevers before the attachment of the glass beads, and then corrected for the shift of the loading point (Equation (8)), with the spring constants k_{CP}^{LP} of the CPs calibrated after the

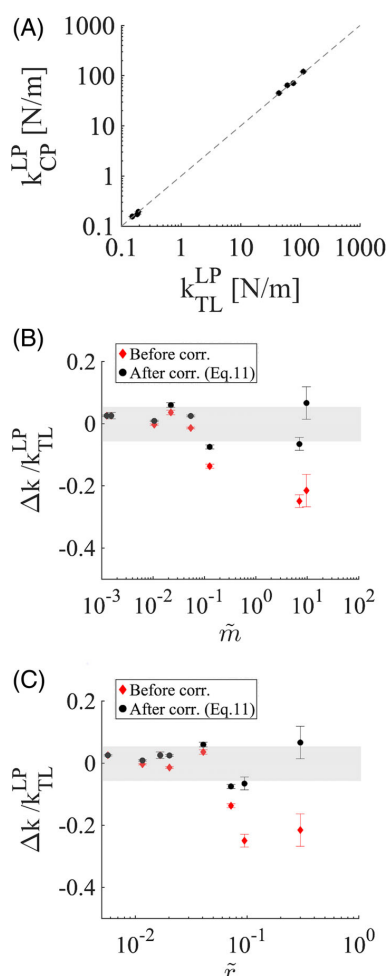


FIGURE 8 A, Spring constant k_{CP}^{LP} of CPs corrected according to Equations (8) and (11) vs the reference spring constant k_{TL}^{LP} of the tipless cantilever calculated at the loading point (Equation 8), measured before the sphere attachment. The dashed line represents a linear regression of the data, with slope 1.0090 ± 0.0006 . B and C, The relative difference between k_{CP}^{LP} and k_{TL}^{LP} as a function of the reduced mass and radius of the CP, respectively, without (red filled diamonds) and with (black filled circles) the correction by Equation (11). The shaded area represents the $\pm 5\%$ interval

attachment of the spheres, corrected for the loading point shift, and eventually for the added mass effect (Equation 11). A linear regression of the data provides a slope of 1.0090 ± 0.0006 , which confirms the effectiveness of the correction procedures.

Figure 8B,C shows the relative difference between the spring constant k_{TL}^{LP} and the spring constant k_{CP}^{LP} as a function of $\tilde{m}$ and $\tilde{r}$, respectively. The spring constant values are shown before (red) and after (black) the β -factor correction according to Equation (11).

As shown in Reference 27, the effect of the added concentrated mass of the sphere is to induce an underestimation of the spring constant, if the standard thermal noise formula (Equation 7) is used, with $\beta = \beta_0 = 0.817$. When $\tilde{m}$ and $\tilde{r}$ are larger than approximately 0.1, the spring constant starts to decrease, reaching a maximum relative deviation from k_{TL}^{LP} of about 25% (Figure 8B,C, red filled diamonds); these deviations are effectively corrected by Equation (11) (Figure 8B,C, black filled circles). For $\tilde{m} < 0.1$, the correction is not very relevant, and falls below the typical noise level.

The small differences between k_{TL}^{LP} and k_{CP}^{LP} reported in Figure 8 could be due, among other factors, to errors in the determination of the deflection sensitivity of the CPs, as discussed below in Section 3.6, in the determination of the loading point, as well as in the possible rigidification effect of the cantilever, due to the attachment of the sphere, which could influence the dynamics of the last part of the cantilever.

3.6 | Deflection sensitivity and CPs

When big spheres are attached to the cantilever, the standard approach for the calibration of the deflection sensitivity S_z (described in Section 2) can be inaccurate, due to phenomena occurring during the loading phase of the force curve.

In particular, the cantilever tilt angle θ towards the surface may determine a tangential force component, which is due to friction, between the sphere and the substrate. As in the case of normal forces,^{37,38} also tangential friction forces applied at the loading point of the cantilever induce a torque^{53,65,66,71-73}; this torque in turn induces a modification of the cantilever curvature that impacts on the measurement of S_z .⁷²⁻⁷⁴

The friction-related effect is stronger in the high-friction limit, when the probe is pinned on the surface instead of sliding.⁷⁵ The friction, and therefore the torque, is stronger when the effective tip height is larger, as in the case of large CPs, and when the normal force is higher, as in the case stiffer cantilevers are used. Noticeably, for mechanical measurements stiffer cantilevers can be used, in combination with large spheres, in order to achieve reasonably high indentations.

Since the AFM optical lever detection scheme is sensitive to the inclination of the cantilever rather than to its deflection, it turns out that the measurement of S_z can be particularly inaccurate for large CPs, especially in conditions of strong adhesion and friction (as in air). In addition, larger adhesion related to the larger radius of CPs in general makes the acquisition of force curves in air, but also in liquid, critical.⁷⁶

The direct calibration of the deflection sensitivity of large CPs by the standard method based on the acquisition of force curves can therefore be problematic. In this case, alternative approaches should be adopted. One possibility is to consider the mean of the slope the loading and unloading portion of the force curve to effectively subtract the deflection artefact, and then inverting the mean slope to obtain the invOLS, as proposed by Chung et al.⁷⁶ The same authors proposed an electrostatic method to calibrate the deflection

sensitivity, which however requires to build a dedicated micro-device and the metallization of the CP.⁷⁶ In the following sections, we will propose an alternative method to overcome these limitations.

4 | BEST PRACTICES FOR THE CALIBRATION OF (LARGE) CPS

In summary, the thermal noise method can be applied directly to the CP, according to the following rules:

1. S_z is measured carefully, neglecting the initial region of the FC after the contact point, as the inverse of the mean of the slopes of the loading and unloading curves, to minimize friction-related effects, as proposed by Chung et al.⁷⁶
2. The deflection signal of the cantilever is sampled over time with sufficient resolution and number of points, in order to obtain, when applying the thermal noise method, a well-defined peak in the Fourier space; this will allow to accurately estimate the power P_V , despite the typical peak sharpness in air. Alternatively, the thermal noise calibration can be carried out in water, in order to obtain a wider resonance peak. In this case, also the deflection sensitivity must be characterized in water.
3. The β -factor correction (Equation 11) must be applied to the intrinsic spring constant calculated by Equation (7). To this purpose, the sphere radius, the cantilever dimensions, and therefore the reduced mass $\tilde{m}$ and the reduced gyration radius $\tilde{r}$, must be accurately determined.
4. The corrected intrinsic spring constant k_{CP} is transformed into the apparent spring constant, including also the effect of the shift of the loading point, using Equation (10) (with k_{CP} in place of k_{TL}).

According to the results reported in the present study, we suggest an alternative accurate and reliable approach for the calibration of CPs, and especially of large CPs. This approach can be applied when the CP is produced in house, using tipless cantilevers. In this case, the following procedure can be followed:

1. The intrinsic spring constant k_{TL} of the tipless cantilever, before the attachment of the sphere, is calibrated by the thermal noise method, measuring the deflection sensitivity in the standard way, that is, from a FC acquired on a hard substrate, or using a deflection sensitivity-free approach, like LDV (and thermal noise)⁵⁶ or the Sader method.^{45,54,77}
2. Once the sphere is attached, measure the position of the loading point using an optical microscope and correct the intrinsic spring constant k_{TL} into the apparent $k_{CP,app}^{LP}$, using Equation (10).

It must be considered that, while the interferometric approaches can be used, together with Equation (11), to calibrate CPs (in fact, LDV is a technique of choice for calibrating the spring constant of

AFM probes, but at present it relies on expensive instrumentation), the applicability of the global calibration initiative launched by Sader⁴⁵ should be checked. This approach is based indeed on the statistical assessment of the hydrodynamic function for a given cantilever geometry and may not account for the contribution of the large attached sphere. The general Sader method for the calibration of cantilever of arbitrary shape and dimensions⁵⁴ could in turn work well, provided the calibration of the hydrodynamic function is performed in house.

Once the intrinsic spring constant k_{CP} of a CP has been accurately calibrated, the deflection sensitivity S_z of the current experiment can be calculated assuming that the thermal noise method, through Equation (7), must provide the known value of k_{CP} (see References 78 and 79 and the SNAP procedure⁸).

5 | CONCLUSIONS

Colloidal probes are used in a wide range of experimental conditions. Their dynamical behaviour presents peculiar aspects,²⁷ which also pose issues for their calibration, since the modal shapes are significantly affected.

In this work, we have demonstrated that the addition of a large mass to a tipless cantilever concentrated at its end, besides significantly decreasing the resonant frequency, boosts the quality factor and the amplitude of the first resonant peak, so that a large CP mimics a single-mode mass-on-a-spring system better than a standard AFM probe. These findings may have an impact on the application of CPs in spectroscopic dynamical applications.

Despite the relevant modifications of the cantilever dynamics of CPs, the accuracy of the calibration of CPs is not severely affected until the added mass of the sphere becomes comparable to that of the cantilever, which typically happens for diameters of the glass sphere of about 10 μm .

In the case of large CPs, it is possible to accurately calibrate the spring constant by applying a correction factor (Equation 10) to the value obtained according to the equipartition theorem for an ideal tipless rectangular cantilever. We present two alternative detailed procedures, including one allowing to minimize the impact of the inaccurate determination of the deflection sensitivity.

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CONFLICT OF INTEREST

The authors declare there are no conflicts of interests.

AUTHOR CONTRIBUTIONS

All authors have read and approved the final manuscript. Alessandro Podestà and Ludovic Bellon designed the research plan and contributed essential reagents, materials and tools. Luca Puricelli and Matteo Chighizola carried out the experimental activity and analysed the data with the support of Alessandro Podestà and Ludovic Bellon. Alessandro Podestà, Matteo Chighizola and Ludovic Bellon wrote and revised the paper.

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3.1 Additional discussion

Even though the results presented in the previous section demonstrate a good accuracy of the model and the applied corrections, some further investigations should be carried out in order to better understand some specific issues.

3.1.1 The rigidification effect

The calibrated spring constant k should always be related to the loading point of the cantilever, irrespective to where the laser is aligned.

When a sphere is attached to a tipless cantilever, an extended neck could be present at the sphere-cantilever interface (Figure 1d-f, pg 30), either because of excessive softening of the glass bead, or due to a large residual amount of solidified glue. The corresponding portion of the cantilever therefore rigidifies, and may not contribute anymore to the cantilever bending, nor to the thermal noise-driven oscillation. Rigidification of the cantilever can be clearly identified by inspecting the profile of the CP using an optical microscope.

In Figure 1, pg 30, the distance ΔL of the loading point from the cantilever end is shown. When the microsphere is small compared to the cantilever dimension and none or little amount of glue is used, negligible rigidification is present on the cantilever (Figure 1b). If these assumptions fail, the rigidified portion of the cantilever cannot bend anymore (Figure 1d,f), and the loading point needs to be shifted to a different position of the cantilever.

Reasonably, the last portion of the cantilever able to bend is located at the beginning of the fused basement of the sphere, on the side of the sphere facing the cantilever fixed end. As a consequence, the system dynamics would behave in a totally different manner: a portion of the cantilever of length L_{eff} (see Figure 13) would be free to oscillate dynamically, while the second portion (the rigidified one) will follow rigidly the tilt of the final part of L_{eff} . This configuration would resembles those exanimated by Edwards et al.⁷¹ so called “Sphere on a tip” with an angle $\alpha_0 < \theta$.

This peculiar configuration is not accounted for by any special theoretical model and further calculations should be made with the aim to describe the above-mentioned static and dynamical behaviour.

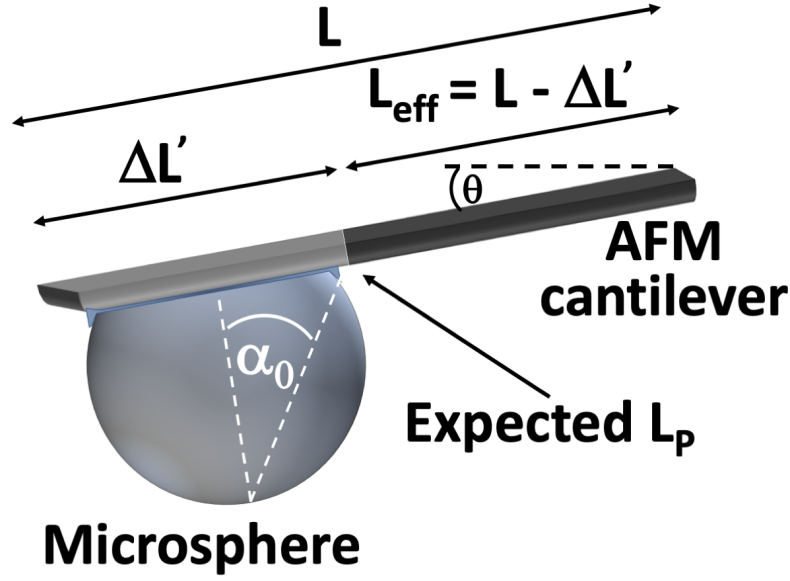


Figure 13. Scheme representing the configuration of a rigidified cantilever with all the related variables outlined.

3.1.2 Added-mass away from the free-end

The corrections developed by Laurent et al.⁷² and tested in the above paper, consider a configuration where the microsphere is attached perfectly to the free-end of the cantilever, but this condition is not always satisfied. Indeed, even if no rigidification is present, it could happen (in particular with home-made probes) that the sphere is attached several microns or tens of microns away from the free-end. Once again, an added mass located at distance ΔL away from the cantilever's end would affect the amplitude and position of the normal modes and the parameters $\hat{\alpha}$ and χ should be modified accordingly.

3.1.3 Finite laser-spot

If we consider a rigidified configuration, also the effect of the finite spot size of the laser should be taken into account⁸⁴, in particular if the laser spot dimension are compatible or even larger of the rigid portion of the cantilever.

3.1.4 Rectangular Vs triangular cantilever shape

Like most of the theoretical model developed for the description of AFM probes, the added-mass correction has been modelled on the rectangular cantilever shape. The triangular cantilever oscillation dynamics is different from the rectangular one, and so the resulting correction parameters $\hat{\alpha}$ and χ . How a mass

attached to the free end of a triangular cantilever affects the oscillating dynamics is not investigated. The triangular cantilevers may be described by the “*parallel beam approximation*” (i.e. a rectangular cantilever wide as the sum of the widths of the two arms and long as the effective length of the triangular cantilever⁸⁵), and with this approximation all the consequent corrections would follow straightforward. But the “parallel beam” is an approximation and a more detailed description should be done, in particular considering that the approximation fails more and more with the cantilever shortening⁸⁶.

3.1.5 Standardization of the correction parameters for thermal noise

It has been recently published a preprint by J. Rodriguez and F. Rico⁸⁷ where they present a finite-element simulation to reproduce and measure the cantilever bending shape in the loaded and freely oscillating condition. Exploiting these simulations, they are able to directly calculate the correction factors of the thermal noise for every geometrical and peculiar configuration of the probes. This powerful approach would allow to directly obtain the operative (apparent) force constant with a single thermal noise. Nevertheless, in their calculation it is evident that not every dependence of the geometrical configurations (highlighted in the previous sections) has been taken into account, and some others has been embedded in a more general definition. In order to compare and merge our and their approaches some detailed calculations should be provided, indeed if we want to provide a standardized and reliable protocol, useful for the scientific community, we need first to find a common definition of all the parameters used by everyone.

Chapter 4

Nanotopographical Colloidal Probes mimicking the disordered morphology of the ECM for the investigation of the cell reaction to morphological cues

The first interaction step in the mechanotransductive cascade takes place at the cell membrane level, i.e. at the interface between cell and microenvironment. Starting from the ECM, the adhesive structures at the cell membrane that enable the cell to perceive the biophysical configuration of its microenvironment and to translate the information into appropriate cellular responses are the integrin adhesion complexes (IAC), which can mature into bigger structure like focal complexes (FCs, cluster of integrins) and focal adhesions (FAs) under suitable microenvironmental conditions (in terms of rigidity and spatial organization of ligands). The integrin family consists of heterodimeric transmembrane receptors, always build up by one α - and one β -subunit that can assemble in different combinations. As discussed in the introduction, they possess large extracellular domains and short cytoplasmic tails (see Figure 14). Once the ECM-binding connection take place, mechanosensing of the biophysical and structural features begins which determines whether the maturation process occur or not.⁹.

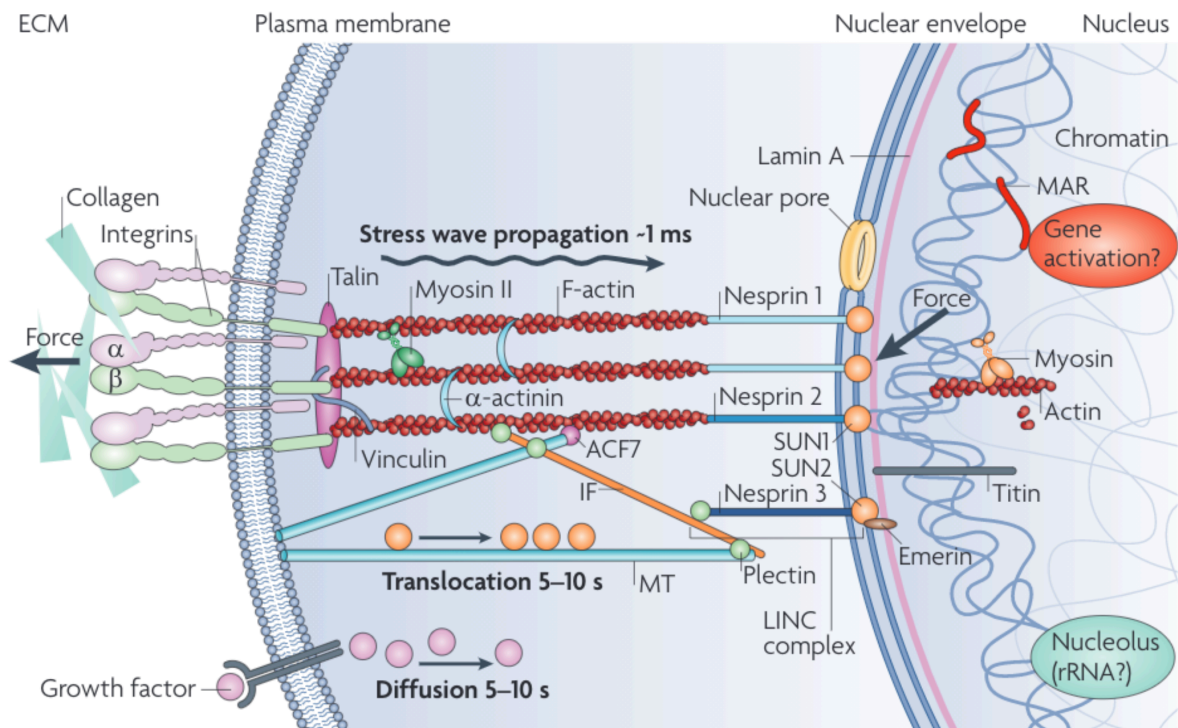


Figure 14. Molecular connectivity from the ECM to the nucleus. A local force applied to integrins through the ECM is concentrated at focal adhesions and channelled to actin filaments that are eventually connected to the nuclear envelope. Image adapted from Wang et al⁸⁸.

The spatial organization of the integrins adhesion sites plays a fundamental role in the integrin clustering and eventually in the ability of cells to react to the external stimuli. A better understanding of the adhesion dynamics at the cell membrane level would bring an insight of the early steps of cellular mechanosensing in response to different topographical stimuli of the ECM.

Albeit the specific structural organisation can strongly vary between the ECMs of different tissues (from relatively ordered fibrillar-dominated ECM to much more crosslinked, reticular, disordered features^{89–91}), the ECM structure is always characterised by a very complex nano-3D topography that is not easy to mimic *in vitro*, as the existence of a myriad of biomaterial approaches attest.

Previous works have shown that nanostructured thin films, grown by assembling of zirconia nanoparticles (ns-ZrO₂) on a substrate through Supersonic Cluster Beam Deposition technique⁵⁷, possess a morphological disorder on the nanometer scale with structural features that mimic topographical properties of the ECM, in particular those of basement membranes (see Figure 15). Experiments performed with PC12 cell line demonstrated a link between specific nontopographical properties (like roughness and correlation length) of the ns-ZrO₂ films and specific mechanotransductive events, which eventually foster neuronal differentiation^{30,32}.

Rhesus macaque corneal epithelial basement membrane in scanning electron microscopy and atomic force microscopy imaging

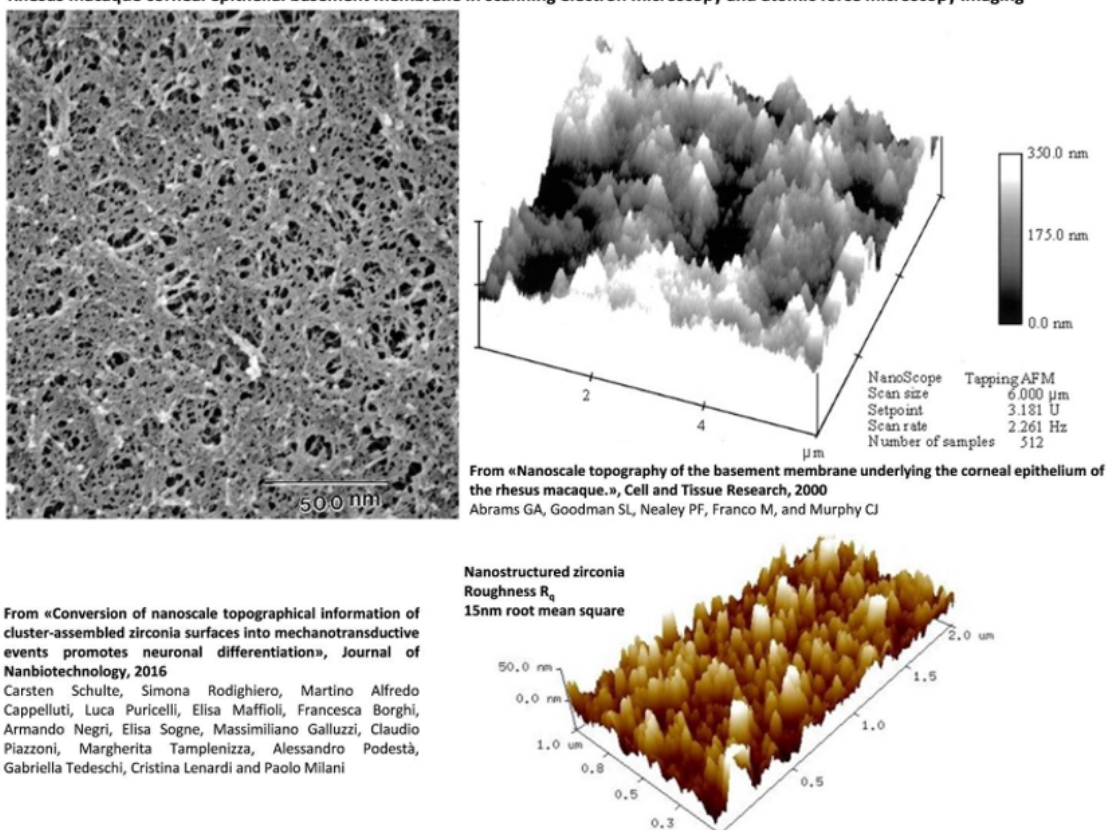


Figure 15. (top-left) SEM image of rhesus macaque basement membrane, image adapted from Liliensiek et al.⁹⁰. (top-right) 3D-AFM image of ECM from corneal epithelium, image adapted from Abrams et al.⁴⁷. (bottom) 3D-AFM image of ns-ZrO₂ thin film growth with the SCBD technique, image adapted from Schulte et al.³⁰.

The work presented below represents the core of my PhD project and consists in the development of protocols for the fabrication and characterization of a new kind of AFM CPs: the *nanotopographical colloidal probes (nt-CPs)*, and in the study of the early step of the mechanotransductive pathway in relation to specific morphological cues.

Starting from the considerations of the previous chapter I functionalized the CPs by growing nanostructured thin films of Zirconium-Oxide (Zirconia, ZrO₂) via cluster-assembling in an extremely controlled manner. The purpose of this functionalization was to move the 3-dimensional complexity of the ECM on the interaction region of the CPs. By means of these nanotopographical CPs, I characterized the adhesive behaviour, in particular with respect to the force dynamics, of the PC12 cell line through *AFM Force Spectroscopy* experiments. Here below, the whole paper is reported including the “Supplementary Information”, which provides also technical details on the creation of the *nt-CPs*.

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Adhesion force spectroscopy with nanostructured colloidal probes reveals nanotopography-dependent early mechanotransductive interactions at the cell membrane level†

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Mechanosensing, the ability of cells to perceive and interpret the microenvironmental biophysical cues (such as the nanotopography), impacts strongly cellular behaviour through mechanotransductive processes and signalling. These events are predominantly mediated by integrins, the principal cellular adhesion receptors located at the cell/extracellular matrix (ECM) interface. Because of the typical piconewton force range and nanometre length scale of mechanotransductive interactions, achieving a detailed understanding of the spatiotemporal dynamics occurring at the cell/microenvironment interface is challenging; sophisticated interdisciplinary methodologies are required. Moreover, an accurate control over the nanotopographical features of the microenvironment is essential, in order to systematically investigate and precisely assess the influence of the different nanotopographical motifs on the mechanotransductive process. In this framework, we were able to study and quantify the impact of microenvironmental nanotopography on early cellular adhesion events by means of adhesion force spectroscopy based on innovative colloidal probes mimicking the nanotopography of natural ECMs. These probes provided the opportunity to detect nanotopography-specific modulations of the molecular clutch force loading dynamics and integrin clustering at the level of single binding events, in the critical time window of nascent adhesion formation. Following this approach, we found that the nanotopographical features are responsible for an excessive force loading in single adhesion sites after 20–60 s of interaction, causing a drop in the number of adhesion sites. However, by manganese treatment we demonstrated that the availability of activated integrins is a critical regulatory factor for these nanotopography-dependent dynamics.

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1. Introduction

A complex crosstalk between cells and their microenvironment, *i.e.* the extracellular matrix (ECM), governs the development and maintenance of multicellular tissues. The biophysical properties of the microenvironment were therein identified as critical factors for the regulation of many cellular responses, such as proliferation, migration, and differentiation. The ECM is a complex meshwork of intertwined macromolecules (with protein and sugar components) characterised by the presence of fibrillary and reticular structures, pores and asperities at the nanoscale. The configurations can

be relatively ordered, *e.g.* in the fibrillary collagen-dominated ECM, or instead rather disordered, as in basement membranes or the brain ECM. However, at the local nanoscale level often irregularities, anisotropies and density gradients are present.^{1–6}

In recent years, it has become evident that mechanical stimuli of the ECM, such as the rigidity and/or spatial organisation and dimensionality of adhesion sites (*e.g.* in terms of geometry and topography), alter intrinsic cellular properties, such as the actin cytoskeletal organisation/mechanics and the signalling status.^{7–10} The intricate processes through which the cell perceives and reacts to mechanical and structural stimuli in its microenvironment were termed mechanosensing and mechanotransduction.^{11–18}

Mechanotransductive processes are involved in virtually all aspects of the cellular life and tissue organisation^{11–19} and aberrations in components that participate in mechanosensing and mechanotransduction have been linked to various

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diseases, in particular cancer and metastasis. A detailed comprehension of how biophysical ECM characteristics modulate mechanotransduction would promote new approaches for treatment of diseases, drug therapies or diagnostic approaches, exploiting and targeting identified mechanotransductive key regulators or structures.^{11,20–26}

Cells sense the biophysical microenvironmental information at the nanoscale, and nanotopography has emerged as a crucial parameter in the regulation of mechanotransductive processes and signalling.^{7–9,27–31} The mechanotransductive pathway is primarily mediated by specific transmembrane proteins, called integrins, and modulated by the extent to which these integrins cluster together into integrin adhesion complexes (IACs) and mature into bigger structures, such as focal complexes and focal adhesions (FAs). The extent of integrin clustering and FA maturation, in turn, depends on the force loading within the so-called molecular clutches of nascent adhesions, *i.e.* the initial connection of the ECM-binding integrins to forces generated by the actin cytoskeleton *via* adaptor proteins (in particular talin and vinculin). The spatial organisation of integrin adhesion sites (ligands) exerts a fundamental influence on the integrin clustering and eventually on cellular behaviour and responses. These effects are mediated in particular through the principal elements of mechanotransductive signalling, such as RhoGTPases, the actin cytoskeleton and mechanosensitive transcription factors (*e.g.*, YAP).^{11–19}

However, a better understanding and quantification of the dynamics at the cell/microenvironment interface and the force development in the early steps of cellular mechanosensing in response to different topographical stimuli is required. In particular, although the presence of nanotopographical disorder (due to the impact on the spatial distribution of integrin ligands) has been shown to have a strong influence on protein adsorption, cell adhesion, integrin clustering and differentiation,^{7,26,31–33} the systematic characterisation of the influence of disordered configurations with different nanoscale three-dimensional features is still in its infancy.

To unravel the molecular mechanisms of cellular mechanosensing and the mechanotransductive responses it provokes in the cells, versatile biophysical approaches are essential.^{10,17,30,34–42} Smart biomaterials with complex structural architectures and/or tuneable physical properties are needed to create controllable cellular microenvironments that mimic the *in vivo* ECM situation.^{30,31,34,36,37,41} In addition, sophisticated techniques are required that enable the mapping of mechanobiologically relevant alterations in cells.^{17,35,38,42}

In this context, atomic force microscopy (AFM) represents a powerful tool due to its capacity to allow an accurate probing of cell surfaces, determining cellular mechanical properties^{42–46} (and mechanotransduction-related alterations^{47–49}), and measuring adhesion forces down to the single-molecule contribution.³⁸ The standard techniques used to test the cellular adhesion properties are single-molecule force spectroscopy (SMFS)⁵⁰ and single-cell force spectroscopy (SCFS).^{51,52} SMFS consists of the functionalisation of the AFM

probe (*e.g.*, coating with ECM proteins) in order to permit the interaction with specific transmembrane proteins. SCFS instead uses the cell itself as a probe (attached to the cantilever) that interacts with the substrates of interest (including other cells).

SCFS has been widely used to study in detail the cooperative action of integrins in early cellular adhesion to fibronectin or collagen^{53–55} and their connection to internal cellular biochemical signalling.⁵⁶ This technique has furthermore been exploited to test the biocompatibility of materials for implants in orthopaedic surgery,⁵⁷ or the role of ligand spacing in the cell adhesion using substrates decorated with suitably functionalised nanoparticles, separating integrin ligands with different specific distances.^{58,59} A limitation of SCFS, as explained in detail by Naganuma,⁶⁰ is that the actual contact area between the cell and the substrates cannot be measured. As a matter of fact, the cell, once captured and immobilised on the tipless cantilever, will evolve its own adhesion on the probe itself, changing its morphology during time and with it the adhesion properties. These dynamics will introduce a bias in the force spectroscopy experiment and, since different cells behave differently, make results less comparable between each other.

To address these issues, we propose a reversal of the conventional SCFS approach. Our novel strategy for the study of early mechanotransductive interactions at the cell membrane level is based on the use of functionalised colloidal probes (CPs) mimicking the peculiar nanoscale topographical features of the *in vivo* ECM for force spectroscopy experiments on living cells. The probes represent the source for the mechanical cues regulating the cascade of the mechanotransductive events. By inverting the typical cell-microenvironment interaction geometry (Fig. 1), we take in particular control over the cell/substrate contact area and obtain a more accurate assessment of the forces and molecular interactions that develop at the cell membrane during the early mechanotransductive events.

Our technological approach for the fabrication of nanotopographical surfaces consists of growing a cluster-assembled thin film of nanostructured zirconium oxide (ns-ZrO₂, zirconia) with disordered, yet controlled, topographical features by means of supersonic cluster beam deposition (SCBD).^{61–63} Zirconia is a biocompatible material used in various clinical applications, particularly due to its chemical inertness and structural properties.⁶⁴ SCBD is an additive technique and the disordered surface morphology of the deposited films is characterised by nanoscale roughness and other morphological parameters, like the surface area or the correlation length, which can be accurately tuned and reproduced by controlling the film thickness through the deposition time.⁶⁵

The obtained disordered nanostructured films possess nanotopographical features that resemble those that can be observed in the *in vivo* ECM (*e.g.* in basement membranes or in the brain^{5,6}) and with nano-3D configurations (in terms of asperity dimensionality and distances) that have the potential to modulate integrin-dependent mechanotransductive processes and signalling.^{32,47,61,66–70} (Fig. 1a). Indeed, asperities

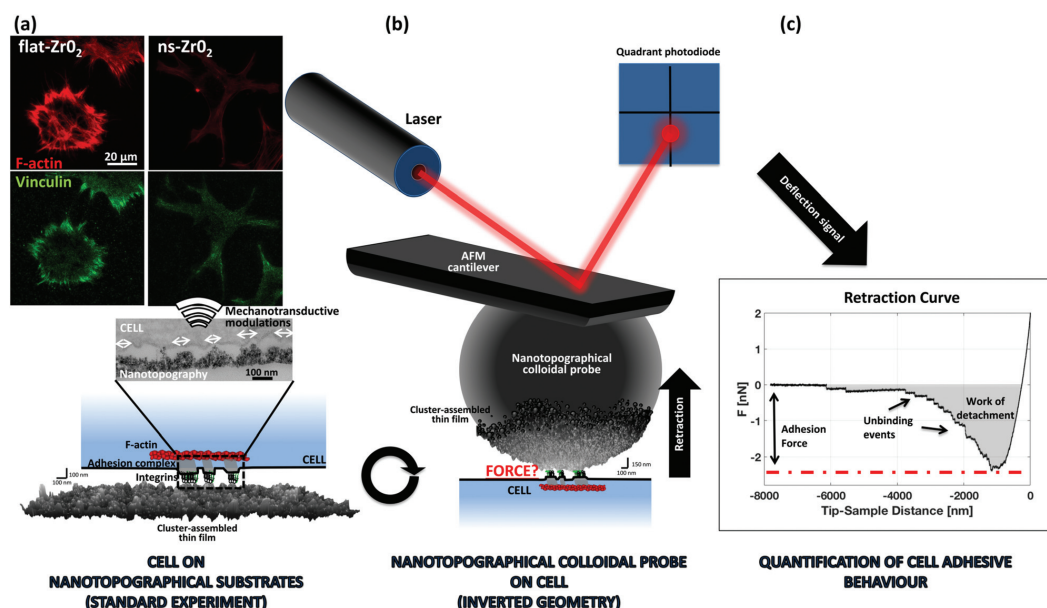


Fig. 1 Approach of adhesion force spectroscopy by means of colloidal probes functionalised with extracellular matrix-mimicking nanopotographical features. (a) The scheme at the bottom left illustrates the conventional approach used to study the impact of nanopotographical features on mechanotransductive processes. The image inset shows a transmission electron microscopy (TEM) image of interaction sites between a PC12 cell and the asperities of cluster-assembled ECM-mimicking nanopotographical zirconia substrate (indicated by the white arrows) with an rms roughness $r_q = 15$ nm (ns-ZrO₂), for which we have shown that it induces mechanotransductive modulations.⁴⁷ The immunofluorescence (IF) images in the panel above (in green, total internal reflection recordings of vinculin staining; in red, epifluorescence recordings of the actin filaments marked by phalloidin staining) demonstrate that, after 4 hours of interaction on the nanopotographical ns-ZrO₂ substrate, only small punctate focal complex size adhesion sites formed and stress fibres were absent, whereas on the flat zirconia (flat-ZrO₂), focal adhesions and stress fibres are present.⁴⁷ TEM and IF images were adapted from Schulte *et al.*⁴⁷ (b) Schematic illustration of the approach to measure adhesion forces to colloidal probes with a nanopotographical surface ($r_q = 15$ nm) produced by SCBD (the scale bars close to the nanostructured surfaces refer only to the nanopotography, other graphical icons are symbolic and not in scale). (b and c) Examples of the deflection signal extracted with the AFM and used to quantify the cellular adhesive behaviour in response to different nanopotographies.

and pore sizes of ECMs with reticular, crosslinked structures are well represented by the nanopotographical features of the ns-ZrO₂, but also in those ECMs whose structure is dominated by fibrillar features, there are often irregularities at the nanoscale, *e.g.* with regard to the distances and heights of fibres.^{6,47}

Using these nanostructured thin films as substrates (Fig. 1a), we have recently shown that the interaction of cells, in particular neuronal cells, with the nanopotographical features impacts decisively mechanotransductively relevant events, such as FA maturation, cytoskeletal organisation/mechanics and integrin signalling, as well as the cellular program and differentiative behaviour.^{47,61,66,67,71}

Instead of plating the cells on nanostructured substrates, here we exploited nanopotographical colloidal probes (nt-CPs) to stimulate the cells and to characterise the interfacial integrin adhesion dynamics by means of adhesion force spectroscopy (Fig. 1b, c). Compared to our previous experiments, this approach provided access to phenomena that occur at the cell-microenvironment interface that would otherwise be buried below the cell body.

To date, only a few protocols have been developed to functionalise AFM probe surfaces either with ECM proteins, like collagens, laminins, or cellulose nanofibers^{72,73} or with nanoparticles.^{74–76} In the former case, natural biomaterials were typically deposited onto CPs, but poor or null control over the nanoscale topography was possible. In the latter case, AFM standard sharp tips were decorated with nanoparticles in order to study their interaction with cells; in other experiments the AFM tip apex was used to model a single nanoparticle.⁷⁷ Our nt-CPs were instead used to study the early steps of the cell adhesion to an ECM-mimicking nanopotography with the sensitivity of adhesion force spectroscopy measurements. Following this innovative approach, we were able to follow the temporal evolution of early integrin-related adhesion events at the interface between the cell and a nanopotographical microenvironment at the level of single adhesion events and piconewton force range. To the best of our knowledge, in this framework of the cell/nanopotography interaction such a resolution has not been achieved to date.

2. Results and discussion

2.1 Characterisation of the nanopotographical colloidal probes

Fig. 2 shows the representative AFM images of a clean borosilicate glass sphere before (Fig. 2a) and of the same sphere after deposition of the ns-ZrO₂ film (Fig. 2b) used for the production of a nt-CP (Fig. S1†).

Ns-ZrO₂ grows on the curved CP surface, as it does on conventional flat smooth substrates (see the ESI for details, Fig. S2†).

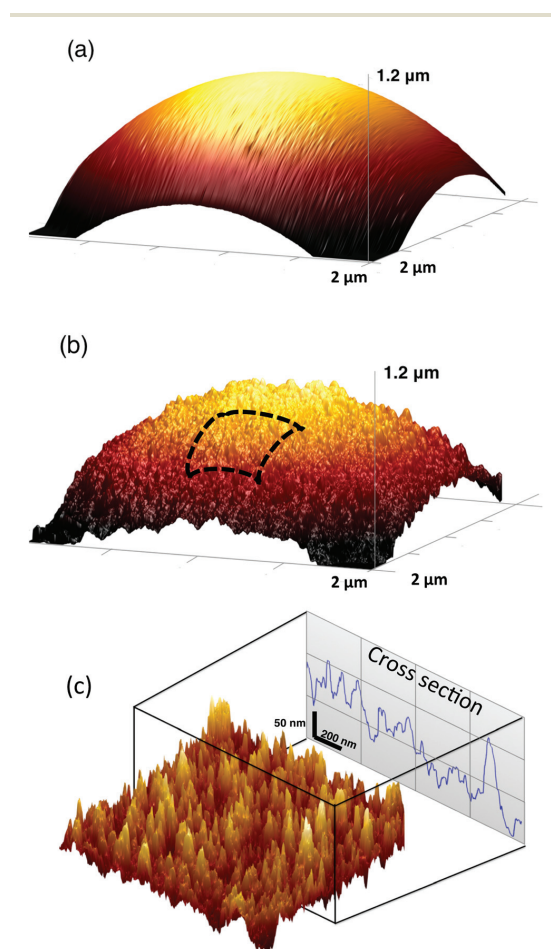


Fig. 2 Comparison of the surface morphology of the colloidal glass probes before and after the deposition of the zirconia nanoclusters. The surface morphology characterised by AFM of a borosilicate glass sphere. (a) Before, and (b) after the deposition of ns-ZrO₂ ($r_q = 15$ nm). (c) A higher resolution AFM image (the baseline curvature was removed) from the nt-CP shown in (b); the cross section highlights distinct peaks and valleys at the nanoscale.

The structure and morphology of these cluster-assembled films result from the random stacking and aggregation of impinging nanometer-sized building blocks (the ZrO₂ clusters) into larger and larger units. The surface profiles of nanostructured zirconia films are characterised by peaks and valleys (see Fig. 2c), defining a complex random pattern of nanoscale features, whose dimensions and spatial distribution resemble those found in natural ECM topographies.^{5,6} The specific surface area, the rms roughness, the average lateral dimensions of the largest morphological features (the correlation length ξ), and the interfacial porosity of the films typically increase with film thickness.^{62,63,78} The interfacial open pores, delimited and defined by the surface asperities,⁴⁷ can accommodate proteins (including fibronectin and vitronectin, which are present in the serum supplement of the culture medium we used) and nutrients;^{33,78–81} asperities evolve in height, area, and surface charge density.⁸²

In this work, we concentrated our attention on nt-CPs with the rms roughness of the ns-ZrO₂ film $r_q = 15$ nm. This particular value of r_q was chosen because we recently demonstrated that it induces in PC12 cells mechanotransductive modulations at the level of integrin adhesion complexes and cytoskeleton (Fig. 1a), as well as differentiative events, such as neuritogenesis, and a vast change in the cellular program.^{47,61,67}

2.2 Cell adhesion dynamics at nanopotographical interfaces

To investigate in which way nanopotographical features influence the characteristics of cellular adhesion processes, we performed adhesion force spectroscopy on PC12 cells with four CPs, possessing different types of functionalisation:

- (1) ns-ZrO₂-coated CP, produced by SCBD, with $r_q = 15$ nm (surface: ns-ZrO₂).
- (2) flat ZrO₂-coated CP, produced by ion gun sputtering, without nanopotographical features, $r_q < 1$ nm (surface: flat-ZrO₂).
- (3) borosilicate glass CP (surface: glass).
- (4) poly-L-lysine-coated CP (surface: PLL).

The choice of the PC12 cells as a cellular system and the surface conditions were based on our previous work, where we found that the PC12 cells formed FA and stress fibres on PLL-coated glass and flat zirconia substrates, whereas on the ns-ZrO₂ ($r_q = 15$ nm) substrate the adhesion sites remained at focal complex dimensions and the stress fibre formation was reduced⁴⁷ (Fig. 1a). PLL coatings represent the canonical substrate condition for PC12 cell experimentation, and are also routinely used to facilitate protein absorption and cell adhesion to solid surfaces (in particular glass) in biological applications (by providing positively charged sites favouring electrostatic interactions). The untreated glass CP instead served as a (negative) control because PC12 cells adhere scarcely to nude glass.

The dependence on the contact time ct of the measured parameters (maximum adhesion force, work of detachment, number of unbinding events for jumps and tethers, see Fig. S4†) is discussed in the next paragraphs.

Maximum adhesion force F_a . Fig. 3a shows that the cells are capable of creating stronger adhesion on all functionalised surfaces, compared to the untreated glass (PLL vs. glass, $p < 0.05$, except 0 s; ns-ZrO₂ vs. glass, $p < 0.05$, except 0 s; flat-ZrO₂ vs. glass, $p < 0.05$, except 0 s and 60 s). Nevertheless, the extent of these differences compared to the reference glass surface, and the temporal evolution of the adhesion, are different on each surface.

On glass and flat zirconia, F_a rapidly reaches a plateau, although on flat-ZrO₂ the final value is higher. On ns-ZrO₂ and

PLL, F_a follows a similar growing trend in the first 60 s, reaching much higher values than flat-ZrO₂ (both $p < 0.05$). PLL is the only surface where adhesion increases during the whole time interval, achieving its maximum values at 240 s (with the highest value of all the conditions). On the nanotopographical surface, the F_a maximum is reached at 120 s, and then adhesion decreases to a value similar to that on flat-ZrO₂.

Work of detachment W . The temporal evolution of the work of detachment W provides additional information about the level of complexity and the maturation of the cellular adhesion

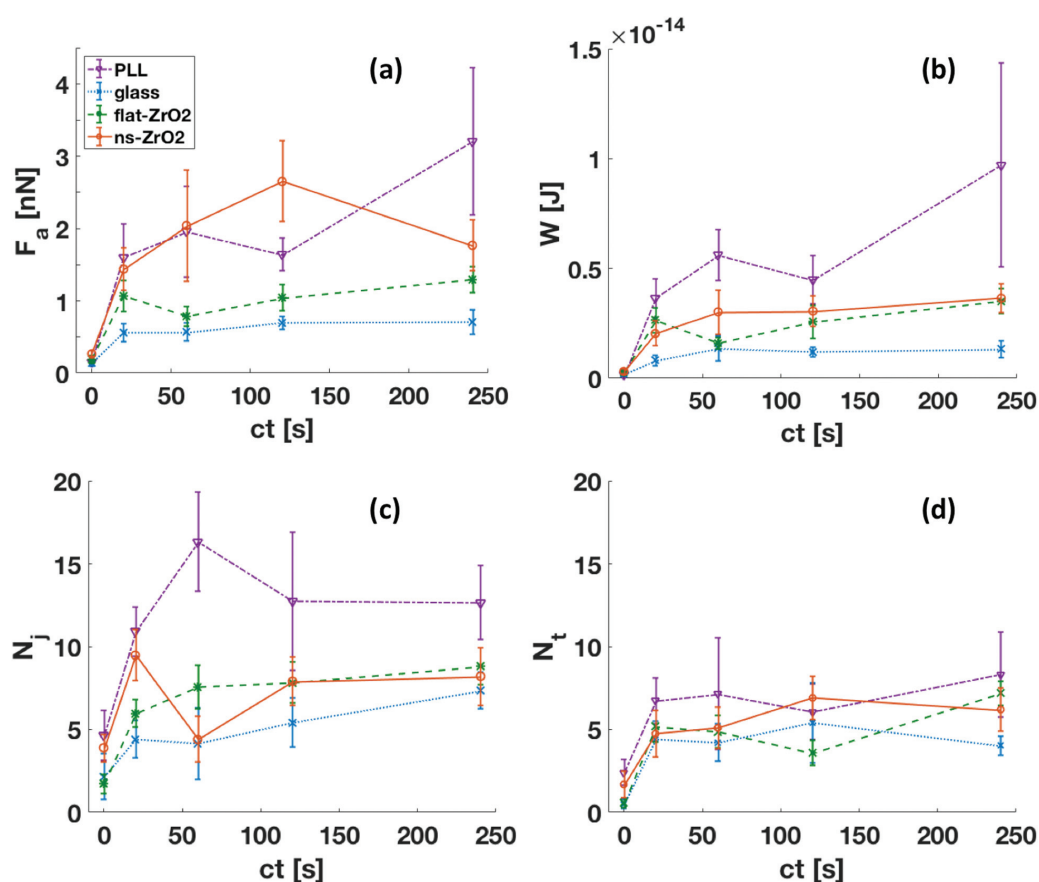


Fig. 3 The nanotopographical features provoke specific effects on the adhesion dynamics, in particular with respect to jump events. The panel shows the results of the adhesion spectroscopy measurements performed with the four different probe surface conditions: untreated (glass, blue line) or with PLL functionalisation (PLL, violet line), zirconia films, flat (flat-ZrO₂, green line) or with nanotopographical features with a roughness $r_q = 15$ nm (ns-ZrO₂, orange line). Four different parameters were extracted from the force curves (see Fig. 1c and Fig. S4†) which have been analysed for 5 different cell/probe contact times (ct = 0 s, 20 s, 60 s, 120 s and 240 s), i.e. the maximum adhesion force (F_a), work of detachment (W), mean number of unbinding events, identified as jumps (N_j) or tethers (N_t) (for details see Fig. S4†). (a) Dependence of the adhesion force F_a and (b) work W on the contact time. F_a of PLL and ns-ZrO₂ are significantly higher ($p < 0.05$) than that of glass for all cts (except 0 s) and higher than flat-ZrO₂ at 60 s (ns-ZrO₂ also at 120 s); flat-ZrO₂ > glass at 20 s, 120 s, and 240 s ($p < 0.05$). W of PLL is higher than that of glass at 120 s and 240 s ($p < 0.05$). (c) N_j counted per contact time; N_j for PLL is higher than ns-ZrO₂ and flat-ZrO₂ at 20 s, 60 s and 240 s ($p < 0.05$), at 20 s ns-ZrO₂ is higher than flat-ZrO₂ ($p < 0.05$), and N_j of ns-ZrO₂ at 20 s is higher than that at 60 s ($p < 0.05$). (d) Mean number of tethers per contact time. The error bars represent effective standard deviations of the mean (details in the ESI†).

(Fig. 3b). While the trends of W for glass and PLL-coated glass are similar to those of F_a (significantly higher at 120 s and 240 s, $p < 0.05$), this is not the case for zirconia surfaces; while the evolution of F_a was different for the two zirconia surfaces, the evolution of W is similar. Moreover, the measured work for both flat-ZrO₂ and ns-ZrO₂ never reaches the value of the PLL-coated glass. Since the work is a force times a distance, a different trend of W compared to F_a can be attributed either to different numbers of bonds (detected as unbinding events), or to different bond lengths, or both.

Mean number of unbinding events N_j , N_t . The mean number of detected unbinding events for cells interacting with different surfaces (Fig. 3c and d) revealed distinct differences in the temporal adhesion dynamics between PLL, ns-ZrO₂ and flat-ZrO₂, in particular with respect to the jump events N_j , which we discuss first (Fig. 3c).

The jump events in force curves are predominantly attributed to receptors in the membrane that are anchored to the cytoskeleton (e.g. integrins in molecular clutches *via* talin).^{51,54–57,83–85}

PLL-coated glass (Fig. 3c) and ns-ZrO₂ show a similar progression of N_j in the first 20 s (with both reaching 10 events), after which they develop differently. The difference between the ns-ZrO₂ and the flat-ZrO₂ condition is significant at 20 s ($p < 0.05$). The N_j of PLL reaches a maximum at 60 s, and also later on the cells created significantly more jump adhesion spots on the PLL compared to flat-ZrO₂ and ns-ZrO₂ ($p < 0.05$ at 60 s and 240 s).

Under the nanotopographical surface condition, N_j decreases strongly (–53%) from 20 s to 60 s ($p < 0.05$). Intriguingly, this drop is a recurrent and specific phenomenon that appears systematically for all investigated cells interacting with the nanotopographical surface. Under the flat-ZrO₂ condition, there is instead a progressive rise of N_j in the first 120 s (reaching 8 events).

The evolution of the number of tethers N_t (Fig. 3d) instead is more similar for all four conditions.

The nature of the tethers has been poorly investigated, but they are usually associated with receptors that are not anchored to the internal actin cortex, which results in membrane extrusion from the cell reservoir.^{83–85} Another hypothesis is that tether events could, at least partially, be related to the unfolding of glycocalyx sugar chains.⁸⁶ It has been demonstrated that the tethers do not respond as a catch bond,⁵⁵ and also in our experimental set-up they seem to participate in a negligible manner to the maturation of the adhesion (with a generally low contribution to F_a , see ESI, Fig. S6†), showing only minor divergent reactions towards the different surface conditions.

In the following, we will concentrate our attention mainly on the N_j parameter.

The interaction time window between 20 and 120 s seems to be the most interesting for the dynamics of the adhesion spots on different surfaces, in particular when looking at the combined evolution of the different parameters (e.g., F_a and N_j).

PLL and ns-ZrO₂ have a comparable development of F_a from 20 to 60 s, whereas N_j evolves in a converse manner, *i.e.* it increases markedly for PLL, and decreases for the nanotopographical surface (Fig. 3a and c). Glass and flat-ZrO₂ instead show more moderate alterations.

In order to better investigate these dynamical phenomena, we have calculated the mean adhesion force per jump $\langle F_j \rangle$ (see the ESI for details, Fig. S6†). The result is shown in Fig. 4. An interesting outcome specific for the ns-ZrO₂ surface is visible. While the average force per single jump (between 20–120 s) is similar for all the surfaces without nanotopographical features, *i.e.* glass, PLL, and flat-ZrO₂, a sudden 3.3-fold increase of the single jump strength ($p < 0.05$ for ns-ZrO₂ 20 s vs. 60 s; $p < 0.05$ for the comparison between flat-ZrO₂ and ns-ZrO₂ at 60 s and 120 s) is evident for ns-ZrO₂. At the regime, after 240 s, the force $\langle F_j \rangle$ converges down to the value of the other surfaces, nevertheless it remains significantly higher compared to glass and flat-ZrO₂ for most of the time.

Interestingly, these divergent dynamics happen in the critical time window for molecular clutch reinforcement, the initiation of integrin clustering and nascent adhesion growth.^{55,87}

2.3 Adhesion dynamics to the nanotopographical probe are influenced by the availability of activated integrin and depend, at least partially, on $\beta 1$ integrin

Due to our previous data on the effects of the nanotopography on the configuration of integrin adhesion sites⁴⁷ and the interesting chronology of the jump adhesion events and force development observed for the ns-ZrO₂ surface, we tested in which

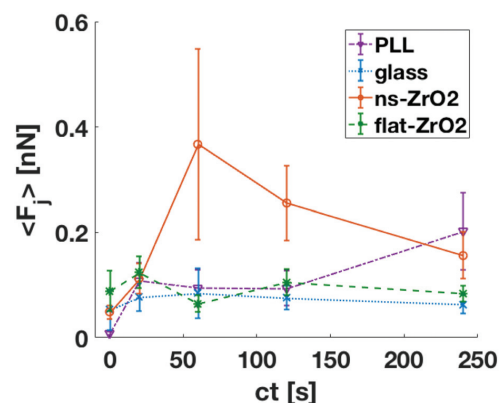


Fig. 4 Nanotopography-specific increase in jump adhesion force loading. The graph shows the evolution of the mean jump force $\langle F_j \rangle$ in dependence of the contact times for the different probe surfaces (glass: blue line; PLL: violet line; flat-ZrO₂, green line; ns-ZrO₂, orange line; the difference between ns-ZrO₂ 20 s vs. 60 s is significant ($p < 0.05$) and ns-ZrO₂ is higher than the other surfaces at 60 s and 120 s ($p < 0.05$). The specifics of the $\langle F_j \rangle$ calculation can be found in the ESI (Fig. S6†). The error bars represent effective standard deviations of the mean (details in the ESI†).

way these dynamics towards the nanotopography depend on the integrin activity.

In the first step, we examined how an excess of integrin activation would affect the early adhesion dynamics towards the nanotopographical surface. To this purpose, we activated the integrins with Mn^{2+} . This treatment kept N_j from dropping after 60 s ($p < 0.05$), as was observed on the ns-ZrO₂ surface without Mn^{2+} activation (Fig. 5a).

This impact of the abundant availability of activated integrins might be explained by two effects (or a combination of

both). In nascent adhesions, Mn^{2+} -induced integrin activation is known to increase the density within integrin clusters;⁸⁸ moreover, unligated activated integrins could favour the bridging between separated, but adjacent adhesion sites.⁸⁹ In any case, the force loading can be distributed over more active integrins, confirmed by the decrease of mean force per jump event at 60 s due to the Mn^{2+} treatment (Fig. 5b, $p < 0.05$ for 60 s), which stabilises the adhesion sites.

Furthermore, we treated the cells with an allosteric inhibitory antibody against $\beta 1$ integrin (4b4) to see how this would impact N_j . As shown in Fig. 6, in the presence of the 4b4, N_j decreased at all contact times (from 36% (ct = 60 s) to 60% (ct = 240 s), $p < 0.05$ for all cts). Since $\beta 1$ integrin represents the most common, but not the only, β integrin subunit, this result demonstrates that the recorded jump interaction events depend, at least partially, on the activation of $\beta 1$ integrin subunit-containing integrin receptors. This outcome is in line with the involvement of the ($\beta 1$) integrin activation and signalling in the nanotopography-sensitive modulations in PC12 cell mechanotransduction and differentiative behaviour (in particular neuritogenesis) we have previously reported.^{47,67}

Altogether, these results indicate that the availability of activated integrins seems to be an influential regulatory factor for spatial sensing of adhesion sites at the nanoscale.

These results provide new open questions in regard to nanotopography mechanosensing to be addressed in the future. It has been shown that the interaction of distinct integrin types with ECM ligands contributes differently, and in a force-dependent manner, to mechanotransduction.^{90–95} Although the inhibition of the $\beta 1$ integrin subunit covers a variety of ligand specificities, further experiments (e.g. with other inhibitory integrin antibodies or RGD peptides) are needed to dissect more precisely how integrins with different

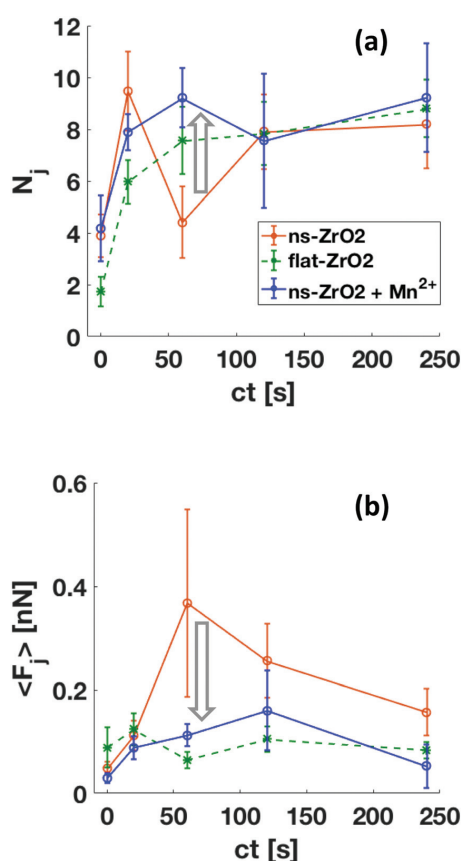


Fig. 5 Mn^{2+} -Induced global integrin activation averts the nanotopography-specific drop in jump events and excessive force loading. The graphs demonstrate the (a) mean number of jumps N_j detected on the ns-ZrO₂ surface before (orange) and after (blue) Mn^{2+} treatment (1 mM for >10 min to obtain global integrin activation) versus the contact time, and the (b) mean jump force $\langle F_j \rangle$ measured on ns-ZrO₂ before (orange) and after (blue) Mn^{2+} treatment versus the contact time. The flat-ZrO₂ values (green, reproduced from Fig. 7) are also shown for the sake of comparison. After the Mn^{2+} treatment, N_j was higher and $\langle F_j \rangle$ was lower at 60 s (both $p < 0.05$) in the ns-ZrO₂ condition, highlighted by the grey arrows. The error bars represent effective standard deviations of the mean (details in the ESI†).

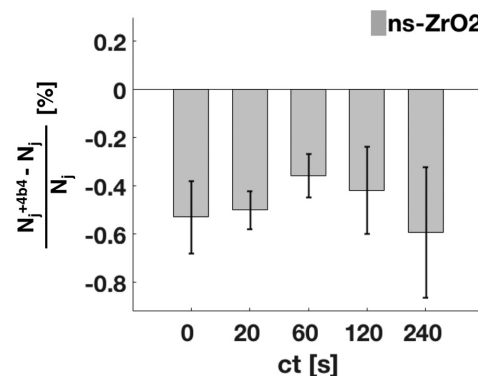


Fig. 6 Inhibition of $\beta 1$ subunit-containing integrins strongly reduces the number of jump events. The graph shows the relative change [%] of the mean number of jumps N_j in the ns-ZrO₂ condition after the 4b4 antibody treatment (5 $\mu\text{g mL}^{-1}$) compared to the untreated ns-ZrO₂. All changes are significant ($p < 0.05$). The error bars represent the effective standard deviations of the mean (details in the ESI†).

ligand affinities are involved in nanotopography mechanosensing. In addition, nt-CPs were not functionalised with a specific ECM ligand (the same is true for the substrates used in our previous experiments with PC12 cells^{47,61,67}), but due to the presence of serum in the medium, *e.g.* fibronectin and vitronectin can adsorb onto the probe surfaces (the >30 min pre-incubation of the probes with the medium before starting the measurements was done exactly for this reason). It will be important to study in the future (by coating the probes *e.g.* with RGD peptides, laminins, fibronectin, vitronectin, or collagens) in more detail how specific ECM ligands modulate the adhesion dynamics as a function of the topography. These combined experiments will lead to a better understanding of the contribution of different integrin types and ECM ligands to nanotopography-dependent mechanotransduction, and how the force loading dynamics is involved in this.

2.4 Evolution of jump force distribution over time

For a better understanding of the dynamics of the adhesion force development on different surfaces, we analysed in greater detail the distribution of the measured strengths of the single jumps at different contact times *ct*.

At *ct* = 0 s, the distributions of jump forces are similar for all surfaces, with a median value detected around 40 pN for zirconia surfaces and 20 pN for glass and PLL (see Table 1). The immediate appearance of a force in this range is consistent with the recently shown very fast integrin adhesion response (in that case for $\alpha 5 \beta 1$ integrin/fibronectin binding).⁵³ The range is also compatible with the reported peak tension of single integrin–ligand bonds during initial adhesion^{54,96–100} and furthermore, it coincides with the force thresholds for the extension of the talin rod, vinculin recruitment and molecular clutch reinforcement.¹⁰¹

With the increase of *ct*, we observed that on glass the median jump force remains almost constant, with the appearance of few events at higher forces around 120 pN (Fig. 7a). On PLL (Fig. 7b), a constant increase of the jumps forces can be noted, even if the lower force values (around 20 pN) remain always present. The distribution of flat-ZrO₂ (Fig. 7c) is similar, although the events are more scattered and there are more counts at higher forces at small contact times.

On ns-ZrO₂ (Fig. 7d), the distribution of forces, already after 20 s, shifts towards higher forces, even above 200 pN. High force bonds (>100 pN) appear almost with the same frequency (~45%) as the weaker ones (<100 pN) at every contact time (0 s excluded).

Table 1 Most probable jump force at *ct* = 0 s

Surfaces	Most probable jump force at <i>ct</i> = 0 (pN)
Flat-ZrO ₂	42 ± 15
ns-ZrO ₂	36 ± 13
Glass	21 ± 10
PLL	19 ± 17

Regarding the adhesion force F_a (Fig. 3a), this increased strength of the single jumps actually compensates for the lower number of adhesion spots compared to the PLL-coated glass (in particular at *ct* = 60 s, see Fig. 3c). In other words, on the nanotopographical surfaces the adhesion spots are exposed to higher forces. The decrease of high-force unbinding events on ns-ZrO₂ observed at *ct* = 240 s, with respect to earlier *cts*, is compatible with the general decrease of F_a (Fig. 3a).

We point out how the increased occurrence of higher-force events (Fig. 4 and 7) happens simultaneously with the drop of N_j (Fig. 3c), which depends on the availability of activated integrins (Fig. 5), under the ns-ZrO₂ condition. This is peculiar, in particular because it happens during the critical time window for nascent adhesion growth and integrin clustering.⁸⁷ These complex nanotopography-specific adhesion dynamics are not explainable by increased protein adsorption because of the higher roughness of the nanostructured surface (as reported by Scopelliti *et al.*⁸⁰ and Gailite *et al.*,⁷⁹ partially due to the increase in surface area and nanoporosity). If ligand adsorption were the decisive factor, we would expect for the flat-ZrO₂ versus ns-ZrO₂ comparison similar dynamics as we have observed for the glass ± PLL situation. The observed temporal course of the nanotopography-specific adhesion dynamics could rather indicate that too small and/or too separated adhesion sites (critical thresholds for integrin clustering have been determined to be ≥60–70 nm) disintegrate, due to exposure to excessively high forces. Instead, when the conditions are suitable, bigger clusters of integrin form and succeed to reinforce and mature^{58,59,68–70} (Fig. 8). These clusters might need to exceed at least the size of the minimal adhesion unit of a few integrins in sufficiently close, *i.e.* tens of nanometres, vicinity;^{89,102} which is consistent with the appearance of the second peak at 120 pN.

These results are compatible with the dimensions of the nanotopographical features and the confinement action at the nanoscale, provided by the small contact area offered by the asperities of the nanostructures and the distance between them.

We have demonstrated previously that the PC12 cells are in contact only with the apical part of the nanotopographical asperities, which restricts the size of the adhesion sites to the nanometric level, and inhibits the maturation of bigger adhesion structures on a larger scale (see Fig. 1a). Analyses of TEM images (an example in Fig. 1a) showed that the contact areas between the cells and the asperities of the nanotopography (with $r_q = 15$ nm) have an average width of 53.2 ± 48.0 nm (median: 40.4 ± 21.6 nm). Moreover, the distances (mean: 99.1 ± 101.4 nm, median: 60.4 ± 30.4 nm) between the asperities of the disordered nanotopography effectively oscillate around the critical ligand spacing threshold (≥60–70 nm).⁴⁷ The results reported in this work are consistent with the previous observations, and confirm, at the level of single binding events and at the pN scale, the crucial role of force loading in the molecular clutches in the nascent adhesions to accomplish nanometric spatial sensing of adhesion sites, reported recently by Oria *et al.*¹⁹

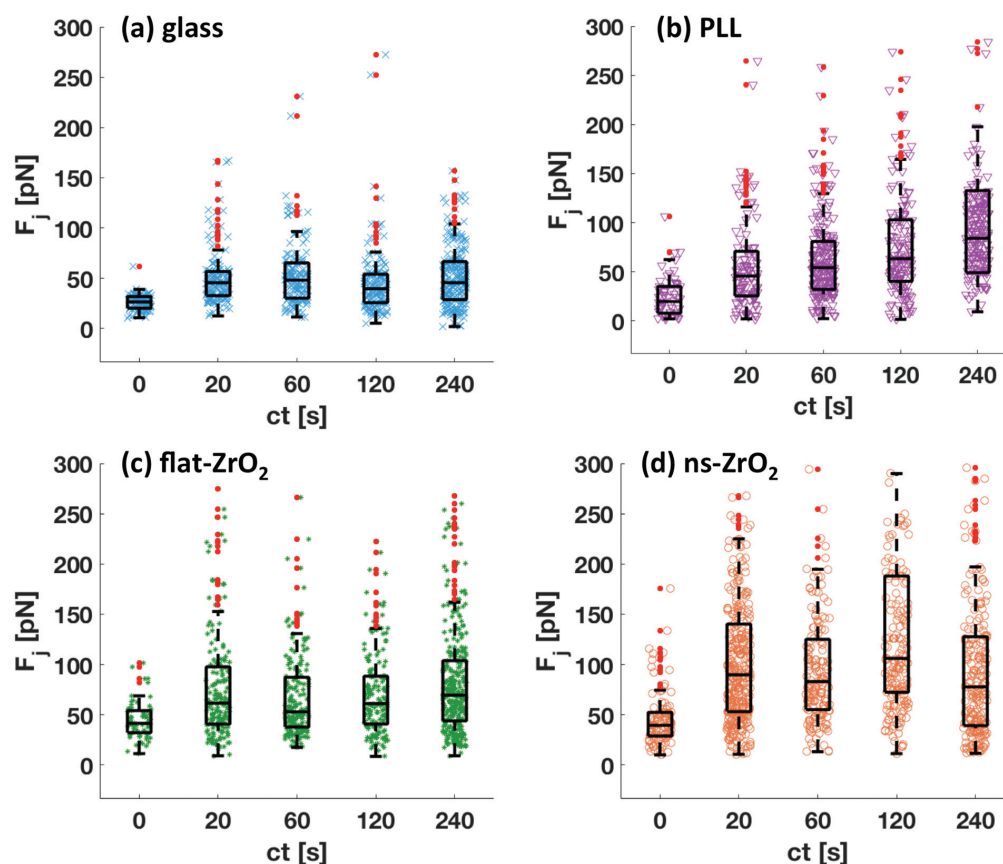


Fig. 7 Early and persistent occurrence of high jump forces specific for the nanotopography condition. This panel plots the evolution of the distribution of the forces of the single jump events (symbols) for the different contact times for (a) glass (blue), (b) PLL (violet), (c) flat-ZrO₂ (green bars) and (d) ns-ZrO₂ (orange). The boxes indicate the inter-quartile range (with the base representing the first and the top the third quartile, respectively), the lines in the boxes show the median, and the dashed lines highlight the highest and lowest values (red dots mark the outliers).

3. Materials and methods

3.1 Fabrication and calibration of the colloidal probes

Fabrication of the colloidal probe. The procedure for the fabrication of colloidal probes is based on the approach described in detail in ref. 103. Borosilicate glass spheres (Thermo Fisher Scientific), with radius $R = 10 \pm 1 \mu\text{m}$, are first cleaned to remove surface contaminants. The cleaning procedure consists of three sequential 60 seconds centrifugations (10 000 rpm) in a 1 : 1 water and ethanol solution, carefully replacing the old with new solution after each centrifugation. The cleaned spheres are then dispersed in toluene and deposited on a microscopy glass slide coated with a thin Au film (with thickness 100 nm) deposited by sputtering. The Au film is used to reduce the capillary force between the sphere and the substrates with respect to that between the sphere and a tipless cantilever¹⁰³ (Micromash HQ:CSC38/tipless/no Al, force constant $k = 0.02\text{--}0.03 \text{ N m}^{-1}$). The capture of the sphere by the canti-

lever is done using the XY motorised stage of the AFM microscope, integrated in the optical inverted microscope. The probe is then transferred in a pre-heated high-temperature oven and kept for 2 hours at 780 °C. This temperature is slightly below the softening point of borosilicate glass, which is qualitatively defined as the temperature at which a solid object begins collapsing under its own weight. After two hours, the microsphere is covalently attached to the cantilever. Due to the monolithic character of the resulting CP, any gold residue, as well as other contaminants, can be effectively removed by washing the probe in aqua regia (a mixture of nitric and hydrochloric acids, in a molar ratio of 1 : 3), or any other aggressive solution.

Determination of the probe radius. The characterisation of the CP radius is performed by AFM reverse imaging of the probe on a spiked grating (TGT1, Tips Nano), as detailed in ref. 103. Upon scanning the CP on the spiked grating, hundreds of independent replicas of the probe apical region are

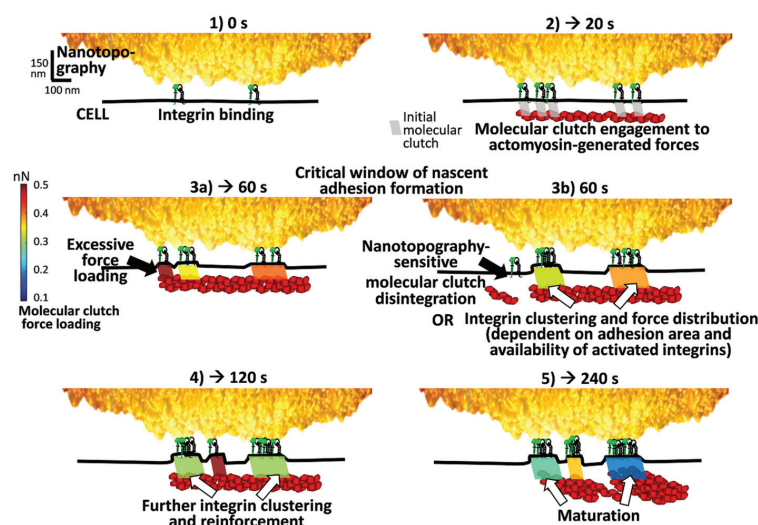


Fig. 8 Suggested mechanism (integrating the experimental results) of nanopography-dependent adhesion site disintegration, due to excessive force loading, or reinforcement by integrin clustering and force distribution. These graphics are a visual representation of our actual experimental data integrated into a potential mechanism of nanopography-sensitive force loading dynamics and consequential adhesion site disintegration or reinforcement. (1) After the almost immediate binding of integrins (0 s contact time), (2) the molecular clutches engage to the actomyosin-generated forces (~ 20 s). (3a) This leads to increasing force loading within the molecular clutches (20–60 s) in the critical window of nascent adhesion formation. (3b) Depending on the nanotopographical conditions (*i.e.* existing adhesion area and distance to other adhesion sites) and the availability of active integrins (see Mn^{2+} treatment, Fig. 5), either adhesion site disintegration (excessive force loading because of insufficient integrin clustering, black arrows) or reinforcement (integrin clustering and force distribution, white arrows) is induced around 60 s. (4, 5) Where the nanotopographical conditions are suitable the adhesion sites are sustained further on and mature (white arrows). The colours of the parallelogram icons (symbolising the force loading within molecular clutches due to the engagement of the integrins and the actomyosin-generated forces) code for the force intensities measured by means of our approach at the different contact times (see the colour bar on the left of the middle row, compare values with Fig. 4). In the schemes decisive events are highlighted, such as integrin binding, the molecular clutch engagement to actomyosin-generated forces, integrin recruitment/clustering, force distribution, and integrin adhesion complex (IAC) reinforcement/maturation. The length scales on the top left refer to the morphology of the nt-CP only; the other graphical objects (such as integrins) are not in scale and also their number is symbolic to visualise the development.

obtained. From the measured geometrical properties (like the volume V and the height h), it is possible to determine the value of the radius R by fitting a spherical cap model $V = \frac{\pi}{3} h^2 (3R - h)$ to the data. The evaluated probe radius has an accuracy as good as 1%.

Calibration of the cantilever spring constant. The spring constant is calibrated using the thermal noise method^{104,105} where a special correction factor is applied in order to take into account the relevant dimension and mass of the glass sphere.¹⁰⁶ For large CPs, the conditions that both the dimension and mass of the sphere are small compared to the length and mass of the cantilever are not always satisfied. Since the mass of the microsphere scales with the cube of the radius R , beads with radii larger than $5 \mu\text{m}$ possess a mass comparable to the mass of the cantilever, and in some cases, especially with stiffer, shorter cantilevers, even larger. These conditions lead to the failure of the assumption that the mass of the cantilever is uniformly distributed along its length, resulting in an underestimation of the spring constant.

According to ref. 106, it is possible to correct the apparent spring constant K_0^{th} measured by the thermal noise method using the formula:

$$K^{\text{th}} = \frac{\beta}{\beta_0} K_0^{\text{th}}$$

where K^{th} is the corrected spring constant, while for rectangular cantilevers $\beta_0 = 0.817$. β depends on the reduced mass of the sphere $\tilde{m}$, *i.e.* the ratio of the mass of the sphere m_s to the mass of the cantilever m_c , and on the reduced gyration radius of the sphere $\tilde{r}$, proportional to the ratio of the radius R of the sphere to the length L of the cantilever. For small tips, $\tilde{m}$ and $\tilde{r}$ are negligibly small, and $\beta = \beta_0$, therefore $K^{\text{th}} \cong K_0^{\text{th}}$. In our case, since $L = 350 \mu\text{m}$, $m_c = 2.65 \times 10^3 \text{ ng}$, and $m_s = 1.05 \times 10^3 \text{ ng}$, we had $\tilde{m} = 0.4$ and $\tilde{r} = 0.03$, therefore corrections were needed.

3.2 Production of nanotopographical and reference CPs

Deposition of the ns-ZrO₂ film on the CP. For the production of nt-CPs, ns-ZrO₂ films are deposited on the colloidal probes

exploiting an SCBD apparatus equipped with a pulsed microplasma cluster source (PMCS)^{107,108} (see ESI, Fig. S1†). Partially oxidised zirconia clusters are produced within the PMCS and then extracted into the vacuum through a nozzle to form a seeded supersonic beam. Clusters are collected directly on the CPs intercepting the beam, in the deposition chamber. Upon landing on the probe surface, which is locally flat due to the large radius, clusters form a nanostructured, highly porous, high-specific area, biocompatible ns-ZrO₂ film.^{47,61,62,82,109} The oxidation of the nanostructured film further proceeds upon exposure to air, up to an almost complete stoichiometry, although rich of local defects. The crystalline phase is cubic at room temperature.⁶⁵

The root mean square (rms) roughness r_q of the deposited film, defined as the standard deviation of surface height values, evolves with the film thickness h , according to the power law^{62,63} $r_q \sim h^b$, where $b = 0.35$ approximately (details about the evolution of the roughness–thickness relation and on the mechanical stability of the nanostructured thin film can be found in the ESI, Fig. S2 and S3†).^{63,65} Therefore, by controlling the thickness of the film deposited using a quartz microbalance placed inside the deposition chamber, it is possible to produce nanotopographical CPs with very high reproducibility. The typical thickness of the ns-ZrO₂ films deposited on CPs was in the 70–250 nm range, corresponding to roughness values in the range of 13–20 nm.

Deposition of smooth ZrO₂ films on the CP. Thin, compact and very smooth coatings of ZrO₂ (flat-ZrO₂) with the rms roughness below 1 nm were deposited by ion sputtering on the CPs, in order to produce reference interacting surfaces, without any nanotopographical cues. To this purpose, a Kaufman ion gun (Cyberis 40-f) was used to sputter a Zr target. The produced coating is partially oxidised in the deposition chamber; oxidation further proceeds upon exposure to ambient air.

Production of poly-L-lysine-coated CPs. Also for reference purposes, CPs were coated with poly-L-lysine (PLL). PLL is a poly-amino acid routinely used to facilitate protein adsorption and the attachment of cells to solid surfaces in biological applications, including our previous experiments with PC12 cells.^{47,67} For the PLL coating, the probes were incubated with a 0.1% (w/v) PLL solution (Sigma-Aldrich) at room temperature for 30 min, and washed thoroughly afterwards with milliQ water several times before the measurements.

3.3 Force spectroscopy experiments and data analysis

Force spectroscopy. The force spectroscopy experiments were performed using a Bioscope Catalyst AFM (Bruker). During the AFM measurements, the temperature of the medium was maintained at 37 °C using a perfusion stage incubator and a temperature controller (Lakeshore 3301, Ohio, USA). The colloidal probes were incubated with the cell culture medium for >30 min at 37 °C before the actual measurements.

The deflection sensitivity was calibrated *in situ* and non-invasively before every experiment by using the previously characterised spring constant as a reference, according to the

SNAP procedure described in ref. 44. The standard approach, *i.e.* pressing the probe on a stiff surface and measuring the inverse of the slope of the force curve in the contact region, could likely cause contamination of the nt-CP surface and damage of the nanotopographical asperities. Moreover, friction-dependent issues can influence the accuracy of the determination of the deflection sensitivity by the standard contact method, when using large CPs.¹¹⁰

Sets of raw deflection *versus* approaching distance curves were acquired at locations on the cells' body selected by means of an optical microscope. The raw curves were converted into force *versus* distance curves (shortly force curves, FCs), rescaling the deflection axis by multiplication by the deflection sensitivity and the cantilever spring constant, and summing the cantilever deflection to the Z-piezo displacement axis¹¹¹ (see Fig. 1b, c and S4†). FCs containing 8192 points each were recorded on cells, with ramp length $l = 8 \mu\text{m}$, maximum load $F_{\text{max}} = 1 \text{ nN}$ and retraction speed at $v_r = 16 \mu\text{m s}^{-1}$. The pulling speed was kept relatively low to reduce hydrodynamics effects.¹¹²

To measure the early steps of cellular adhesion, we selected five contact times (cts): 0, 20, 60, 120, 240 s, accordingly. During the contact time, the Z-piezo position was kept constant using the Z closed-loop feedback mode. Long contact times require a very stable system; this condition is obtained by means of both an active anti-vibration base (DVIA-T45, Daeil Systems) and an acoustic enclosure for the AFM (Schaefer Italy), by controlling the environmental temperature in the laboratory, and by allowing for a long equilibration time (30 min, which served also to allow protein adsorption from the medium, such as fibronectin and vitronectin which are present in the serum supplement, to the probe) before starting the experiments. Fig. S5† shows that the drift along the vertical direction during contact of the CP with the sample is very small (max. 20 nm in 240 s), and determines a negligible variation of the applied force.

To reduce the stress on the cells and not to alter their adhesive behaviour, a maximum applied load of 1 nN, corresponding to a pressure of the order of 10 Pa, was set during the acquisition of the FCs. For the same reason, the number of FCs collected per cell was limited (not only because of the very long acquisition time of each curve).

Data analysis. Data processing of the sets of curves was carried out in the Matlab (Mathworks) environment using custom routines.

The values of several parameters were calculated for the analysis of the cell–probe detachment mechanisms (Fig. S4†). In particular, we inferred the maximum cell–probe adhesion force F_a , defined as the maximum force needed to start the detachment of the probe from the cell, with respect to the zero level of the baseline. The work of detachment W required to separate the nt-CP from the cell, W describes the energy dissipated during the detachment and is calculated by integrating the area between the zero level and the profile of the force *versus* distance curve. The maximum detachment force F_a depends on different properties of the cell, such as overall

rigidity, cortex tension, cell shape, single binding strength and spacing. Compared to F_a , W provides more complete, integrated information about the cell adhesion, related to the numbers, lengths and strengths of all bonds formed between the nt-CP and the cell. Moreover, we extracted the mean number of $N_{j,t}$ and strength $F_{j,t}$ of the single unbinding events, defined as the difference in force measured before and after the detaching event, of every FC measured³² (for both jumps j and tethers t). The unbinding events must be identified in the retraction section of the FCs:^{113,114} we exploited the numerical derivative of the curves with respect to the tip-sample distance¹¹⁵ (see ESI, Fig. S4†) to detect the location of the events.

Statistics and error analysis. Mean values ψ_{mean} and associated errors σ_{mean} (based on the standard deviation of the mean) were calculated for each observable $\psi(F_a, W, N_{j,t}, \dots)$, as described in detail in the ESI.† These values and errors represent a population of cells in a given condition. To this purpose, first mean values and errors have been calculated for the single cells tested, and then these values have been averaged and the resulting error calculated.

Every condition/probe has been tested twice, in two separated days. For every condition/probe at every experimentation day, 5 cells per contact time have been measured by collecting 3 FCs on each cell. The reason behind this procedure is that the measurements for obtaining complete datasets for a long interaction time (*i.e.* contact time) are quite time consuming. Therefore, we decided to collect this number of force curves per condition to be able to secure at each day a consistent and complete volume of data, and to repeat the measurements during the second day under the same conditions for statistical purposes. Every condition/probe has thus been tested twice, in two separated days, with physically different, yet equivalent, in terms of surface properties, probes. This results in 10 cells per probe/condition per contact time, *i.e.* 30 single force curves per probe condition per contact time. Once we have tested the equivalence of sets of data acquired under the same conditions in different days, we have averaged over the collection of equivalent FCs (*i.e.* the 30 FCs acquired with the same probe/condition, the same contact time). In the case of inhibition of the $\beta 1$ integrin activity 7–10 cells per condition per contact time have been tested.

We performed two tailed *t*-tests for assessing the significance of differences of mean values, as indicated in the main text, where we report the *p* values. For the sake of clarity of the figures, significance levels were only mentioned in the captions, but not indicated in the figures.

3.4 Cell culture and preparation for the force spectroscopy experiments

As a cellular model we used neuron-like PC12 cells (*i.e.*, in particular the PC12-Adh clone, ATCC catalogue no. CRL-1721.1TM). The choice of this cellular model was made due to the fact that we knew from previous studies that these cells interact with ns-ZrO₂ films and react with mechanotransductive responses to the provided nanotopographical stimuli (*i.e.* $r_q = 15$ nm).^{47,61,67}

For routine cell culture (subculturing every 2–3 days), the cells were kept in an incubator (Galaxy S, RS Biotech) at 37 °C and 5% CO₂, in RPMI-1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 units mL⁻¹ penicillin, 1 mM pyruvic acid, and 100 μ g mL⁻¹ streptomycin (all reagents from Sigma Aldrich, if not stated otherwise).

For the force spectroscopy experiments, the cells were detached from the cell culture flasks with trypsin/EDTA solution, counted with an improved Neubauer chamber, and plated in a low concentration of 4000 cells per cm² (to guarantee the presence of single separated cells) on Ø 40 mm glass-bottom dishes for cell culture (Willco Wells) the day before the experiment. Phenol-red free solutions were used for the experiments, since this molecule was found to be harmful for the AFM tip holder. Directly before the cell plating, the Ø 40 mm glass-bottom dishes were coated with PLL (incubation with a 0.1% PLL solution for 30 min at RT, followed by several washing steps with PBS) and sterilised with UV light for 10 min. After cell plating, the cells were kept overnight in an incubator to guarantee good cell attachment before the force spectroscopy experiments.

For the integrin activation, the cells were pre-incubated with manganese chloride (MnCl₂) at a concentration of 1 mM for >10 min before the measurements (the treatment has been labelled Mn²⁺ in figures). In the case of inhibition of the $\beta 1$ integrin activity, the cells were pre-incubated with the inhibitory 4b4 antibody (Beckman Coulter) at a concentration of 5 μ g mL⁻¹ for >15 min before the measurements (the treatment has been labelled 4b4 in the figures).

4. Conclusions

Integrin-mediated mechanosensing and mechanotransduction are regulated by the biophysical properties of the cellular microenvironment, such as *e.g.* the nanotopography of the ECM, as it influences the spatial and geometrical organisation of potential cellular adhesion sites.^{7–10,30} However, a precise understanding of the spatiotemporal dynamics and molecular actions at the cell/microenvironment interface during adhesion is still elusive, because these events take place at the nanometre scale and involve pN range forces, whose exploration requires sophisticated methodologies. Moreover, an accurate control over the nanotopographical features of the microenvironment is essential, in order to systematically investigate and precisely assess the influence of the different nanotopographical motifs on the mechanotransductive process.

In this framework, we were able to study and quantify the impact of the microenvironmental nanotopography on early cellular adhesion events by means of adhesion force spectroscopy based on novel colloidal probes mimicking the nanotopography of natural ECMs. Our approach merges the sensitivity of AFM-based force spectroscopy^{38,51} with the possibility of controlling the nanotopographical features of the ECM-

mimicking substrate provided by Supersonic Cluster Beam Deposition.⁶¹

Thanks to our innovative approach, we could detect nanotopography-specific modulations of the molecular force loading dynamics and integrin clustering at the level of single binding events, in the critical time window of nascent adhesion formation. Following this approach, we found that the availability of activated integrins is a critical regulatory factor for these nanotopography-dependent dynamics.

Our results are in agreement with the reported importance of force loading¹⁹ for cellular spatial sensing of the microenvironment, and integrin nanocluster bridging between adjacent (tens of nm) adhesion arrays.⁸⁹

In the current work, we compared flat and nanostructured zirconia with the specific roughness parameter $r_q = 15$ nm, *i.e.* the two conditions which have shown the most divergent cellular responses in PC12 cells in terms of their impact on differentiative and mechanotransductive processes, according to our previous work.^{47,61,67} After this proof-of-principle, which demonstrated that we can detect meaningful differences with this approach, we plan to compare nanotopographies with more subtle variations in the roughness parameter in the future. For this purpose we can exploit the fine control of the surface topography of the nanostructured colloidal probes to dissect in more detail the precise role of morphological properties (such as, *e.g.*, roughness, asperity diameter/distance, or correlation length) in spatiotemporal cell adhesion dynamics, in particular focusing the attention on the clustering of integrin adhesion complexes⁴⁷ and force loading in the molecular clutches. Nanotopographical colloidal probes could also be used to examine how disease-related aberrations in the components of the mechanotransductive machinery alter the force loading dynamics of cellular adhesion.

Additional features of nt-CPs make them suitable for the investigation of nanoscale phenomena of nanobiotechnological interest. Due to their large area, nt-CPs allow optical probing of the interaction interface, which is particularly useful when using fluorescence microscopy and suitable markers to image the actin cytoskeleton and/or the integrin adhesion complexes. Moreover, nt-CPs could be used as nanotopographical templates for carrying out further functionalisation, for example by grafting to the corrugated surface biochemical moieties relevant to mechanotransduction, such as ligands, ECM motifs, *etc.* Apart from the cell biology framework, we think that these probes have good potential if used in physics of matter experiments, *e.g.* in the context of nanofriction,¹¹⁶ DLVO theory^{82,117} or adhesive contact mechanics at disordered, nanostructured interfaces.^{118,119}

Author contributions

Conceptualisation: CS, AP; methodology – probe fabrication and characterisation: MC, AP, CP; methodology – cell culture and preparation: TD, CS; methodology – AFM spectroscopy: MC, TD, AP; data curation and analysis: MC, CS, AP; original

draft writing: MC, CS, AP; draft reviewing and editing: MC, CS, AP, CL, PM; supervision: CL, PM, CS, AP; resources, funding and project administration: CL, PM, AP. Author contributions were allocated adopting the terminology of CRediT – Contributor Roles Taxonomy.

Conflicts of interest

There are no conflicts to declare.

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Adhesion force spectroscopy with nanostructured colloidal probes reveals nanotopography-dependent early mechanotransductive interactions at the cell membrane level

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SUPPLEMENTARY INFORMATION

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Supersonic Cluster Beam Deposition Apparatus (SCBD)

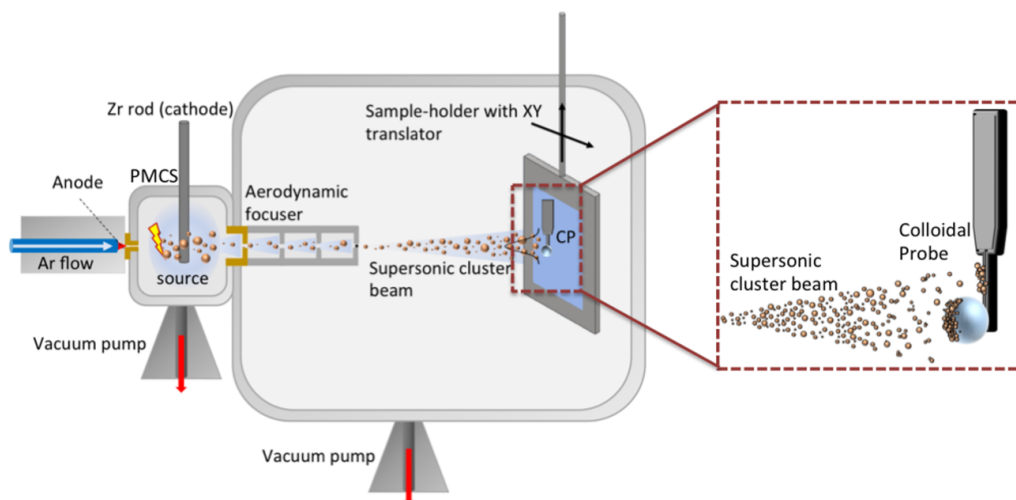


Figure S1. A schematic representation of the SCBD apparatus that was used for the production of nt-CPs. The Zr rod is sputtered by a plasma discharge, triggered by the introduction of argon (Ar) through a pulsed valve into the source cavity and the application of a high voltage between the Zr rod and the anode. The ablated species condense into clusters and the resulting gas-clusters mixture is extracted through a nozzle and an aerodynamic focuser into a high vacuum chamber; during the process, the carrier gas-clusters mixture undergoes a supersonic expansion. The cluster beam impinges on the CP, where a thin, nanostructured ZrO_2 film is formed.

Scaling of the surface roughness of ns-ZrO₂ films

To characterise the growth mechanism of ns-ZrO₂ films on colloidal probes (CPs), we dispersed the glass spheres on a flat glass microscopy slide, and deposited ns-ZrO₂ films varying the deposition times. We then imaged the coated CP surfaces by AFM in Tapping Mode (probe model: NCHV, Bruker), with relative scan speed of the tip $v_{\text{scan}} = 2 \mu\text{m/s}$, and we measured the rms roughness r_q . The film thickness h was measured on the flat glass surface, in a region close to a sphere, by imaging the ns-ZrO₂ film across a sharp step produced by masking the substrate during deposition. By applying a linear regression on a loglog scale to the r_q versus h curve (see Figure S2), the value of the growth exponent b can be determined as the slope of the curve, according to the equation $r_q \sim h^b$.

According to previous results¹⁻³, the b parameter of the cluster-assembled ZrO₂ thin films grown on flat substrates is $b = 0.368 \pm 0.001$ on silicon, or $b = 0.31 \pm 0.09$ on glass coated with a monomolecular PAcrAm-g-(PMOXA, NH₂, Si) layer⁴. These values are compatible with the prediction of the ballistic deposition growth model ($b = 0.32 - 0.25$), which assumes that clusters impinge with a direction perpendicular to the plane of the substrate, and they do not diffuse significantly upon landing⁵⁻⁷. Higher values can be found when the impinging particles possess a distribution of size and different sticking probabilities.

On nt-CPs, we found $b = 0.314 \pm 0.017$, in agreement with the value measured on flat substrates. Therefore, the curvature of the nt-CPs did not influence the growth exponent.

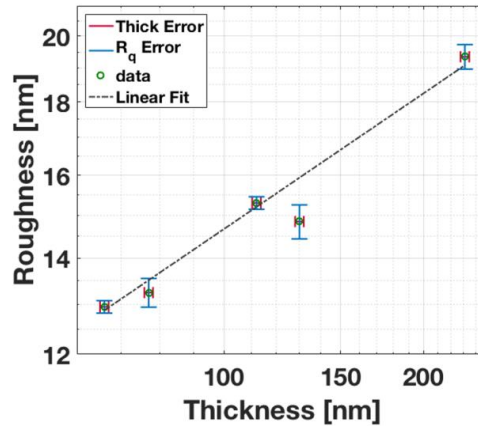


Figure S2. Scaling of the rms roughness r_q of ns-ZrO₂ film (i.e. roughness) on the nt-CPs.

Mechanical stability of ns-ZrO₂ films

To test the mechanical stability of the nanostructured coating we used a stiff AFM tapping mode probe (force constant $K = 50.4 \text{ N/m}$) to apply high forces on the thin film, in order to record at which forces rupture events between ZrO₂ nanoparticles take place.

An example of the force curve (FC) with the rupture events detected is shown in Figure S3a, while the rupture forces measured are represented in Figure S3b. Rupture forces are clustered around specific values and it is possible that the higher forces events represent cascade rupture events, where groups of nanoparticles simultaneously detach, while the lower forces represent the tip slipping across a nanoparticle, or small nanoparticles detaching from low-attachment points. The lowest rupture force detected is around $F \sim 70 \text{ nN}$, that is almost 25 times larger than the highest force measured during the force spectroscopy experiment.

Furthermore, we scanned in Tapping Mode the surface of the contact region of an nt-CP after a whole day of force spectroscopy experiments. The image obtained is shown in Figure S3c, after subtraction of the spherical curvature in order to highlight the morphological details at the nanoscale. The granularity of the surface due to the presence of the ns-ZrO₂ thin film is clearly evident.

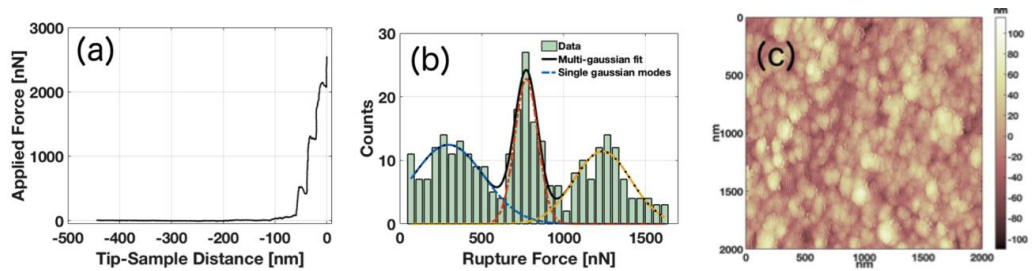


Figure S3. (a) Representative FC with three rupture events detected; (b) distribution of the rupture event forces from all FCs, with multi-gaussian fit highlighted; (c) scan of the contact surface of a nt-CP after force spectroscopy experiment.

Data Analysis

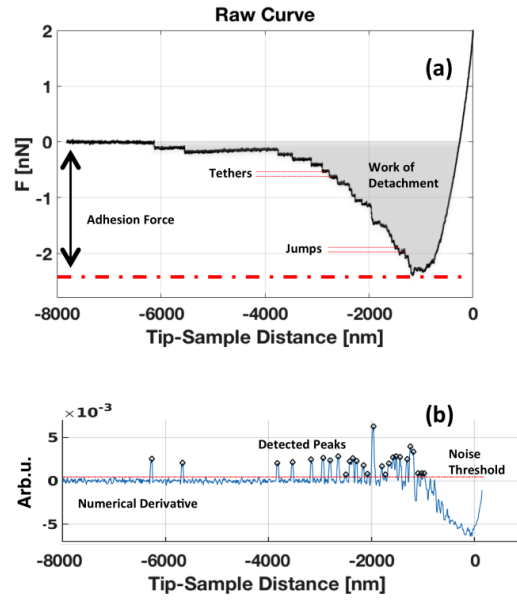


Figure S4. (a) The retraction part of a representative FC, with the two different possible unbinding events, jumps and tethers, total adhesion force and work of detachment shown. (b) Numerical derivative of the FC used to identify and locate the unbinding events.

Statistics and error analysis

For each observable ψ_{FCs} extracted by each force curve (FC) a mean value ψ_{cell} was evaluated for each cell. The error σ_{cell} associated to ψ_{cell} was obtained by summing in quadrature the standard deviation of the mean σ_ψ of the results coming from the single FCs and an estimated instrumental error $\sigma_{instruct}$ ($\sigma_{instruct} / \psi = 3\%$)⁸:

$$\psi_{cell} = \frac{\sum_i^n \psi_{FCs}}{n}$$

where n is the number of force curves per each cell,

$$\sigma_{cell} = \sqrt{\sigma_\psi^2 + \sigma_{instruct}^2}$$

The final mean value ψ_{mean} representative of the cell population behaviour in a given condition was evaluated as:

$$\psi_{mean} = \frac{\sum_i^n \psi_{cell}}{N}$$

where N is the number of cells investigated for the given condition.

The final error σ_{mean} associated to ψ_{mean} was calculated by summing in quadrature the propagated error of the mean σ_s and the standard deviation of the mean of the singles cell values ψ_{cell} :

$$\sigma_{mean} = \sqrt{\sigma_{std}^2 + \sigma_s^2}$$

where

$$\sigma_s = \frac{1}{N} \sqrt{\sum_i \sigma_{cell}^2}, \quad \sigma_{std} = \sqrt{\frac{\sum_i^n (\psi_{cell,i} - \psi_{fin})^2}{N}}$$

Force curves during contact

To test the stability of the Z-piezo upon contact of the CP with the sample in close-loop mode, we recorded the cantilever deflection as a function of the contact time t , up to $t = 240$ s.

The time-dependent deflection is shown in Figure S5. On a stiff substrate, a smooth drift is observed (~ 10 nm in 240 s), corresponding to a maximum variation of the applied force of approximately 0.5 nN after 240 s. On the cell surface, a similar drift is observed superimposed to small and slow (tens of nm in tens of seconds) fluctuations, due to the adjustment of the cell below the probe, probably accompanied by internal reorganisation of the cytoskeleton⁹.

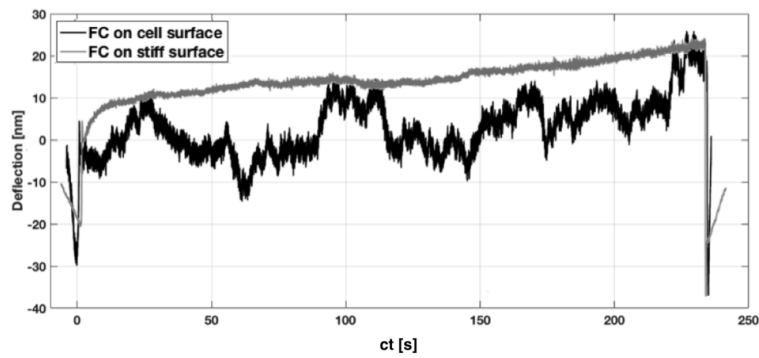


Figure S5. Representative deflection versus contact time curves.

Dependence of the adhesion force on the contact times, with the contribution of the tethers

The evaluation of the tether contribution to the total adhesion force F_a was evaluated as follow: for each contact time, the mean value of the tethers unbinding force has been evaluated and multiplied by the mean number of tethers N_t per FC, to obtain the tether background adhesion value.

The contribution of the tethers then has been subtracted from the adhesion force F_a , in order to calculate the contribution of the jumps. The obtained total jump adhesion force was eventually divided by the mean number of jumps N_j , in order to calculate the mean adhesion force per jump presented in Figures 4 and 5 of the main text.

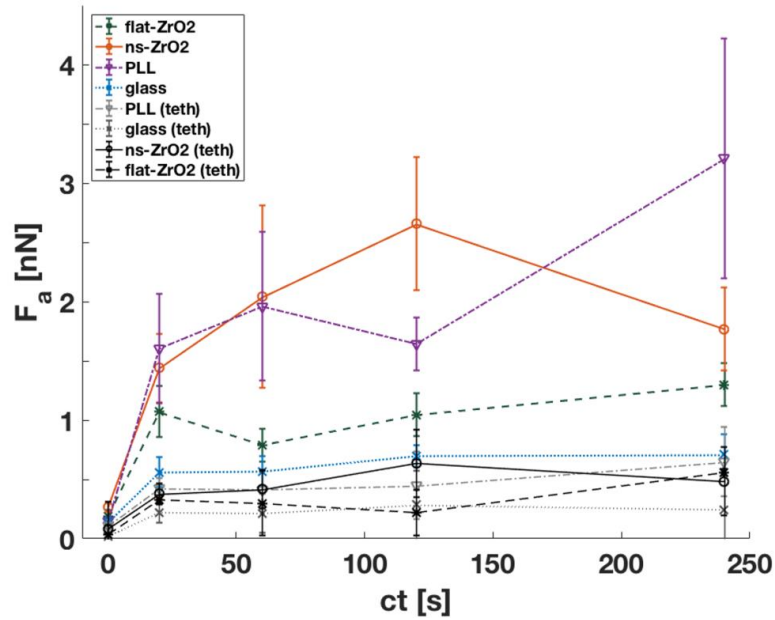


Figure S6. Dependence of the adhesion force on the contact times as in Figure 3a; in addition, the contribution of the tethers (calculated as described above) is shown.

Representative retraction force curves at different contact times

We show representative of FCs of the adhesive behaviour of the cells on the different surfaces for contact time $ct=20s$, $60s$, $120s$.

It is possible to observe the differences in the adhesion force F_a , work of detachment W , and number of jumps N_j , respectively. In particular, it can be seen how the adhesion on the ns-ZrO₂ surface (at $ct=60$ sec) reaches values compatible to the adhesion on the PLL, even in the presence of much less detachment events.

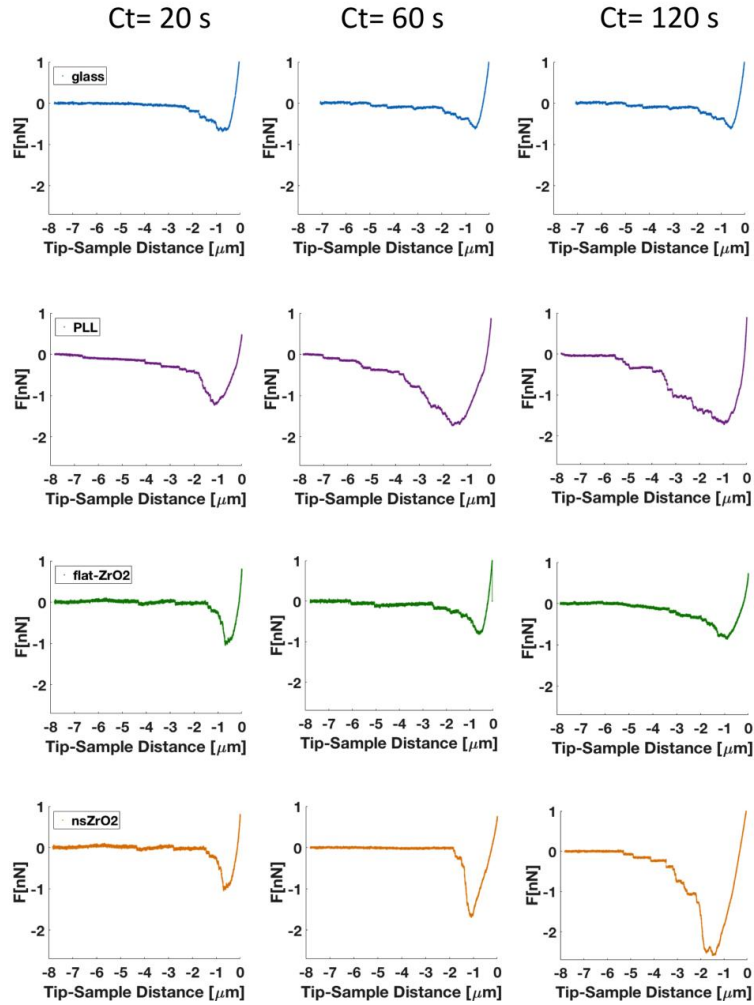


Figure S7. Representative FCs at $ct=20\text{ s}$, 60 s , 120 s for each condition: glass (blue), PLL (violet), flat-ZrO₂ (green), ns-ZrO₂ (orange).

Regarding the occurrence of jump events over a longer distance in the PLL condition, it must be pointed out that the positive charge of the PLL coating may favour on the one hand unspecific, electrostatic binding events of surface receptors, and on the other hand the polymeric nature of PLL with its long chains can unfold and be pulled away from the probe surface. This could favour detachment events occurring at higher separations, compared to the other conditions. In the PLL condition, the jump events could therefore more frequently be caused also by non-integrin surface receptors that are in some way attached to the cytoskeleton, as e.g. CD44 or syndecan-4. However, for the moment being we can only speculate about this.

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Chapter 5

The glycocalyx impacts on the ability of cells to perceive the nanotopographical features*

The glycocalyx (GC) is a biological structure located at the outer surface of the cell membrane, directly at the interface between the cell and extracellular space, where cellular receptors sense their microenvironment^{92,93}. The macromolecules that compose the glycocalyx can be divided into glycolipids, glycoproteins, proteoglycans and glycosaminoglycans (GAGs). The GC therefore shares many elements that can also be found in the ECM.

The most frequent GAG is the hyaluronic acid (HA, see Figure 16); the HA sugar chains are polymers that can be very large (molecular weight over 1 million Dalton) and can reach, if completely extended, a length even up to 10 μm ⁹⁴. Other GAGs are chondroitin sulphate (CS), heparan and keratan sulphates (HS, KS) which are covalently linked to protein scaffolds to form proteoglycans. The terminal sugar is often the sialic acid⁶¹ and the addition of sialic residues increases the negative charge at the cell surface⁹⁵.

Through its dense polysaccharide network, the glycocalyx acts as a water reserve, it regulates the transport and diffusion of small signalling molecules and ions, affecting therewith cell permeability by functioning as a sieve⁹⁶, and has a role in cell communication processes. It forms a protective barrier against the entry of pathogens and substances harmful to cells^{97,98}.

Overexpression of glycocalyx components can be involved in tumour characteristics⁹⁹, such as high motility¹⁰⁰, drug resistance and inhibition of the apoptotic pathway¹⁰¹. As predominant component situated exactly in the cell/microenvironment interface, the glycocalyx participates also strongly in the

* An advanced draft is under preparation and will be soon uploaded (as a pre-print) on BiorXiv database. The list of authors that contributed to this project are listed in the following.

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regulation of cell adhesive processes¹⁰² and it has been suggested that it contributes to mechanosensitive pathways¹⁰³.

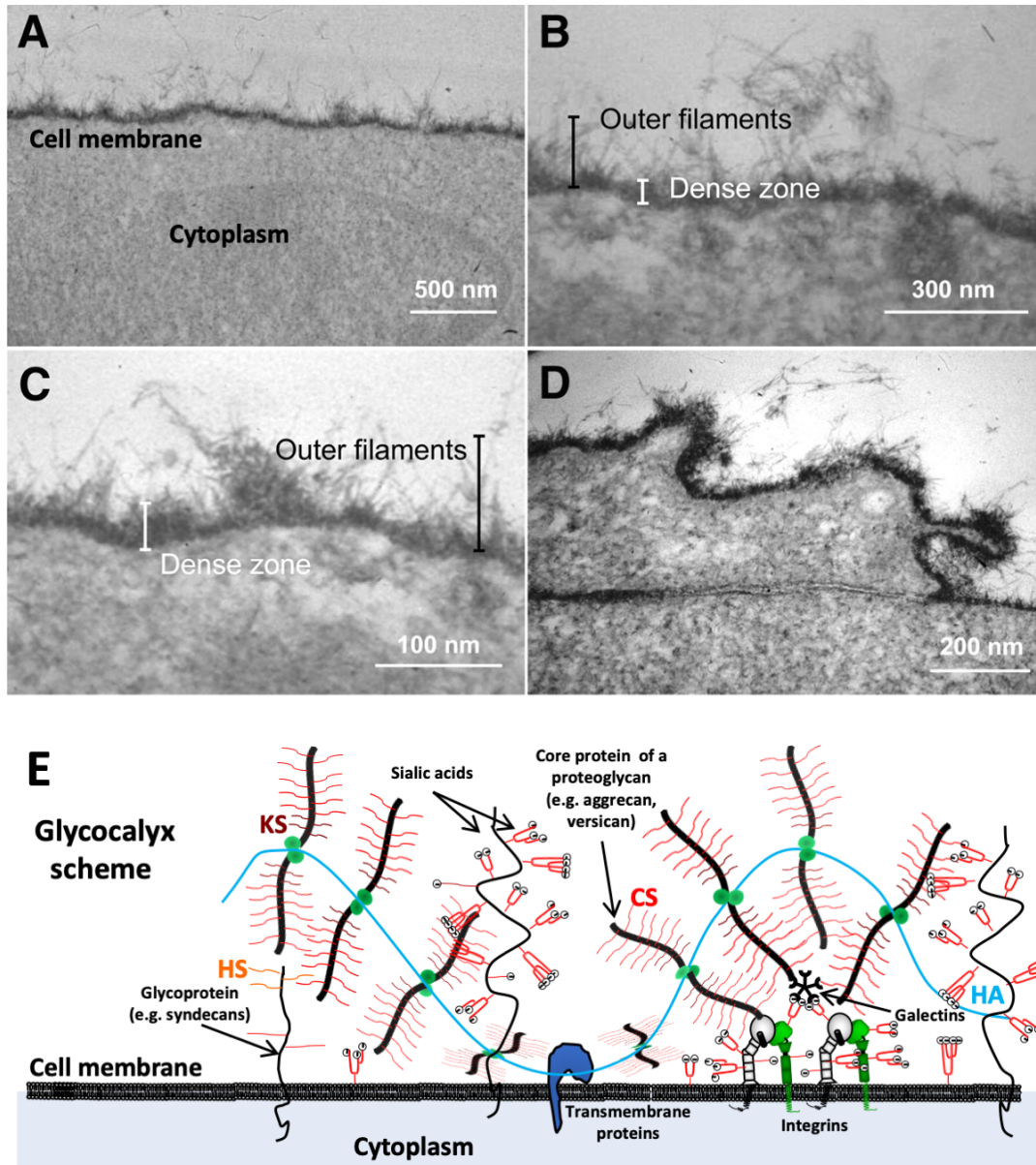


Figure 16. (A-D) SEM images of the endothelial GC on cultured HUVECs (human-umbilical-vein-endothelial-cells), image adapted from Chappel et al¹⁰⁴. (E) Scheme of GC structure and composition with the main components highlighted: Hyaluronic acid (HA), Chondroitin sulphate (CS), Keratan sulphate (KS), Heparan sulphate (HS), and Sialic acids.

The glycocalyx affects the mechanotransductive processes in a complex way, influencing the accessibility of ligands for surface receptors^{62,102} (integrins, cadherins). The thickness of the glycocalyx (from few hundreds of nm¹⁰⁵ to several μm ⁹⁴) and the net negative charge interferes during the initial approach of the cell with the ECM or substrates¹⁰².

An integrin in the active extended configuration reaches ~ 20 nm in length, therefore remaining practically submerged in the glycocalyx network. The cell has somehow to overcome this initial steric and electrostatic repulsion of the glycocalyx to begin to adhere. GC It is not an impenetrable network, though, and

once an integrin succeeds to bind a ligand, the distance between the cell membrane and the ECM is shorter in this initial adhesion spot, due to the already compressed glycocalyx, leading to a funneling of integrins in this region. Furthermore, the compressed adjacent glycocalyx exerts a repulsive force towards the cell membrane which keeps the ligated integrins tensed and thus *extended* which promotes catch bond formation^{7,62,102,106}. The overall effect is therefore a kinetic trap that favours the integrin clustering (see Figure 17). However, the GC is a complex system and its intricate role in mechanosensing and mechanotransduction is far from being understood in detail. Most studies are furthermore focused on the endothelial cells, known to express a particularly thick and easy to detect cellular brush^{94,96,103,104}. Every cell type possesses its own specific pericellular sugar coat, with a different structure, relative abundancies of the components and thickness. It seemed thus necessary to first characterize the structure of PC12 cell line's glycocalyx and the effects of several enzymes able to digest specific GAGs.

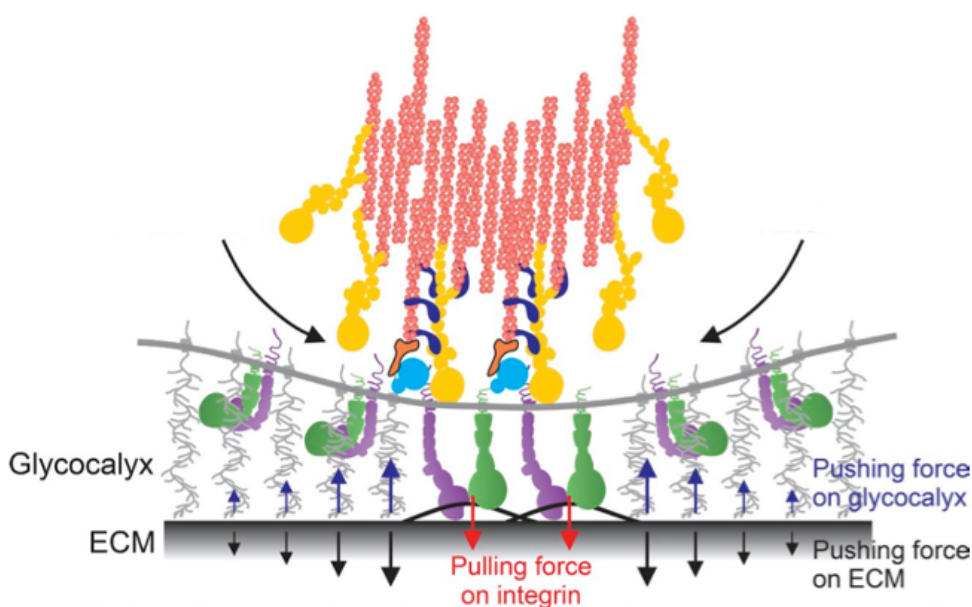


Figure 17. Representation of the kinetic trap model, once the integrins-ligand bond is established, the compressed glycocalyx pulling force keep the integrins activated and, at the same time, the pushing force of the glycocalyx at the boundaries drive the integrins accumulation.

Starting from the results presented in the previous chapter, and in the awareness of the above-mentioned model which to date has been obtained through experiments performed on flat substrates, we decided to exploit the *nt*-CPs as a scaffold to investigate whether the glycocalyx is involved in the cells' ability to react to different morphological stimuli. To this purpose, we selected three different roughness values of the nanostructured zirconia (ns-ZrO₂) thin-films to be deposited on the probes ($R_q = 0, 15, 20$ nm) and we performed AFM force spectroscopy experiments (as discussed in the Methods) on cell surfaces with and without the surface glycocalyx layer.

5.1 Methods

5.1.1 Cell culture and preparation for experiments

The experiments were performed with the neuron-like PC12 cells (PC12-Adh clone, ATCC catalogue no. CRL-1721.1TM). This cell line has shown to respond to the nanotopographical cues provided by the substrates produced by SCBD (in particular ns-ZrO₂ with roughness $r_q=15$ nm) by mechanotransductive processes and signalling, which we have profoundly characterised in previous studies^{30,32,56}. For routine cell culture (subculturing every 2-3 days using trypsin/EDTA solution), the PC12 cells were maintained in a humid atmosphere at 37°C and 5% CO₂ in an incubator (Galaxy S, RS Biotech) provided with RPMI-1640 medium (supplemented with 10% horse serum, 5% fetal bovine serum, 2 mM 1-glutamine, 10 mM HEPES, 100 units mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, 1 mM pyruvic acid). All reagents and materials from Sigma, also in the following, if not stated otherwise.

For the experiments, the cell detachment was done on the day before with an EDTA (1mM in PBS) solution to not digest the protein component of the glycocalyx. The cells were counted with an improved Neubauer chamber before plating them in a low concentration (4000 cells per cm², to have mostly single, separated cells) on the indicated substrates. The cells were then maintained in the incubator overnight to guarantee good cell adhesion.

For the experiments involving AFM measurements, the cells were seeded the day before the experiment on Ø 40 mm glass-bottomed plates (Willco Wells) that were coated before with PLL (0.1% w/v PLL solution for 30 min at RT). Solutions without phenol red were used, as it results harmful for the AFM tip holder.

For the actin dynamics measurements, one day before the experiment the cells (after LifeAct transfection) were instead plated on Ø 35 mm glass-bottomed dishes (MatTek, the Ø 13 mm glass was in the center of the plate) on which either a flat zirconia film (produced by ion gun sputtering) or nanostructured cluster-assembled zirconia films (fabricated by SCBD) have been deposited.

In the case of glycocalyx-targeting enzymatic treatment, an enzyme cocktail was applied to the cell culture medium to obtain a final concentration of 13.5 U/mL hyaluronidase II, 150 mU/mL chondroitinase ABC, 300 mU/mL heparanase and 150 mU/mL neuraminidase (these concentrations were inspired by the work of Zeng et al.¹⁰⁷). The cells were incubated with these enzymes for 2 hours right before the experiments. Following the incubation, the cell culture medium was discarded. After washing 3x with PBS (to remove the digested glycocalyx), fresh medium was added containing again the enzymatic cocktail to avoid reassembly of the glycocalyx during the measurements.

Enzyme	Target GAG
<i>Hyaluronidase</i>	<i>Hyaluronic acid (HA)</i>
<i>Heparinase</i>	<i>Heparan sulphate (HS)</i>
<i>Chondroitinase</i>	<i>Chondroitin sulphate (CS)</i>
<i>Neuraminidase</i>	<i>Sialic acids</i>

Table 1. List of enzymes used coupled with the target glycosaminoglycans aimed to digest.

5.1.2 Fabrication and calibration of nt-CPs.

Nanotopographical colloidal probes have been fabricated and calibrated as discussed in previous sections (see Materials and Methods and pg 58-59).

5.1.3 Characterization of the native glycocalyx layer of PC12 cells

Glycocalyx characterisation by wheat germ agglutinin staining and 3D-SIM. The glycocalyx of the living PC12 cells in the indicated experimental conditions was marked 30 min before the recording by adding Oregon Green™ 488 conjugate wheat germ agglutinin (WGA, Thermofisher) to the medium at a final concentration of 25 µM/mL. WGA binds sialic acid and N-acetylglucosaminyl residues. After the 30 min incubation (in the incubator at 37°C and 5% CO₂), the cells were washed 3x with PBS to remove the excess of WGA (to reduce the background fluorescence noise) and new phenol red-free medium (prewarmed to 37°C) was given to the cells (in case of glycocalyx-targeting enzymatic treatment containing again the enzymes). Then the plates were immediately transferred to the 3D-SIM Nikon A1 microscope (equipped with a stage-top incubation system (Okolab), conditions: humid, 37°C, 5% CO₂) to take the images.

The thickness of the glycocalyx determined from cell borders in the plain just above the cell/substrate interaction and analysed by means of ImageJ/FIJI¹⁰⁸ (for better distinction the “Enhance Contrast” tool was applied). The graph was obtained with the help of Plotsofdata web apps¹⁰⁹.

AFM imaging of the cellular surface. Cells were fixed with 4% paraformaldehyde (10 min) after 2 hours of incubation with the enzymes cocktail. The cell morphology was characterized using a Bioscope Catalyst AFM from Bruker. The AFM images (with scan size of 2–4 µm) were acquired in PBS buffer in Peak-Force-Tapping (PFT) mode, ramp frequency $\nu=0.5$ KHz. Because of the significant height variations within the samples (up to several micrometers), short cantilevers with a tip height of 14 µm were used (VistaProbes CS-10). The scan rate was set to 0.6 Hz, and the sampling resolution was set to 4096 × 1024 points. The accurate alignment of the optical and AFM images was obtained using the Miro module integrated in the AFM software.

AFM indentation measurements: the brush model. To get quantitative information about the thickness of the glycocalyx the Sokolov's brush model was implemented^{94,110,111}.

Looking at Figure 18 it is evident that the cell and the glycocalyx can be thought as a two-layer system with two different elastic properties. The brush model was developed with the purpose to decouple the single contributions of the glycocalyx compression and the cell deformation. As shown in the geometry of the indentation experiment (Figure 18) the **probe-membrane** distance h can be described as:

$$h = Z - Z_0 + i + d \quad (1)$$

Where Z is the relative piezo position, Z_0 is the undeformed position of the cellular membrane, d the deflection of the cantilever, which is positive when the cantilever is bent upward (in repulsive regime), and i the indentation calculated using the Hertz model

$$i = \left(\frac{3k(1-\nu^2)}{4E} R^{1/2} \right)^{2/3} d^{2/3} \quad (2)$$

Here E is the Young's modulus of the cell body, k is the spring constant of the probe, $\nu=0.5$ is the Poisson coefficient.

Assuming that the glycocalyx layer is much softer than the cell body, the cantilever reaches an indentation force where the glycocalyx can be considered completely squeezed ($h=0$) before reaching the maxim load set. This assumption depends obviously from the value of the force threshold applied, which should be sufficiently large. In this condition $(Z - Z_0) + d = -i$, as in conventional indentation experiments.

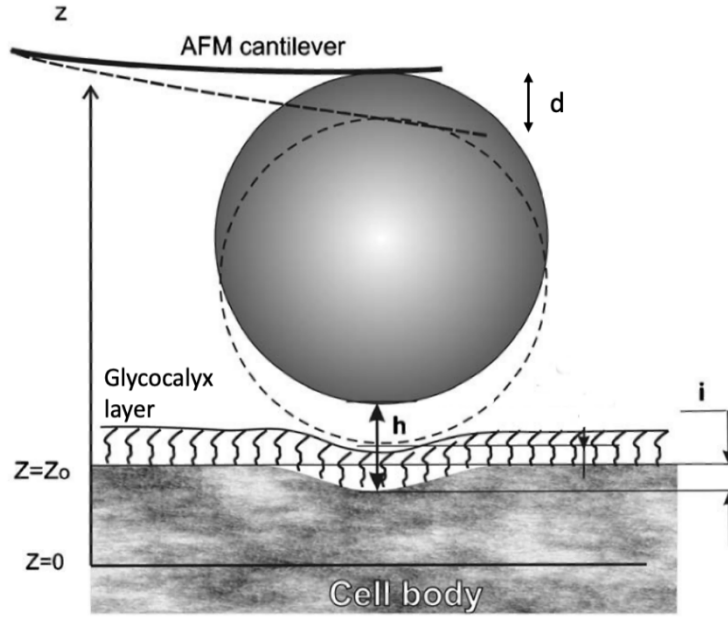


Figure 18. Interaction of the colloidal probe with the two-layer cell structure. Image adapted from Sokolov et al.¹¹⁰.

The force needed to completely squeeze the glycocalyx cannot be known a priori and must be found. Our approach was to divide the total effective deformation δ_{eff} (squeezing of the glycocalyx plus cellular deformation) in intervals $s=200$ nm and then apply the hertz model in those intervals. The idea beneath this approach is that the first mechanical response perceived by the probe would be the resistance to compression the pericellular brush, then applying more and more force the CG will squeeze and progressively transfers rigidly the applied force to the cell below. Once the GC will be completely compressed the resulting effective Young's modulus E_{eff} will stabilize to the cell body-bulk values. The E_{eff} was plotted as function of the δ_{eff} and then look a discontinuity in the E_{eff} and reaches a plateau. We interpreted the plateau as a situation where the glycocalyx is fully compressed and what is measured is the Young's modulus of the cell body. The result of this analysis made on a population of ~ 20 cells in standard conditions is represented in Figure 19.

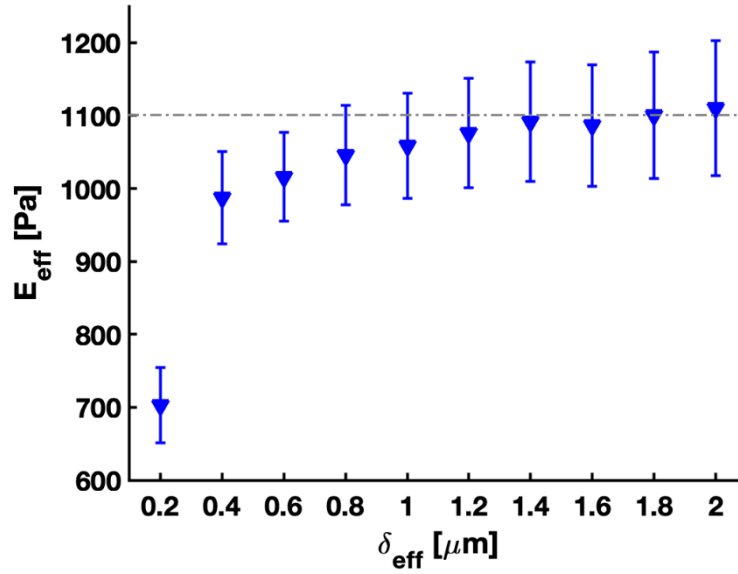


Figure 19. Effective YM E_{eff} evaluated per each intervals s as a function of the effective indentation δ_{eff} . Data represent the mean YM averaged over the cell population, while the error bars represent the standard deviation.

As it is possible to observe, after the first 200 nm a sudden increase of the E_{eff} is present. The subsequent smooth increase of E_{eff} can be attributed to inhomogeneity in the elastic properties of the cellular interior (e.g. cortex vs nucleus) and to finite-thickness effects (see Appendix B). The corresponding force threshold, at which the GC is fully compressed was set at $F_{thr} = 0.1-0.2 \text{ nN}$. Fitting the hertz model in a region just above F_{thr} (where $h \cong 0$) would provide a value of E in the superficial region, through the intersection of a back extrapolation of the hertz model and with the horizontal line ($F=0$) would give the value of the parameter Z_0 , that represents the contact point between the probe and the cell membrane. Then using Eq. 2 it's possible to evaluate the indentation i (that actually is the real deformation of the cell, beneath the GC compression). With all these parameters it is possible to calculate the probe-membrane distance h at every position of the piezo (see Figure 20).

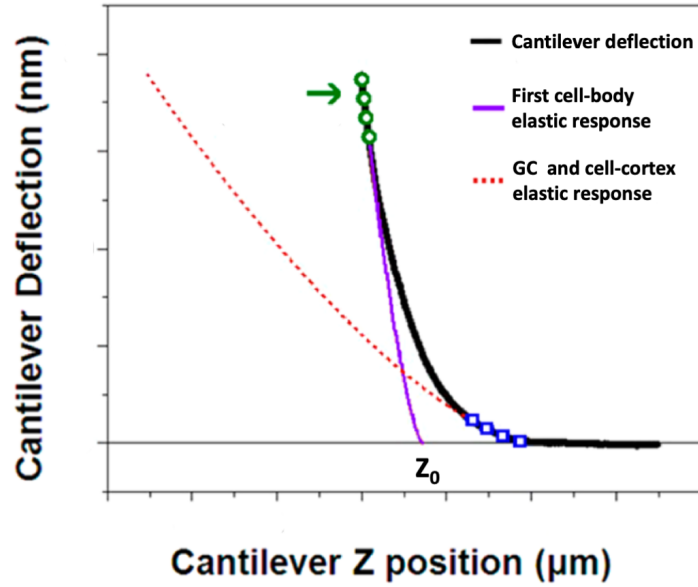


Figure 20. Representation of AFM approaching curve and fit with the brush model. The green circles show the region of the curve where the cellular YM E is fitted to extract Z_0 . The extrapolated curve with Hertz model is shown in purple solid line, while the red dotted curve represents E_{eff} , where GC and cell are compressing simultaneously. Image adapted from Simon et al¹¹².

Note that care must be taken in the choice of the indentation range for the fit of the Hertz model; as it is evident from Figure 19 the inner cell's body is anything but uniform and isotropic and this inhomogeneity is reflected by the change of the YM. When choosing a fitting region, one has to take into account that, looking at Eq. 1, the aim of the procedure is quantify the cellular deformation within the effective indentation δ_{eff} , in order to obtain residual deflection of the probe that is the effect only of the brush steric resistance. So, the YM should be extracted from the first indentation range above the threshold identified in Fig. 19, otherwise the probe-membrane distance and then glycocalyx thickness would be inaccurately estimated.

Once the probe-membrane distance is evaluated the FCs must be rescaled on this new distance as shown in Figure 21.

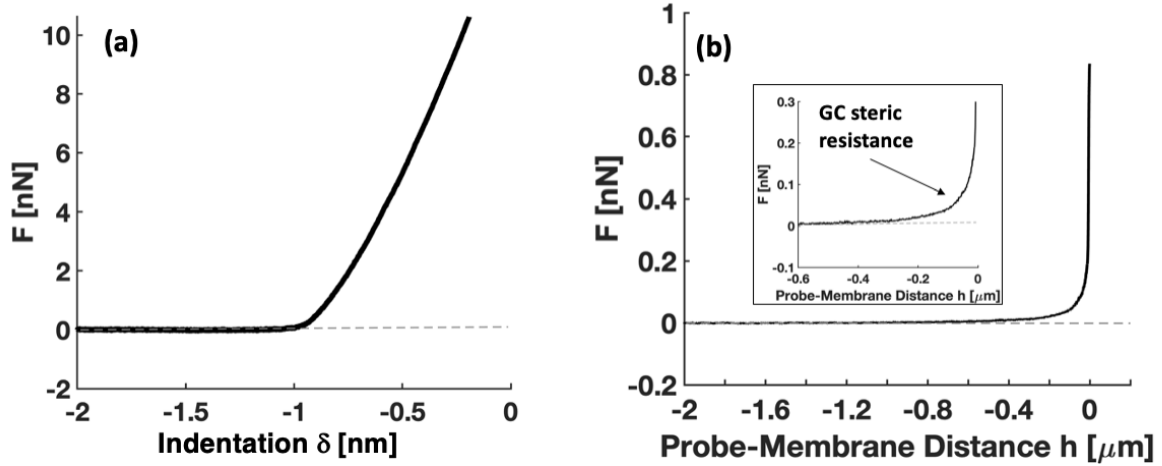


Figure 21. (a) Original Force Vs Indentation curve taken over the cell body. (b) Same curve rescaled over the new distance h . After the glycocalyx complete compression ($F = 0.2$ nN), the probe is contact with the membrane and the rest of the curve collapse on the $h=0$ vertical axis. The inset shows the appearance of the GC steric resistance few hundred if nm before the probe get into contact with the cell membrane.

The dependence of the force due to the glycocalyx compression is represented in Figure 21b ($F=F(h)$). Because of the specific force dependence and its physical nature (actually intertwined polymer chains) the glycocalyx layer cannot be described by an effective YM. The glycocalyx layer increases its stiffness during the compression. To describe the glycocalyx behaviour quantitatively the Alexander-de Gennes model was used. It describes the steric interaction between a spherical indenter of radius R and a flat surface due to the existence of superficial *entropic brush*¹¹³ :

$$F(h) = 50k_B T R N^{3/2} L e^{(-2\pi \frac{h}{L})} \quad (3)$$

Where k_b is Boltzmann's constant, T is the medium temperature, R is the probe radius, N the brush grafting density and L the brush length-thickness. From the fitting of the force rescaled data with Eq (3) it is possible to extract the thickness parameters L .

AFM force spectroscopy experiments. AFM force spectroscopy experiments has been performed as indicated in the previous chapter⁵⁶ and in Materials and method.

5.1.4 Visualisation, recording and quantification of the actin dynamics

Transfection with LifeAct and recording. To visualise the actin dynamics, the PC12 cells were transfected with mCherry-LifeAct (the vector was a kind gift by Prof. Giorgio Scita). For this purpose, the cells were plated on petri dishes (TRP)

at a low confluency (10-15%) the day before the transfection. At the beginning of the next day, the transfection was done with Lipofectamine LTX® performing the following steps:

- Solution 1: 6 µl of Lipofectamine LTX® was added to 150 µl RPMI base medium (incubation for 5 min at RT).
- Solution 2: 2 µl of the mCherry-LifeAct vector DNA (concentration 1 µg/µL) was added to 150 µl RPMI base medium, then 2 µl Plus™ reagent was added.
- The two solutions were mixed and incubated for 20 min at RT and then added dropwise to the cells in a homogeneous way.

The success of the transfection was controlled the next day with a Zeiss Axiovert 40 CFL with epifluorescence.

After transferring the transfected cells onto the indicated substrates, the actin dynamics (before and after glycocalyx removal) were then recorded the next day with a Nikon A1R confocal microscope at the UNI^{TECH} NOLIMITS Imaging facility of the University of Milan. The microscope is equipped with a stage-top incubation system (Okolab) to guarantee humidification, 37°C and 5% CO₂ during the live cell imaging. The recordings were done with a frame rate of 0.5 frames/sec for 2 minutes.

Particle image velocimetry. Particle image velocimetry (PIV) was performed to quantify the actin dynamics. This analysis results in a vector field, highlighting the local movement of the actin fibres in time, from which the average flow speed can be obtained.

Pre-processing – For each movie, a 400 x 400 px (~ 20 x 20 µm) area was selected at the cell edge. A temporal resolution of 6 s between frames was used (intermediate frames were removed).

Analysis – PIV analysis is described in detail elsewhere¹¹⁴. Briefly, a region of interest (source box) is searched for in a larger area (search box) of the subsequent frame by two-dimensional cross-correlation. A grid of regions of interest is drawn over the image, to obtain a full local tracking of the actin filaments. For this analysis, the PIV parameters were optimised as follows: source box 0.6 µm, search box 1 µm, distance between regions of interests (grid) 0.4 µm, correlation coefficient threshold of 0.3. Spatial and temporal convolutions are subsequently performed to interpolate the vector field. The defined kernels were 5 µm (sigma = 1 µm) and 30 s (sigma = 12 s), respectively. Colour maps display flow velocity within a 0-2 µm/min scale.

5.2 Results

The glycocalyx has an acknowledged and essential role in mechanotransduction, which however is rather complex and not yet fully understood. In addition, to date the abovementioned effects of the glycocalyx on mechanotransductive structures and processes were studied only on flat substrates, neglecting the relevance of the ECM morphology in this context. Indeed, the surface asperities of the nanotopography have a role in the spatial confinement of the integrins³⁰, and through this mechanism the cell perceives the morphological properties of the surrounding microenvironment. This confinement is driven also by the GC compression adjacent to the integrin adhesion sites (as discussed by Paszek et al⁶²) and therefore different GC thickness/structure could lead to a different perception of the same morphological cues, e.g., by affecting cell membrane bending and/or diffusion of integrins.

5.2.1 AFM imaging of PC12 cell surface

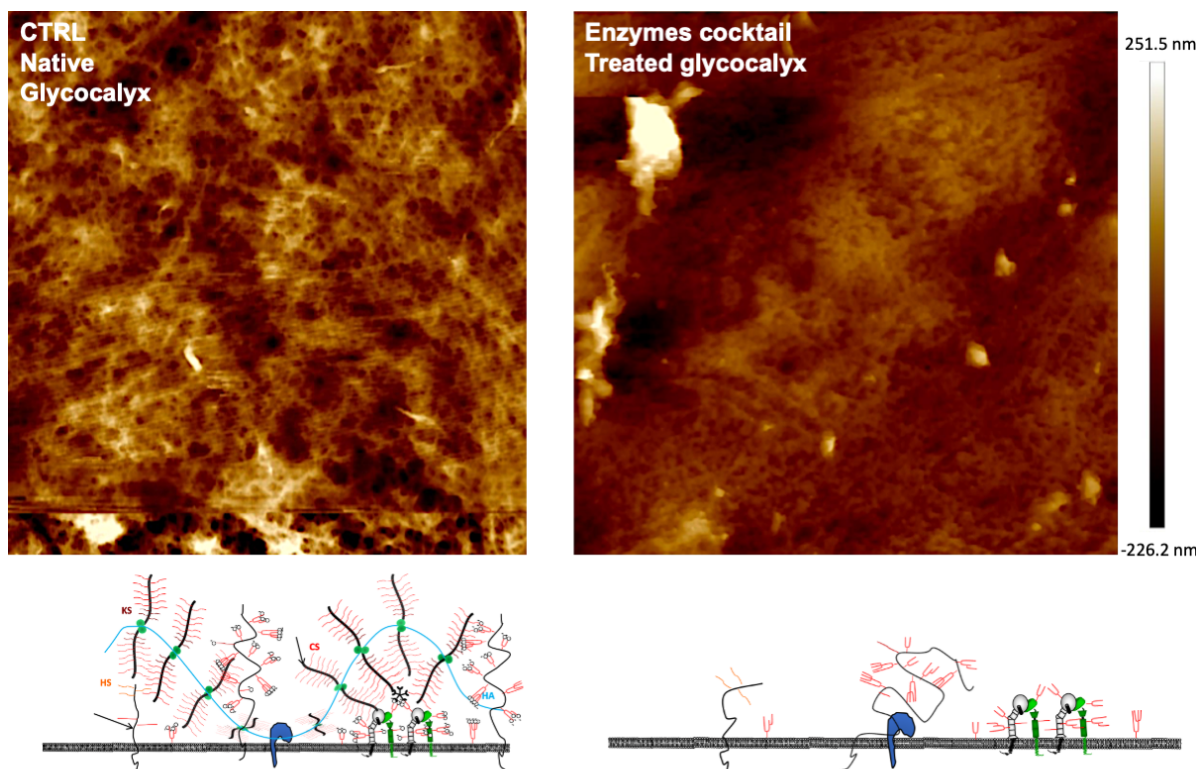


Figure 22. (on the top) AFM Peak-Force-Tapping images of the membrane of fixed cells, before the enzymes treatment (left) and after (right). (on the bottom) Scheme representing the native glycocalyx composition (left) and after the digestion of the enzymes cocktail (right).

Figure 22 shows representative scans made in Peak Force Tapping on the surface of fixed cells. On the untreated cells it is possible to observe a complex network of intertwined and cross-linked fibrillar structures, with some of them being several microns long. In the cells that have been treated enzymatically to remove the glycocalyx, the network disappears instead completely, leaving the

naked cell membrane exposed with some residual and transmembrane proteins visible.

5.2.2 Quantification of the glycocalyx pericellular thickness via 3D-SIM imaging of fluorescent staining with wheat germ agglutinin Oregon green 488

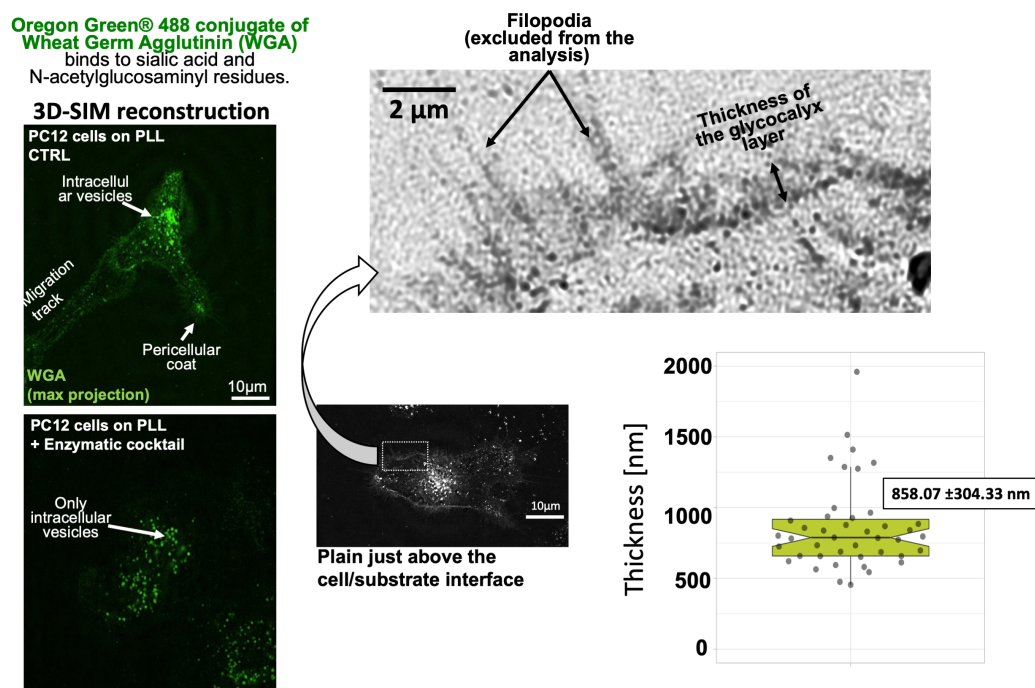


Figure 23. (on the left) fluoresce 3D-SIM images of PC12 with pericellular components stained in green, before (top) and after (bottom) the enzymatic treatment. (centre) Representative image with the focus on the plan just above the substrate, (centre top) magnification used for the quantification of the thickness of the glycocalyx layer, (bottom right) boxplot with results of the analysis of 43 different images.

In the reconstructed 3D-SIM images of PC12 cells in which the glycocalyx was stained with wheat germ agglutinin Oregon green 488 conjugate (Figure 23) show vesicles that presumably transport the components of the glycocalyx to the cell surface. Furthermore, in these native untreated cells also a pericellular fluorescent coat is visible, while in the cells that were treated with the glycocalyx removing enzymatic cocktail, the only present staining is in the intracellular vesicles.

The analysis on the thickness of the pericellular glycocalyx coat in the native cells has been done by quantifying the fluorescent signal at the boundaries of the cells (in the plane just above the cell/substrate interface). The results provide a mean thickness $t=858\pm304$ nm for the native glycocalyx. It was impossible to quantify the residual thickness of the treated cells' pericellular coat due to the weak intensity of the signal, which in any case indicates an almost complete digestion of the GAGs.

5.2.3 AFM Indentation measurements

The results obtained from the implementation of the Sokolov's brush model are shown in Figure 24. The rescaled FCs from the native untreated cells show a bilinear regime in the semi-log scale, this behaviour is commonly associated to the presence of two components of glycocalyx: one long and sparse (red line) and one short and dense (black line). This bimodal behaviour seems to be typical of cancer cells⁹⁹. The fitting of these two linear regions provides a two-peak distribution of the glycocalyx length, the short one $L1=330\pm126$ nm and the long one $L1=1011\pm78$ nm.

After the treatment with the enzymatic cocktail, we recorded an almost total disappearance of the long component of the glycocalyx and an additional shortening of the short one. The nature of the so-called *short-brush* is still unclear, it could be an inner layer composed mostly by transmembrane proteins and core proteins of the proteoglycans (the complete loss of signal in the abovementioned fluorescent images might be indicative of this), less sensitive to the action of the enzymes, or a residual response of the cell body deformation not completely accounted by the model. Overall, the measured value of the GC thickness is in good agreement with 3D-SIM fluorescence-based quantification, confirming the presence of the glycocalyx for the PC12 cell line with a thickness of several hundreds of nm and the efficiency of the enzyme cocktail treatment.

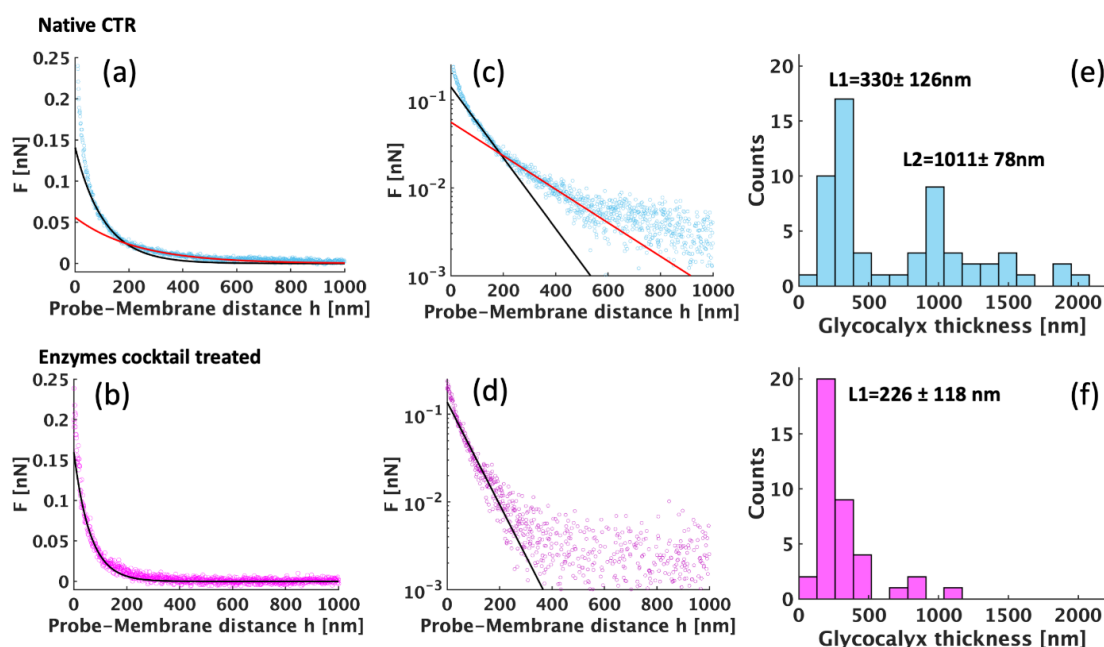


Figure 24. Examples of FC data recorded in the indentation experiment, made on native PC12 and treated cells, and results obtained from the analysis. (a,b) Indentation curves rescaled over probe-Membrane distance, dots are the experimental data and solid lines are the fit of the brush model (Eq.3). (c,d) Same curves shown in semi-log scale in order to highlight the linear regimes (used for the fitting) where the glycocalyx response is purely entropic. (e,f) Distribution of the glycocalyx thickness obtained from the experiments, 1 count corresponds to one cell.

5.2.4 Force Spectroscopy experiments

To investigate in which way nanotopographical features influence the characteristics of the cellular adhesion, and how the glycocalyx layer is involved in such processes, we performed adhesion force spectroscopy on PC12 cells with *nt-CPs* with three different types of morphological functionalization (roughness values of the ns-ZrO₂ thin film):

1. Flat ZrO₂-coated CP, produced by ion gun sputtering, without nanotopographical features, $r_q < 1$ nm (surface: flat-ZrO₂).
2. Ns-ZrO₂-coated CP, produced by SCBD, with $r_q = 15$ nm (surface: ns-ZrO₂_15nm)
3. Ns-ZrO₂-coated CP, produced by SCBD, with $r_q = 20$ nm (surface: ns-ZrO₂_20nm)

The dependence on the contact time of the observed parameters (Detachment force, work, number of jumps and tether events) is discussed below.

Detachment Force. Figure 25 shows that native PC12 cells (solid line) are capable of creating stronger adhesion on surface with the specific roughness value $r_q = 15$ nm. This roughness value is the same that is known to trigger strong mechanotransductive modulations at the level of integrin adhesion complexes and the cytoskeleton, as well as the neuronal differentiation in PC12 cells^{30,32}. On flat-ZrO₂, the force reaches a plateau after 20s and then fluctuates around the same value. On ns-ZrO₂_20nm, the force increases monotonically for all the contact times. On ns-ZrO₂_15nm, instead the adhesion force increases rapidly in the first 20 sec, reaching values higher than the other two conditions at all contact times, then the growth slows down between 20s and 120s and at 240s the force decreases to values compatible of those of the other two conditions.

What is really striking is the change of the adhesive behaviour of the cells **after** the glycocalyx layer removal. The cells' capability of creating adhesion is totally inverted, with the ns-ZrO₂_15nm surface that becomes the least adhesive, the flat-ZrO₂ that is the most adhesive and almost no differences are present on the ns-ZrO₂_20nm.

∇ $r_q = 0$ nm
 $\circ$ $r_q = 15$ nm
 $\times$ $r_q = 20$ nm

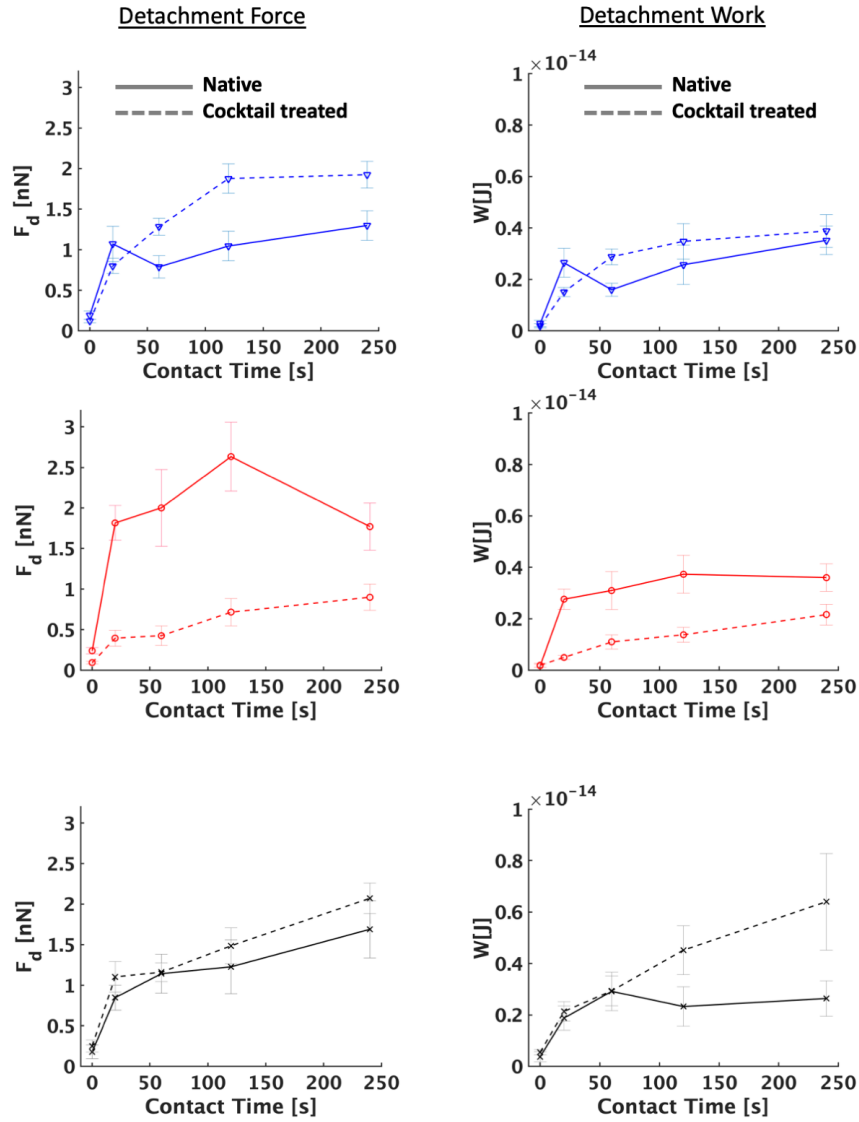


Figure 25. The panel shows the results of the adhesion force spectroscopy measurements regarding detachment force and work performed in the three different conditions, before (solid lines) and after (dashed lines) the enzymes treatment.

Detachment Work. A similar behaviour is shown by the work of detachment even if the differences are less pronounced compared to F_a with almost identical behaviour in the control conditions. However, again the effect of the enzymatic treatment was to reverse the trends, moving the ns-ZrO₂_15nm from the most to the least adhesive surface.

Mean number of unbinding events N_i , N_t . The mean number of detected adhesions events provides additional information about the cell interacts with the different nanotopographical surfaces (Figure 26). The so-called jump events (see pg 54) are usually associated to receptors in the membrane that have their

cytoplasmatic tail anchored to the internal cytoskeleton^{11,52,115,116}, while the tethers are attributed to receptors that are not bound the actin cortex^{117,118}.

The mean numbers of jumps for flat-ZrO₂ and for ns-ZrO₂_20nm, in the control condition, are almost identical between 0s and 120s. In the latter case, the cells are able to create more bonds only at 240s.

The peculiar dynamics of the adhesive behaviour of cells to ns-ZrO₂_15nm has been widely discussed in Chapter 4⁵⁶.

After the glycocalyx digestion, we recorded intriguing modulations. A marked increase (~60% at 20s, 100% at 60s, 105% at 120s and 56% at 240s) can be noted on the flat-ZrO₂ surface.

A similar increase was recorded on ns-ZrO₂_20nm, even if less pronounced and with a bigger dispersion of the data. In both cases, the effect of the enzymes was opposite to the ns-ZrO₂_15nm surface, for which the number of jumps showed an average decrease of 50% (except at 20s where the number halved). It is interesting to notice that the same dynamics recorded for the control condition (the number of bonds N_j drops between 20s and 60s) is observed after the treatment even if less evident.

The results concerning the mean number of tethers did not shows striking differences between the two conditions (with or without glycocalyx) on all the surfaces (flat-ZrO₂ at 120s excluded). This mostly invariant N_t is still noteworthy, though, as tethers have been proposed to be, at least partially, events originating from the unfolding of glycocalyx sugar chains¹⁰². With no significant difference detected in the mean number of tether events before and after the enzymatic treatment, we can now reject this hypothesis. Indeed, if a portion of these events would have come from the GC unfolding, we would have registered a significant decrease of the mean number of tethers events.

∇ $r_q = 0$ nm
 $\circ$ $r_q = 15$ nm
 $\times$ $r_q = 20$ nm

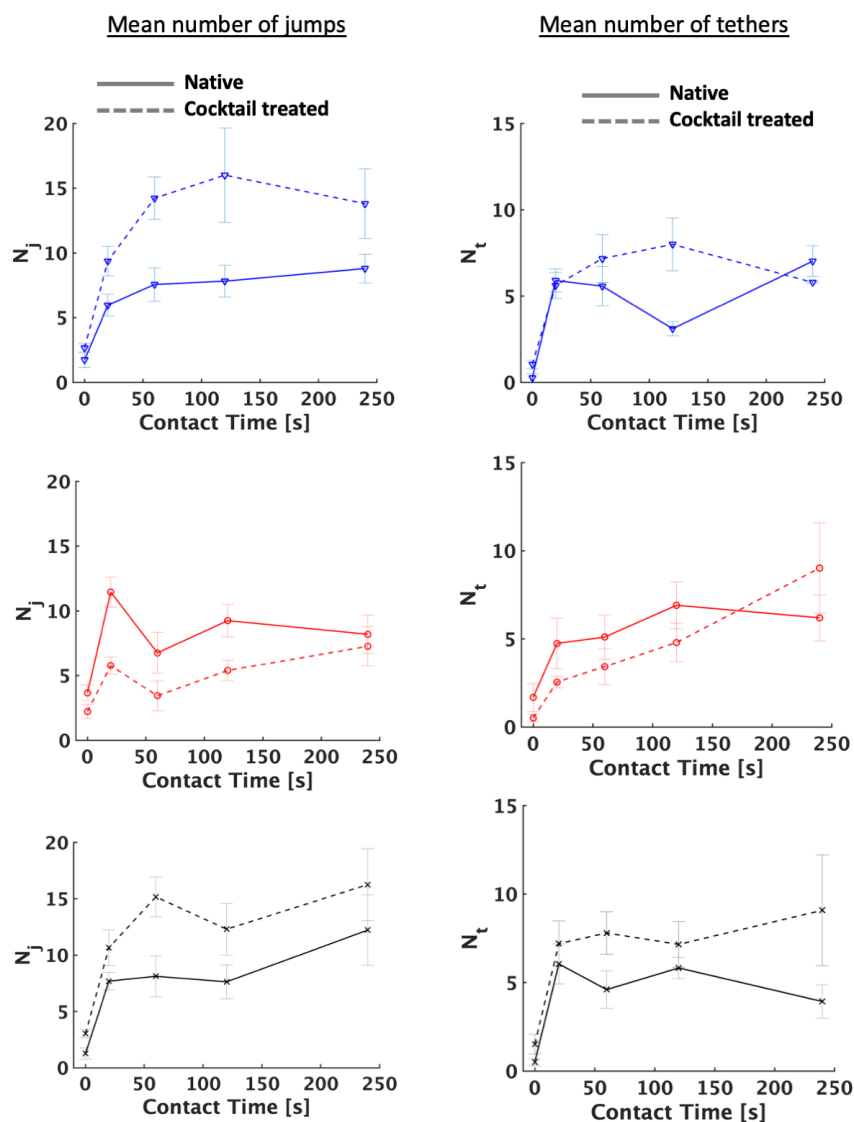


Figure 26. The panel shows the results of the adhesion force spectroscopy measurements regarding the mean number of unbinding events (jumps and tethers) performed in the three different conditions, before (solid lines) and after (dashed lines) the enzymes treatment.

Mean force per single jump. It has been already demonstrated that the higher adhesion developed by the cells on the ns-ZrO₂_15nm originates from the cells capability to create single stronger bonds compared to the other conditions⁵⁶ and this ability finds its source in the availability of activated integrins. Following the same approach, we evaluated the mean force per jump for the three surfaces in both the conditions. Results are shown in Figure 27, before and after the enzymatic treatment.

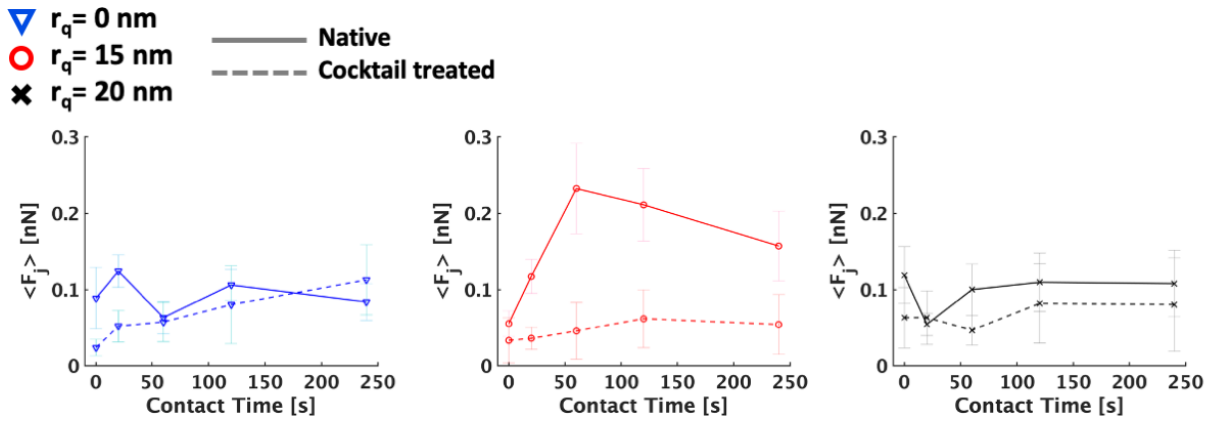


Figure 27. The panel shows the results of the calculation of the mean force per single jump bond at every contact time.

The mean jump force for the conditions flat-ZrO₂ and ns-ZrO₂_20nm does not significantly change after the treatment, if we couple this information with the increase of number of bonds and adhesion force (Figure 25,26), it comes out that the absence of the glycocalyx gives the chance to the cells to create more bonds of the same kind with the surface and the increase in the detachment force originates from this increase of bonds. On ns-ZrO₂_15nm, instead the mean bond strength drops, suggesting that the glycocalyx contributes actually to the reinforcement of the integrin clustering as claims the model suggested by Paszek et al.^{62,106}.

5.2.5 Force spectroscopy control experiment

We have already demonstrated that the peculiar adhesive dynamics seen on the ns-ZrO₂ ($R_q = 15$ nm) are regulated by the availability of activated integrins⁵⁶. To understand, whether this behaviour depends on the internal cytoskeletal forces driven by actomyosin contraction, we treated cells with the protein ROCK inhibitor Y27632. The Rho/ROCK signalling governs the actomyosin contraction and stress fibres formation.

Force spectroscopy measurements with the ns-Zr15 probes on PC12 cells treated with the ROCK inhibitor revealed a clear change of the adhesion dynamics with respect to the untreated cells (see Figure 28a). Differently from the adhesion dynamics towards ns-Zr15 of untreated cells, after Y27632 treatment N_j progressively increased over time, reaching particularly high levels at 120 s and 240 s. With this course, the dynamics in the first 60 s resembled more the flat-Zr condition, but then developed differently towards higher N_j values at the later time points in the range of flat-Zr after glycocalyx removal. There was furthermore a drop of $\langle F_j \rangle$ and a reduction of high jump forces (Figure 28b), making the profiles for N_t and N_j more alike to the flat-Zr and ns-Zr20 conditions. These data indicate that with the excessive force loading the molecular clutch disassembly (detected in the ns-Zr15 condition) and this behaviour depends indeed on the actomyosin contraction-powered forces regulated by Rho/ROCK

signalling. This confirms the importance of force-related processes for later stages of IAC growth on the level of single binding events and for the nanotopography sensing.

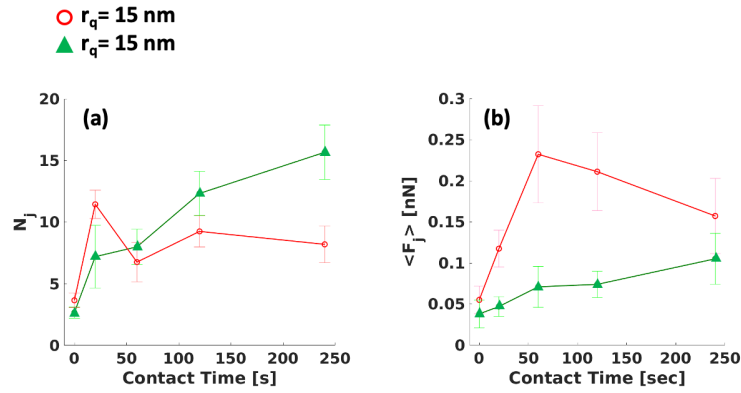


Figure 28. The panel shows the number of jump bond N_j , and the results of the calculation of the mean force per single jump bond at every contact time.

5.2.6 Quantification of the retrograde actin flow

Integrin mediated mechanotransductive processes strongly depend on the way the actomyosin-generated forces of the retrograde flow are transmitted to the substrate and consequentially the force loading in the molecular clutches^{6,27,119}. To dissect the molecular clutch engagement to the actin retrograde flow, we ran a preliminary campaign of measurements aimed to visualise and quantify the velocity of the retrograde actin flow on flat-ZrO₂ and on ns-ZrO₂_15nm, with and without the GC.

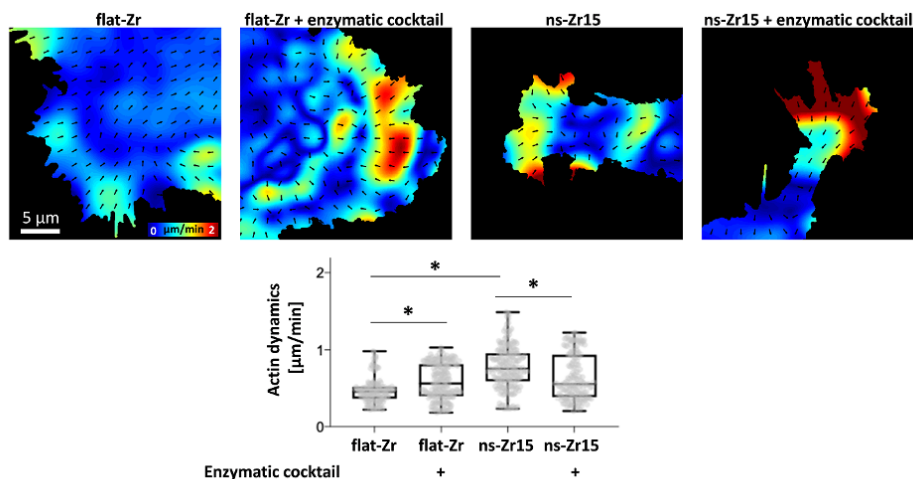


Figure 29. The nanotopography and the glycocalyx influence the lamellipodial molecular clutch engagement and actin retrograde flow. Particle image velocimetry and kymographic analysis, flat-Zr, ns-Zr15 $\pm$ enzymatic treatment. Experiments has been performed by Dott. C. Schulte (Cimaina, Dipartimento di Fisica, Università degli Studi di Milano), particle image velocimetry has been performed by Dott. S. Marcotti (Randall Centre for Cell and Molecular Biophysics, King's College London, London, United Kingdom).

It is evident that the flow is faster on the ns-ZrO₂_15nm compared to the flat-ZrO₂ surfaces (Figure 29), this is consistent with the faster disrupting and recreating of bonds observed with the force spectroscopy. Interestingly, the glycocalyx removal continues producing a reverse effect on the two conditions: it increases the velocity of the actin flow on the flat-ZrO₂ surface, whereas it reduces the actin flow on the ns-ZrO₂_15nm, leaving the velocities of the actin flows almost identical between the two surfaces. This good correlation between the force spectroscopy and retrograde actin flow data, indicates that the glycocalyx removal changes the integrin-mediated adhesion dynamics in a different way for flat and nanotopographical substrates with a specific configuration. The condition with the ns-ZrO₂_20nm is still missing, and future experiments need to be done to complete all the relevant conditions. We expect that the GC removal would not provide significant modulations in the actin dynamics.

5.3 Discussion

The spatial configuration of the nanoscale cues is decisive for the cellular mechanotransductive response because of their impact on the state of integrin adhesion complexes and actin cytoskeleton.

What emerges from our experiments is that the glycocalyx layer represent a decisive player in mechanotransductive processes and that its impact is dependent on the substrate nanotopography. The GC influences strongly the cellular adhesion processes. In particular, it affects the cells' ability to perceive the topographical conditions presented by the microenvironment. Our data seem to represent well the model proposed by Paszek et al.^{62,106}: after the glycocalyx removal we observed a decrease of force per single jump at contact time $ct=0$ sec.

However, our data show also that the situation is different for the nanotopographical condition (depending on the specific configuration of the topography), as the removal of the GC layer leads to opposite effects compared to the flat substrate. It affects the molecular clutch and the integrin clustering, resulting in a decrease (in the case of ns-ZrO₂_15nm) in the cell ability to generate stronger bonds.

However, the divergences in the effects of the glycocalyx as a function of the nanotopographical features of the surfaces indicates that more complex mechanisms are present. Our hypothesis is that the driving parameter that one must take into account is the **effective substrate contact area** at the nanoscale available to the cell to create bonds and integrin nanoclusters. Indeed, it has been demonstrated that the cells interact only with the apical parts of the nano-asperities of the ns-ZrO₂ in the presence of the native glycocalyx³⁰. The ns-ZrO₂_15nm possess accessible asperity surface areas that are smaller than those of ns-ZrO₂_20nm, due to the smoother morphology of the high roughness surface⁶⁰ and the distribution of asperities aspect-ratio and relative distance (the

comparison with the flat Zr substrate is superfluous in this regard, since the cell does not perceive any spatial constraining on a flat surface).

We believe that once the glycocalyx is removed, the steric repulsion and the spatial constrain for the integrins (due to the GC) disappear (this is valid for the flat and the nanostructured surfaces). However, in case of nanostructured surface situation, the cell membrane can bend more and can comply better to the morphology of the microenvironment⁶³, allowing more integrins to bound but in a less stable condition, the subsequent actin flow speed-up because of faster bonding creation/disruption. But this adaptation is only possible when the bending of the cellular membrane is large enough to follow the morphologies of the surface below, if not (like in the case of ns-ZrO₂_15nm) the effective contact area would not increase after the glycocalyx removal and the resulting effect is just the partial loss of the cell ability to stabilize integrins, precisely due to the lack of the glycocalyx layer. Clearly, this also suggests that other surface parameters, beyond the simple r_q , plays a role, like the asperity aspect-ratio, curvature and correlation length.

Obviously, this is an interpretation of preliminary results and further investigations needs to be done to try clarify the multiple aspects that affect such a complex and collective dynamic.

5.4 Future experiments

Some the measurements done until now were preliminary, and additional rigorous characterization has to be done:

- Actin flow quantification has been applied on only two of the three reference surfaces ($r_q=20$ nm is missing). Future analysis will be performed to cover all the AFM force spectroscopy experiments conditions.
- Additional morphological analysis must be done on the different surfaces to better characterize those properties that define the interaction region. Like the density of available asperities, the relative distance between the asperity peaks and the aspect-ratio. Furthermore, also biological parameters, such as the length of the extracellular domain of extended integrins and different membrane bending conditions could be integrated in this analysis to model how that might change the cellular perception of the substrates' morphological properties.
- A direct investigation of the cell/substrate interface in all the conditions must be performed to get visual demonstration of the proposed interaction model. In particular, high resolution microscopy techniques, such as transmission electron microscopy (TEM), possess the resolution needed to achieve such an insight. A similar approach has already been applied previously in Schulte et al. but only for PC12 cells in the presence of their native glycocalyx comparing flat-Zr and ns-Zr15.

Section 2

Chapter 6

Cytoskeletal architecture as biophysical marker of cell adaptation to external stimuli: cell-drug interaction

Cells, adhering to the ECM, probe the physical properties of the surrounding environment, anchoring and pulling on their surroundings through integrins, which provide a structural connection between the external cellular contacts and the internal cytoskeleton^{120,121}. It has been widely reported how the microenvironment properties can impact the cytoskeletal organization and eventually triggers specific cellular behaviours through mechanotransductive processes^{24,30,33,122,123}: the external forces, transmitted *via* the cytoskeleton, affects the mechanical properties of the cell and the resulting elastic modifications reflect the cells adaptation to the cues in the microenvironment^{124–127}.

The ability to survive on, react and adapt to different foreign environments, is typical of cancer cells. After diffusing through the blood vessel and lymphatic system, they are capable to adhere in different location of the body creating metastases¹²⁸. The cell ability to metastasize is accompanied by various molecular changes¹²⁹, leading to alterations of the cytoskeleton architecture²¹ and influencing the overall mechanical resistance of cells. As a consequence, the biomechanics of tumoral cells is being studied more and more in the recent years, with the perspective of clinical applications in diagnosis^{15,16} and new therapies¹⁶. The mechanical investigation of tumour cells, in fluid and temperature conditions that imitates the natural environment, revealed alterations in the tumoral cells mechanics (compared to their normal counterpart) and recognized them as indicator of cancer-related modifications^{16,22}.

The systematic investigation of the cells' mechanical response demonstrated the feasibility to correlate these observables in relation to cancer cells' health. This opens up new strategies for the characterization of the effects of anticancer drugs that directly interfere with cells' ability to reorganize the cytoskeleton, or the contribution of the cytoskeleton in the development of drug resistance.

In the following, a preprint by Kubiak et al.⁶⁴ (under revision on Nanoscale Journal), to which I contributed as a co-author is reported. In this work, we applied AFM nanomechanical measurements on prostate cancer cell line DU145 treated with three different anticancer microtubule-targeting drugs (MTDs) at different concentrations. The developed protocol for the AFM measurements, coupled with fluorescent imaging allows to detect fine modifications in the cellular mechanics and morphology, locate them inside of the cell body and identify the actin filament-microtubule crosstalk as a mechanism that confers resistance to the MTDs⁶⁴.

Stiffening of prostate cancer cells driven by actin filaments – microtubules crosstalk confers resistance to microtubule-targeting drugs

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Authors' contributions:

AK conducted the majority of the experiments, analyzed the data, interpret the data and wrote the manuscript; **MC, CS & AP** contributed multilayer-based analysis of mechanical properties of cells, conducted biomechanical experiments on cells treated with colchicine, interpret obtained results; **JW, NB & AK-B** recorded the fluorescence images using confocal microscopy, created 3D movies based on z-stacks, contributed to data interpretation; **MP & DR** performed, analyzed and interpret data from cytometry; **ML & PL & AP** designed the study, decided on the choice of

AFM probes, interpret the data, wrote the manuscript, provided resources and funding for the study.

All authors contributed to manuscript writing.

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Abstract

The crucial role of microtubules in the mitotic-related segregation of chromosomes makes them an excellent target for anticancer microtubule targeting drugs (MTDs) such as vinflunine, colchicine, and docetaxel. MTDs affect mitosis by directly perturbing the structural organization of microtubules. By a direct assessment of the biomechanical properties of prostate cancer cells exposed to different MTDs using atomic force microscopy, we show that cell stiffening is a candidate mechanism through which cancer cells preserve the original phenotype in response to the application of MTDs. While changes in cellular rigidity are typically mainly attributed to remodeling of the actin filaments in the cytoskeleton, here we provide evidence that cell stiffening can be driven by a crosstalk between actin filaments and microtubules in drug-treated cells. Our findings improve the interpretation of biomechanical data obtained for living cells in studies of various physiological and pathological processes.

Introduction

Microtubules (MTs) are filamentous structures constituting one of the major components of the cell cytoskeleton. They are cylinders composed most often of 13 longitudinally arranged protofilaments. Individual protofilaments are composed of tubulin heterodimers built up from α - and β -tubulin linked together by non-covalent bonds forming $\alpha\beta$ -tubulin heterodimer¹. Inside the cell, microtubules start from a microtubule-organizing center (MTOC) and span the entire cytoplasm, ending at a cell membrane. MTs provide functional pathways for the transport of cellular cargo and they are involved in intracellular signaling¹. The most important role of

microtubules is the participation in mitosis. During this process, they form a mitotic spindle used by cells to separate replicated chromosomes into two newly created nuclei^{2,3}. Thus, any agents affecting microtubule dynamics will introduce severe consequences for cell division by disabling the completion of mitosis, and thereby, by an inhibition of cell proliferation. Such effect has been in the center of attention of clinical oncologists for many years, leading to the use of taxanes and vinca alkaloids in anticancer therapy of various cancers including lung, breast, prostate, gastric, esophageal, and bladder, as well as squamous cell carcinoma of the head and neck⁴⁻⁶.

Since the invention of atomic force microscopy (AFM⁷) and its first use for characterizations of mechanical properties of cells⁸⁻¹⁰ the spectrum of possible AFM applications has widened enormously. A variety of AFM-based mechanical measurements provide evidence that changes in cell rigidity are related to the cell cytoskeleton, mainly to actin filaments^{11,12}. Gathered evidence shows that AFM-based mechanical measurements contribute to a better understanding of physical mechanisms involved in the cellular response to various conditions, including chemical treatments.

Antimitotic agents belonging to microtubule-targeting drugs (MTDs) such as docetaxel improve prostate cancer patient's survival¹³ but, during treatment, cancer cells often develop drug resistance. Despite the known antitumor effect, still it is not known why some cells are resistant to the MTDs. Multidrug resistance linked with overexpression of ABC transporters is one of the possible explanations. Another possible explanation is changes in the expression of tubulin isotypes, which prevent interaction between the drug and its target¹⁴. Mechanisms of MTDs binding to α/β tubulin heterodimers affect their dynamics by stabilizing or destabilizing microtubular network¹⁵. Therefore, strong prerequisites indicate that biomechanical cues underlie the cellular response of prostate cancer cells to drugs against microtubules. Molecular mechanisms involved in the binding of MTDs are, theoretically, independent of the cell types indicating that all cancers can be treated with such drugs. However, there exists a group of cancers that do not respond positively to such a treatment, showing cells resistant to the drug treatment, as in the case of prostate cancers. Here, we combine AFM and cell biological methods to study changes in the mechanical properties of prostate DU145 cancer cells treated with three MTDs drugs i.e., vinflunine (VFL), docetaxel (DTX), and colchicine (COL) at concentrations below IC_{50} . We identified the cell stiffening as a process through which cancer cells preserve the original phenotype in response to applied drugs.

Results

Viability study shows that attached DU145 cells are more resistant to low MTDs doses.

We started our analysis with the evaluation of MTDs' effect on the viability and proliferation of DU145 cells (**Fig. 1a**).

To verify how low and high MTDs concentrations deviate from the IC_{50} , the MTS assay was applied to cells exposed to different drug concentrations, i.e. 50 nM – 2000 nM for VFL, 0.1 nM to 20 nM for DTX, and 0.01 nM to 2 nM for COL. MTS assay quantifies the fraction of metabolically active cells (through dehydrogenase activity). Recorded absorbance was normalized to a viability level of NTC cells and plotted against MTDs drug concentration in the logarithmic scale (**Fig. 1 b-d**). IC_{50} , obtained from the fit of a dose-response function, was 450 ± 20 nM (VFL, **Fig. 1b**), 2.9 ± 0.5 nM (DTX, **Fig. 1c**), and 0.2 ± 0.1 nM (COL, **Fig. 1d**), respectively.

The proliferation rate was assessed by counting cells attached to the glass surface after their incubation with MTDs for 24h, 48h, and 72h. Results show a strong decrease in the cell number for high drug concentrations i.e., for 750 nM for VFL, 5 nM DTX and 0.5 nM COL (**Fig. 1 e-g**), regardless of the culture time. The number of cells drops to the level of 10% – 20% with respect to the number of alive, non-treated cancer (NTC) cells. For low MTDs concentrations, i.e. 100 nM for VFL, 1 nM for DTX, and 0.1 nM COL, the number of cells after 24 h and 48 h of culture remained at the same level as for NTC cells regardless of the drug type. The decrease in the number of cells was observed only after 72 hours. A drop by 33%, 48%, and 23% for VFL, DTX, and COL, respectively, was accompanied by the smallest proliferation rate (**Suppl. Note 1**).

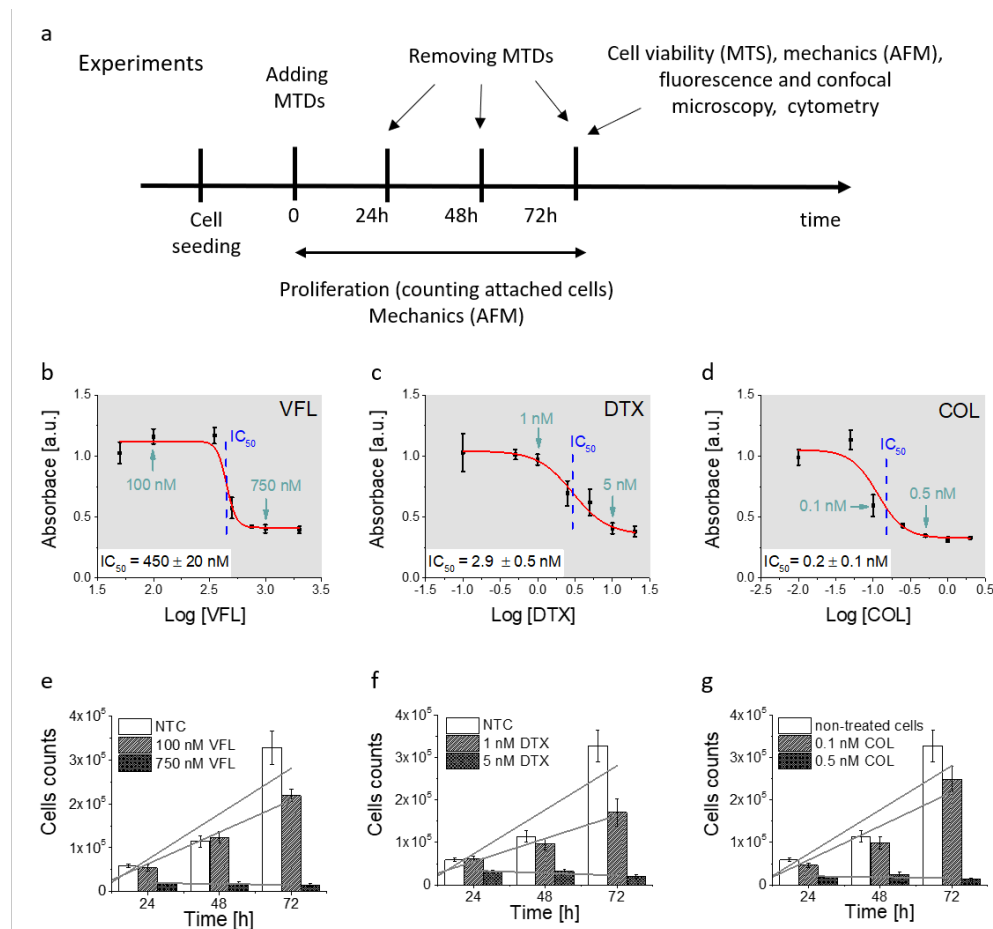


Figure 1. Prostate DU145 cells respond differently to distinct MTDs drug concentrations at different time-points. *a*, Illustration of experimental steps and methods applied. *b-c*, Dose-response curves for cells exposed to VFL (*b*), DTX (*c*), and COL (*d*). Calculated from the fit, IC_{50} were 450 ± 20 nM (VFL), 2.9 ± 0.5 nM (DTX) and 0.2 ± 0.1 (COL). *e-g*, Proliferation level of prostate DU145 cells determined for low and high doses for VFL (*e*), DTX (*f*), and COL (*g*). A slope of linear fits determines a rate of proliferation. NTC cells denote non-treated DU145 cancer cells. Data are shown as mean $\pm$ standard deviation (s.d.) from at least $n = 3$ measurements; IC_{50} is expressed as a fitted value $\pm$ standard error, (s.e.). Arrows indicate the position of low and high concentrations of drugs chosen for further experiments.

In our study, low MTDs concentrations are placed below IC_{50} while high concentrations are above IC_{50} . 72h exposure of DU145 cells to low concentrations of MTDs reveals a cell population that contains a strongly dominating fraction of proliferating cells, while at high concentrations many of the cells died through both apoptosis and necrosis. All cells heavily affected by MTDs are detached from the surface. This is the main reason why the IC_{50} (MTS, bulk measurements) does not fully correlate with the decrease in the number of cells attached to the surface (proliferation). Therefore, our results show that DU145 prostate cells seem to be more resistant to COL, than to VFL and DTX.

NTC and VFL-treated cells show a similar level of calcein efflux

To elaborate on how the viability of cells is linked with the capability of DU145 cells to release MTDs, in our next step, we decided to trace acetoxymethyl (AM) ester derivative of calcein using flow cytometry in NTC and MTDs treated cells (**Fig. 2**).

Calcein-AM is a non-fluorescent, uncharged molecule that can freely permeate the plasma membrane of viable cells. Inside the cell, it is converted to a fluorescent form, calcein, upon cleavage of the lipophilic blocking groups by nonspecific esterase in the cytoplasm^{16,17}. Cytometric study shows that within the analyzed population of DU145 cells (NTC, 5.6%, **Fig. 2a**), there are minoritarian groups of cells actively pumping calcein out, i.e. side population. The side population denotes the shift of fluorescence proportional to the loss of calcein pumped out or accumulated inside the cells¹⁷. The size of this group decreases for cells treated with MTDs. The largest drop, about two-fold as compared to non-treated DU145 cells, was observed for COL (2.7%, **Fig. 2d**) and DTX (3.6%, **Fig. 2c**) treated cells. In the case of VFL treatment, the population of these cells was only slightly smaller (4.9%, **Fig. 2b**).

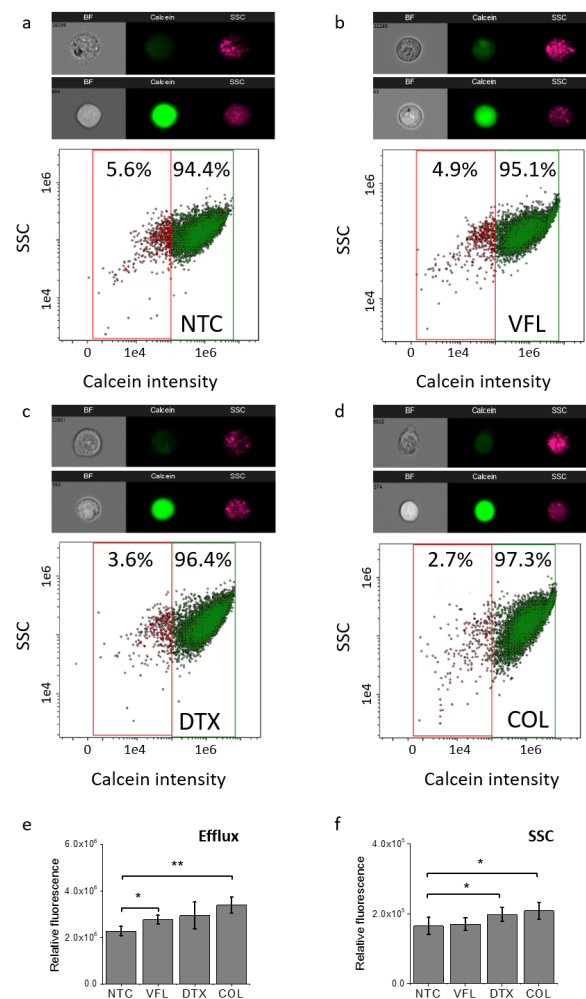


Figure 2. Calcein fluorescence intensity changes with the MTDs type. (a - d) Correlative dot plots of side scatter light (SSC) and calcein intensity within the cell population 72h after MTDs treatment. The high SSC/high calcein intensity subpopulation was observed after cell treatments with VFL and COL. ImageStream obtained cell images are representative of the cells gated in NTC (green) and MTDs-treated (magenta) cell populations. **(e-f)** Average fluorescent intensity of calcein calculated as a mean $\pm$ s.d. ($n = 3$ independent measurements). Statistical significance was estimated by unpaired t-Student test at the level of 0.05.

Together with the decreasing number of cells and the pumping of calcein to the extracellular microenvironment, a shift of a cytometric population towards higher calcein intensity appears. The observed MTDs-related retention of calcein in treated DU145 cells could be indirectly attributed to drug accumulation (**Fig. 2e&f**).

Microtubule rearrangements depend on MTDs binding site

MTDs such as vinflunine, docetaxel, or colchicine are known to bind at distinct locations in the α/β tubulin heterodimer (**Fig. 3a**) and affect the organization of the microtubular network. Thus, we visualized the organization of the microtubules inside the DU45 cells after 72 hours of incubation with a specific drug at low and high concentrations (**Fig. 3b-h**).

In NTC cells, microtubules exhibit multi-branched tubulin networks with clearly visible MTOCs located close to the cell nucleus (indicated by white arrows, **Fig 3b**). Microtubules alone form long fibers that span from MTOC towards the cell membrane. For cells treated with low MTDs concentrations, the degree of microtubule re-organization is a drug-dependent (**Fig. 3c-e**). In cells treated with 100 nM VFL, in which MTOCs are visible, the microtubular network is weakly affected (**Fig. 3c**). Here, proliferating cells are still present (**Fig. 1, Suppl. Fig. S7**).

Around cell nuclei, MTs form a ring, which is strongly or weakly visible under fluorescence (marked by stars in e.g. **Fig. 3c**). The presence of both MTOCs and rings indicate that, after 72h of culture in the presence of MTDs, most of DU145 cells were not at all or weakly affected by these drugs. The microtubular network was strongly preserved for VFL-treated cells (**Fig. 3c**). In the case of prostate cancer cells exposed to 1 nM DTX for 72h, MTOCs became less visible and the microtubular network seems to shrink (**Fig. 3d**). This was accompanied by the aggregation of microtubules in a form of thicker rings surrounding the cell nucleus. Altered organization of MTs is associated with a lower proliferation rate (**Suppl. Note 1**) and cell viability level (**Fig. 1**). The effect of 0.1 nM COL on DU145 cells also induced a rearrangement of the microtubule network (**Fig. 3e**), similar to that obtained for 100 nM VFL. Here, MTOCs were visible only in some cells. These changes in the MT network can be rated as follows (from the strongest to weakest): DTX > COL > VFL. This agrees with the MTS assay, from which one can expect large changes in cells treated with DTX, COL, and VFL (**Fig. 1**).

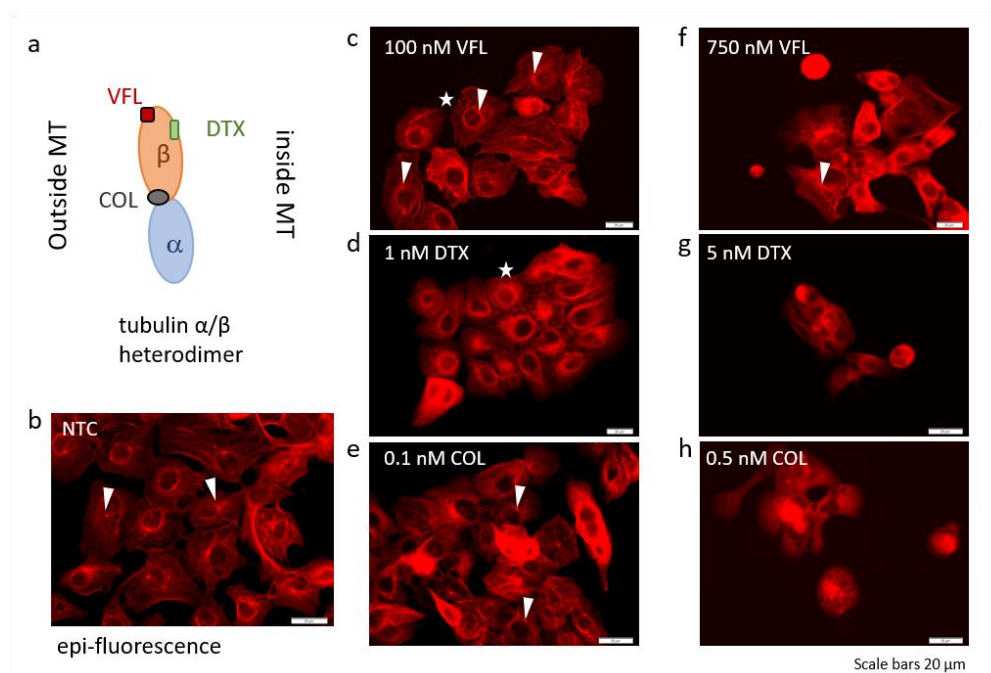


Figure 3. MTDs bind to distinct sites located at the α/β tubulin heterodimer affecting differently the organization of the microtubular network. *a*, A scheme illustrating the binding of VFL, DTX, and COL to α/β tubulin heterodimer. *b-h*, Epi-fluorescent images showing the organization of microtubules in DU145 cells upon treatment for 72 hours with low and high MTDs doses (white arrows show MTOCs while stars indicate MT rings formed around cell nuclei).

For high MTDs concentrations, the clustering of cells was recorded (**Fig. 3f-h**). Although many cells were affected, still single cells were detected that showed organized MTOCs (e.g. **Fig. 3f**). But in the majority of cells, the microtubular network was fully disorganized (disassembled), especially in the case of DTX and COL, for which a destruction of MTs is expected as it could be linked with apoptosis¹⁸. Changes in the microtubular network were accompanied by morphological alterations observed for cell nuclei (**Suppl. Note 2, Suppl. Fig. S1; Suppl. Fig. S2**). High MTDs doses cause a fragmentation of nuclei, especially for DTX and COL, while low MTDs doses reveal a strong decrease of nuclei surface area circularity and roundness for DTX treated cells (**Suppl. Fig. S1c-e**). The results obtained for low doses confirm the postulate that drugs affecting

microtubules at small concentrations are not sufficient to destroy them, but they tend to change MTs dynamics⁶.

DU145 cells stiffen only when treated with VFL

Changes in MT organization roughly correlate with the results of cell viability. At the same time, according to already published results^{19–21}, MT organization also is expected to affect the mechanical properties of cells. To investigate the role of biomechanics in drug resistance of cancer cells, we employed AFM to characterize the mechanical properties of treated cells^{22,23}. In particular, we used AFM to obtain an overview of the temporal evolution of cellular rigidity upon exposure to different concentrations of MTDs. The relative change ΔE_{rel} of Young's modulus E , defined as $\Delta E_{rel} = (E_{drug} - E_{NTC})/E_{NTC}$, is quantified using Hertz contact mechanics (**Materials and Methods, Suppl. Table S1, Suppl. Note 3; Suppl. Fig. S2**).

We first carried out a characterization of the relative changes in the mechanical properties of cells, measured within cell center i.e. above the cell nucleus at different time-points, i.e. after 24h, 48h, and 72h of culture in the presence of MTDs. The results show that MTDs type, dose, and time alter the biomechanical properties of prostate DU145 cells (**Fig.4a-c, Suppl. Fig. S3, Suppl. Table S1**).

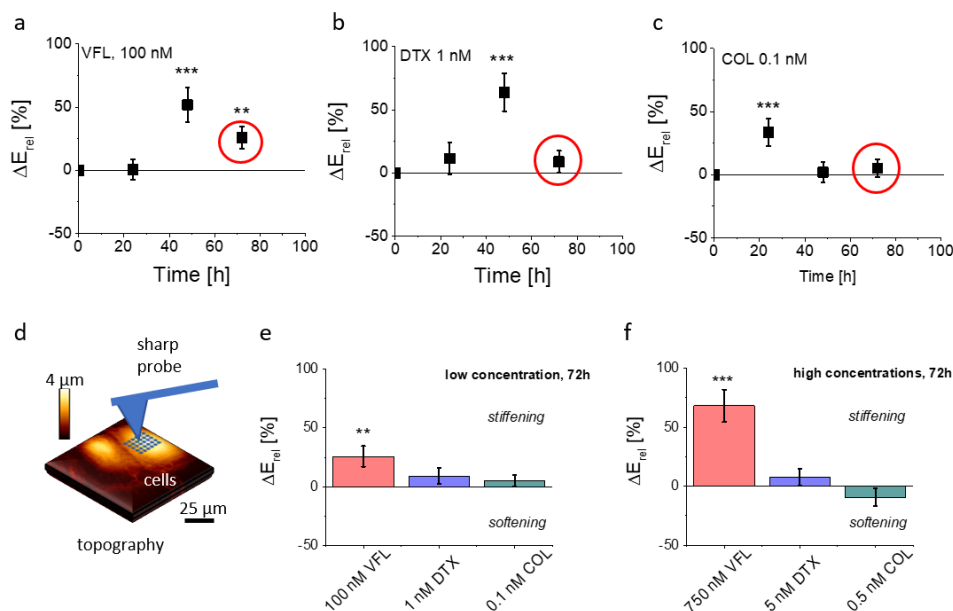


Figure 4. Mechanical properties of DU145 cells depend on the dose and MTDs type. (a-c) Relative Young's modulus change, ΔE , determined for cells treated with low MTDs doses in relation to NTC cells after 24h, 48h, and 72h of the incubation with the corresponding MT drug. Depending on the MTDs, a maximal increase of cell deformability was observed. Circle indicates values, for which a decrease of cell proliferation was observed (**Fig. 1**). **(d)** Measurements were conducted using AFM in indentation mode using pyramidal probes. **(e-f)** The 72h treatment of DU145 cells exposed to MTDs shows that a large change in Young's modulus for cells treated with VFL, regardless of the applied drug concentration, indicating cell stiffening. DTX action leads to weak stiffening regardless of the applied drug concentration while COL treatment to either stiffening (**e**, high drug doses) or softening (**f**, low drug dose) of DU145 cells. All data were normalized by the Young's modulus of NTCs measured at the same time-point. Statistical significance was estimated by unpaired *t*-Student test at the level of 0.05 (** $p < 0.01$, *** $p < 0.001$).

For each MTD, a maximum of cell rigidity was observed. For VFL and DTX after 48h of culture with the drug, while for COL the maximum occurred after 24h of culture. Interestingly, the maximum rigidity is not related to the proliferation rate (**Fig. 1**), showing the decrease in the number of cells after 72h of drug exposure. Also, such an increase was weakly associated with the organization of the microtubular network as MTs re-arranged strongly after 72h of culture with MTDs. Already published data indicate that for high concentrations, microtubule-targeted drugs can be broadly grouped into microtubule-stabilizing agents and destabilizing agents achieved by either promoting microtubule assembly or by triggering their disassembly into tubulin dimers³. The consequence is the re-arrangement of microtubules. Here, high MTDs concentrations cause heavy damages to the microtubular network (**Fig. 3f-h**), for DTX and COL added to cell culture. Observed large effect of MTs damage was not related to relative change in mechanical properties, neither for low nor high MTD concentrations.

Considering the occurrence of maximal cell rigidity, in our study, we focused on cells exposed to low concentration of MTDs after 72h of culture, for which proliferation rate decreased. AFM indentation experiments showed that prostate DU145 cells stiffen (positive ΔE , $p < 0.01$) in response to VFL treatment. After the same time, cells treated with DTX and COL revealed changes in mechanical properties that are statistically not significant ($p > 0.2$). These results are in contradiction with the fluorescence images of microtubular networks, showing that, for proliferating cells, the morphology of 100 nM VFL-treated cells is very similar to that of NTC cells, while the largest morphological changes are observed for 1 nM DTX and 0.1 nM COL-treated cells, for which less obvious changes in cell mechanics are observed.

Stiffening of VFL-treated cells occurs within the nuclear region

To probe more thoroughly the mechanical changes occurring after 72h treatment with MTDs, we performed combined AFM topographical and mechanical measurements, and analyzed the elastic response of the cells at different indentation depths²³ (**Fig. 5, Materials and Methods**). Each recorded force curve was linearized, and two linear regions, corresponding to different Hertzian regimes, were typically identified. The first regime (shallow indentation) corresponding to the 5-15% relative indentation range normalized to the local cell height), is expected to represent mainly the mechanical response of the cell membrane coupled with the actin cortical layer; the second regime (deep indentation) corresponding to the 20–40% relative indentation range, is

expected to be strongly affected by the response of the nucleus and the surrounding MT network. Given the peculiar organization of the MT network around the nucleus, we defined nuclear masks to focus our attention on the nuclear region of the cells (**Fig. 5b-d**). This methodology enabled us to analyze the deformability of different cellular regions and layers, according to the following classification: whole cell, from cell clusters (as DU145 cells tend to grow as clusters), nuclear region probed at the deep and shallow indentations, and cell periphery probed at shallow indentation (**Fig. 5c-d**). The applied finite thickness correction minimized the effect of the underlying glass substrate and local cell height variations (**Suppl. Notes 4&5; Fig. S4**). Elasticity maps revealed that in the case of shallow indentations, a large increase of Young's modulus was observed at the cell periphery (**Fig. 5c**), while the nuclear region appeared softer. At deep indentations, the cell periphery appeared to have lower Young's modulus as compared to the nuclear regions (**Fig. 5d**).

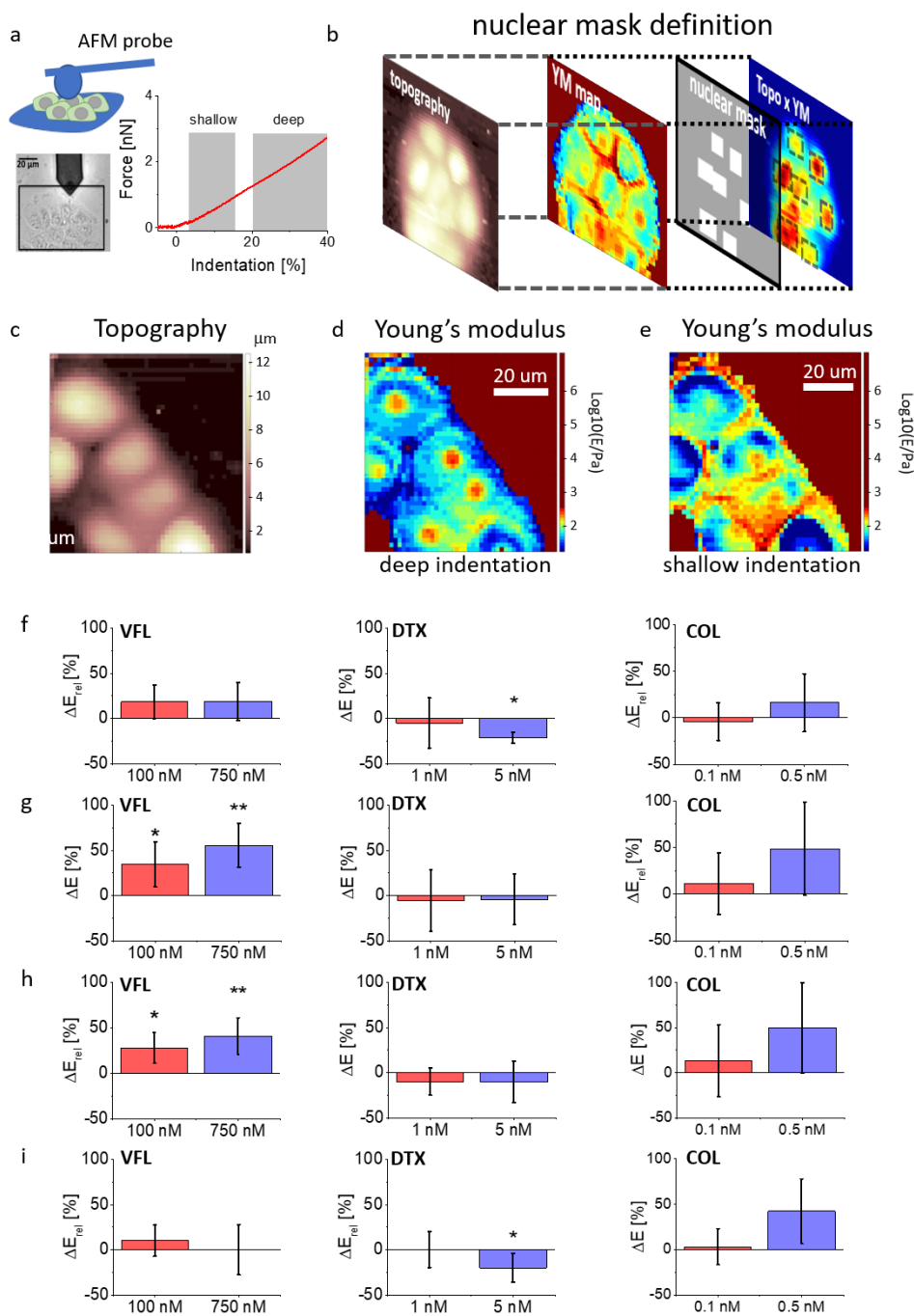


Figure 5. Multicomponent mechanical analysis of cell clusters. (a) Exemplary optical image of prostate cancer cell clusters with highlighted regions selected for the indentation measurements (the colloidal probe at the end of the AFM cantilever is visible as a darker circular shadow). Exemplary linearized force curves collected for VFL treated cells with highlighted shallow and deep indentation regions. (b) Nuclear mask definition used further to sort the data recorded around cell nuclei. (c) Topography of a cell cluster created based on contact point positions. (d-e) Young's modulus maps obtained at (d) shallow and (e) deep indentations. (f-i) Relative Young's modulus change, ΔE , determined for DU145 cells treated with specific MTDs concentrations for the whole cell (f), the nuclear region at shallow (g), and deep (h) indentations, and at the cell periphery at shallow indentation (i). Error bars were calculated using the error propagation method. Statistical significance was estimated by unpaired t-Student test at the level of 0.05 (* $p < 0.05$; ** $p < 0.01$).

The obtained Young's moduli for DU145 cells show changes that are strongly dependent on the cellular regions and the used drug (Fig. 5; Suppl. Table S2). Mechanical properties of COL-treated cells show a very weak tendency to stiffen, especially for 0.5 nM drug concentration, however, experimental errors are large enough to exclude statistical significance (p varies between 0.0584 to 0.8905). This brings the conclusion that colchicine does not affect the mechanical properties of prostate cells. Relative Young's modulus changes in DTX-treated prostate cancer cells do not reveal any alterations in mechanical properties at low (1 nM) concentration. Changes in cell mechanics were observed for high (5 nM) concentration but instead of stiffening, a softening was noted within a peripheral region. No difference as compared to NTC was observed within a nuclear region at both indentation regimes. DTX-induced softening at the cell periphery (Fig. 5i) was dominating in the overall cell softening observed when the whole cells were measured (Fig. 5f). In the case of VFL, changes in cell mechanics were more pronounced for nuclear regions at both applied MTD drug concentrations and indentation regions. Lack of ΔE variations within the cell periphery and at the level of the whole cell indicates that the mechanical properties measured for individual cells independently of the indentation depth are not affected by changes induced by MTD within nuclear regions.

In summary, DTX and VFL have two different effects on DU145 cells. DTX induces cell softening only at the cell periphery while VFL generates the stiffening of cells within a nuclear region.

MT network around cell nuclei is not responsible for the stiffening of VFL-treated cells.

Changes in mechanical properties of DU145 cells induced by the presence of MTDs are supposed to be associated with the alterations of cytoskeletal networks, especially MTs. Rings observed around cell nuclei in epi-fluorescence images (**Fig. 3c-e**) suggest a higher MT density close to cell nuclei, thus, MT crowding around the nuclei could be responsible for deformability changes induced by MTDs. To test this hypothesis, we employed confocal microscopy to visualize whether the spatial organization of microtubules around the cell nucleus is different in treated and non-treated prostate cancer cells. Typically, the images of the cytoskeleton are recorded close to the surface, to which cells are attached. The AFM measurements characterized also the mechanical properties by applying an external load to the apical region of the cells. Therefore, we also recorded an apical section of the cells. We concentrate on the organization of MTs around cell nuclei in cells treated with low MTDs concentrations. At high MTDs concentration, the organization of the microtubular network is heavily affected and can be seen under epi-fluorescent microscopy (**Fig. 3f-h**). Detailed analysis of the three-dimensional (3D) organization of microtubules in DU145 cells treated with low MTDs doses are included in the **Suppl. Note 6**.

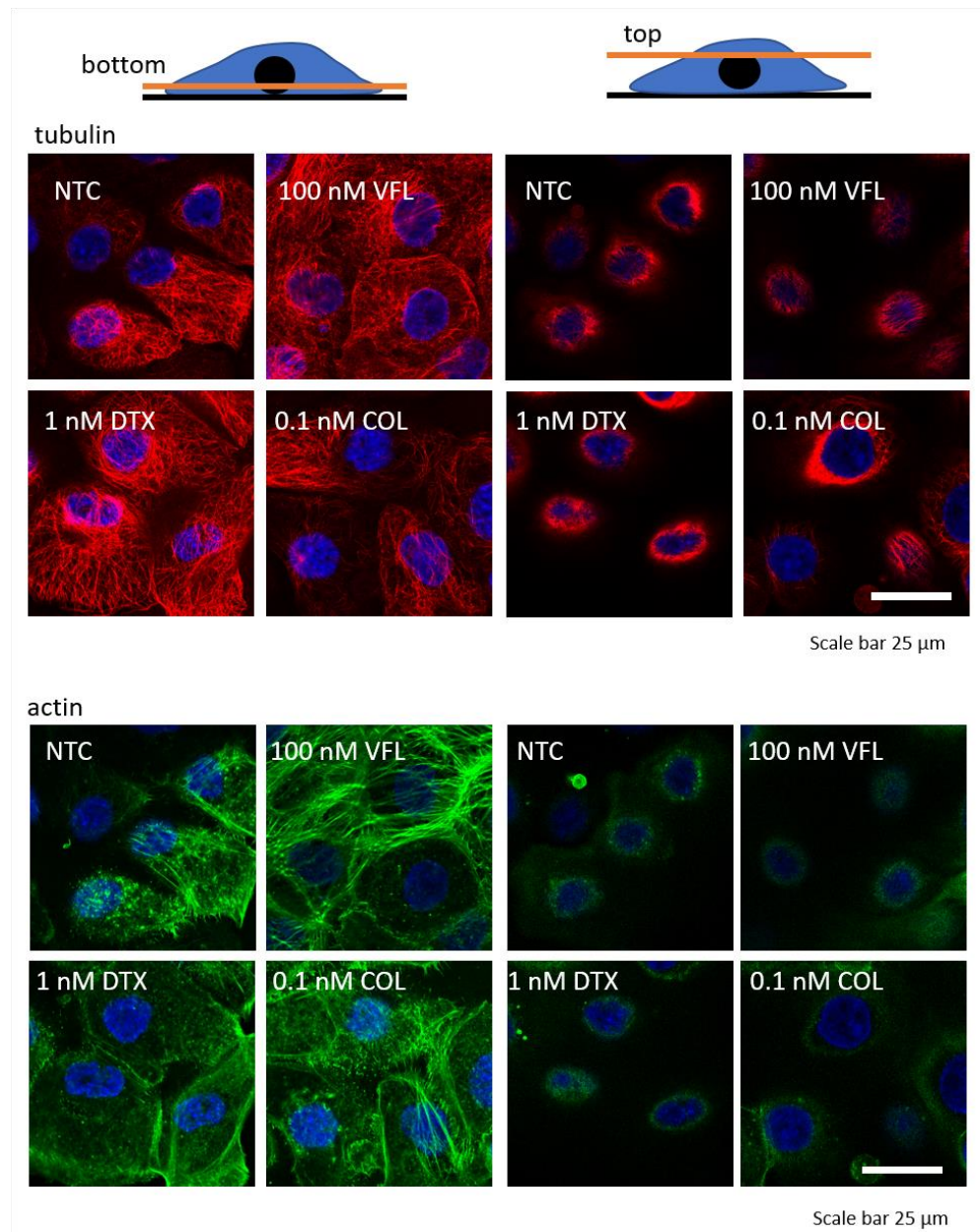


Figure 6. Confocal microscopy reveals a lack of cytoskeleton structural re-organization in microtubular networks linked with changes in mechanical properties of DU145 cells at low

concentrations of MTDs. *Fluorescent images were recorded at two positions along the cell height, top and bottom. The bottom position shows the organization of microtubules and actin filaments and microtubules in a focal plane close to the surface substrate. The top position shows the cytoskeleton organization at the top of the cell where the AFM probe touches its surface (F-actin - phalloidin-Alexa Fluor 488, microtubules β -tubulin antibody - Cy3, cell nuclei – Hoechst 33342).*

Lack of evident MTs re-organization around the cell nucleus guided us towards considering a crosstalk phenomenon between actin filaments and microtubules^{24,25}. Actin filaments play multiple roles regarding the microtubular network by the guidance of filament growth²⁶ or crosslinking and anchoring of microtubules within its network²⁷. Thus, any disruption of the microtubular network will affect the organization of the actin cytoskeleton and vice versa inducing the chemoresistance of cancer cells^{24,28,29}. Thus, we checked whether changes in the mechanical properties of cells might be related to the crosstalk phenomenon by analyzing the organization of actin filaments (**Fig. 6; Suppl. Fig. S5**). After 72 hours of incubation with MTDs, the formation of thick actin bundles was induced in cells. The strongest formation of actin bundles is observed for VFL-treated cells what explains their stiffening.

Discussion

Microtubules, and proteins associated with them, are critical for successful cell division, intracellular transport, signaling, and the regulation of focal adhesion dynamics both in normal and cancer cells. Their deregulation observed in cancer makes both tubulin and microtubules a prominent target for various chemotherapies³⁰.

Numerous studies have reported a cell-, dose-, and time-dependent cellular response to microtubule-targeting drugs^{6,31–35}. Although it is obvious that cells treated with high toxic concentrations are characterized by large damages causing their functional impairments, the effect of low drug concentrations on cells is less visible.

All MTDs interact with microtubules by binding to specific locations at the α/β tubulin heterodimer, thus, affecting the MTs dynamics by stabilizing or destabilizing the microtubular network^{15,26,36,37}. Microtubule stabilizing and destabilizing drugs disrupt microtubule functioning, which is accomplished by various mechanisms.

Vinca alkaloids destabilize microtubules by binding to a site located at the inter-dimeric interface between α and β tubulin heterodimers¹⁵. They induce the formation of alternate microtubules leading to the dissociation of existing microtubules^{38,39}. Taxanes reversibly bind to β -tubulin at the binding site located at the interior lumen of microtubules and enhance the polymerization process⁴⁰. Colchicine binds to a site located at the interface between α and β -tubulin subunits, adjacent to the GTP-binding site of α -tubulin⁴¹. It blocks the availability of α/β tubulin heterodimers for protofilaments or MTs by changing the conformation of the dimers³². As a consequence, colchicine causes microtubule depolymerization by inhibiting lateral contacts between protofilaments⁴². Thus, we could expect that vinca alkaloids and colchicine should increase the softening of cells, while taxanes should enhance the stiffening of cells.

In this study, we have focused on the effect of three selected members of MTDs, i.e., DTX, VFL, and COL, on prostate DU145 cancer cells. Proliferation and MTS assays conducted for cells cultured on glass surfaces for low and high MTDs concentrations show confirmed dose-dependent effects. Applying high MTDs concentrations, regardless of their type, leads to cell death showing a significant decrease in the number of attached cells within a whole-time range (24h–72h). MTS assay carried out for cells substantially confirms the results of the proliferation assay. For low MTDs concentrations, a large drop in the number of attached cells visible after 72h of the exposure of cells to specific MTDs and weakly affected viability of cells indicated that the cell population is not uniform. It contains cells that detached from the surface and cells that still stay attached.

Calcein efflux demonstrated that VFL-treated cells are still able to pump out the drug, while this process is limited in the case of cells treated with DTX and COL (**Fig. 2e&f**). These results indirectly show the accumulation of MTDs inside the cancer cells at drug concentrations below IC_{50} . VFL in contrast to DTX and COL, impacts the cellular bioenergetics very weakly, which does not lead to drug accumulation. Disturbances within MTs and mitochondrial dynamics may be responsible for the diminution of intracellular energy deposits that are necessary for effective drug efflux by transmembrane protein complexes^{43,44}.

The microtubular network alone has already been the object of AFM-based nanomechanical studies. In most cases, research was focused on the mechanical properties of cells treated by taxol-based compounds^{19,20,45–47}. Less frequently, the effect of compounds such as COL or vinca alkaloids, has been evaluated using AFM^{46,48}. Results gathered from a few existing publications report no stiffness change for various cell types treated with COL within a time frame

of 2 h and 4 h for applied concentrations of 500 μ M and 10 μ M, respectively^{46,48}. Vincristine (belonging to vinca alkaloids) induced stiffening of cells treated with 3 nM, 12 nM and 60 nM of drug concentrations for 48 hours⁴⁷. Paclitaxel (belonging to taxanes) affects mechanical properties of cells in various manners – in some cells no effect was observed⁴⁶ while in others an increasing cell rigidity was detected^{21,47,48}. Compared to these results, our findings are different, which might be due to a distinct response of prostate DU145 cells to MTDs as other studies have already demonstrated the susceptibility of cancer cells to cell death induced by these drugs^{33,34}.

According to our results, low concentrations of MTDs applied to prostate DU145 cancer cells induced changes in the organization of the microtubular network and the mechanical properties of cells in a surprising manner. For the VFL, which weakly affected the MTs organization, the largest cell stiffening is observed. Cells became more rigid. DTX-treated cells soften at high (above IC_{50}) concentrations at which large damages of MTs are detected. For COL, which had the strongest effect on the microtubular network, the mechanical properties of cells remained almost unchanged.

From these data, we cannot support the hypothesis that the mechanical resistance of prostate cells is associated with the changes in the organization of microtubules. Nor from the impairments in efflux pumps that transport the drugs out of the cancer cells^{43,44}. We postulate that the stiffening of prostate DU145 cancer cells is likely the mechanism that helps cancer cells to function in a non-disturbed way (like there were no MTDs presence).

Looking at the time-related change of cell deformability (**Fig. 4a-c**), all MTDs induce the maximum deformability changes in cells within a defined time of exposure to them. By analyzing MTDs-induced changes in both actin and microtubular networks, we noticed the formation of actin bundles in the affected cells. In our data, stiffening in VFL-treated cells was accompanied by the enhanced formation of actin bundles (**Fig. 6; Suppl. Fig. S5**). DU145 cells treated with DTX and COL for the same time reveal the mechanical properties of the level of NTC. For these cells, no increased number of actin bundles was observed indicating that the crosstalk between actin and microtubular network was significantly weaker. Our findings show the crosstalk between actin filaments and microtubules is likely the mechanisms that could be involved in the resistance of cells to vinca alkaloids observed in chemotherapy.

Materials and Methods

Microtubule-targeting drugs (MTDs). The MTDs chosen for our study were vinflunine, docetaxel, and colchicine. Vinflunine ditartrate (VFL, Toronto Research Chemicals) was dissolved in deionized water (Cobrabid purification system, $18 \Omega\text{cm}^{-1}$) to prepare stock solutions of 1 mM vinflunine concentration. They were stored in -18°C . Docetaxel and colchicine (DTX and COL, respectively, Sigma-Aldrich) were dissolved in ethanol. Drugs were added to the cell cultures 24 hours after cell seeding, followed by the application of a set of methods (**Fig. 1a**) to characterize properties of prostate cancer cells.

Cell culture. The human prostate cancer cells (DU145, ATCC, LGC Standards) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, LGC Standards) without antibiotics. Cells were grown in 25 cm^2 culture flasks (Saarstedt), passaged every 3-4 days by trypsinization with trypsin-EDTA solution (Sigma-Aldrich) and moved to the corresponding Petri dishes. To study the effect of anticancer drugs targeting microtubules, VFL, DTX, and COL were added to cells cultured in Petri dishes. Final concentrations of anticancer drugs applied to cells were 100 nM and 750 nM for VFL, 1 nM and 5 nM for DTX, 0.1 nM and 0.5 nM for COL. As a control, non-treated cells were used.

Assessment of drugs cytotoxicity by MTS assay. Cytotoxicity of drugs was assessed by Cell Growth Determination Kit, an MTT based colorimetric assay (Sigma-Aldrich). 3000 of DU145 cells/per well were seeded on 96-well cell culture plate (TPP). After the overnight culture of cells, the culture medium was exchanged to that containing the corresponding drug (VFL, DTX, or COL). After 68 hours of culture with drugs, $10 \mu\text{l}$ of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well. After 4 hours of incubation (at time point 72h), formazan crystals were dissolved in DMSO and absorbance was measured by spectrophotometer (ELISA SPECTROstar Nano, BMG LABTECH) at 570 nm wavelength for dissolved formazan and 690 nm for a background.

Proliferation of cells. To assess the effect of anticancer drugs on DU145 cell proliferation, cells were seeded at the density of 3000 cells/ cm^2 in cell culture dishes with $\varnothing = 35 \text{ mm}$ (Sarstedt).

They were kept in DMEM medium containing no phenol red (Sigma-Aldrich), 10% heat-inactivated FBS, and 2 mM L-glutamate (Sigma-Aldrich). The same number of cells was applied, i.e. 3 000 cells per ml for all samples. After 24 hours, the culture medium was replaced by a medium containing a specific drug concentration. Then, cells were maintained in such a medium for 24 h, 48 h, and 72 h. At each time point, a part of the cells was passaged and counted using the Bürker chamber.

Atomic force microscopy – indentation mode. For the measurements of the relative changes of the mechanical properties of the cells at different time-points, AFM indentation experiments were carried out using both pyramidal and spherical probes. It has been already shown that indentation data of living cells collected with sharp and spherical probes can both detect changes between two cell populations⁴⁹. Mechanical properties of DU145 cells were determined by analyzing force curves (additional experimental details are included in **Suppl. Notes S3 and S4**). The raw force curves (raw cantilever deflection versus z-piezo displacement) were converted into force versus indentation curves in the proper units (nN and nm, respectively) according to consolidated procedures⁵⁰. The spring constant of the cantilever was measured using the thermal noise calibration⁵¹. The deflection sensitivity was calibrated by recording raw force curves on non-deformable substrates (coverslip without cells in this case) and evaluating the inverse of the slope of the uncalibrated contact region⁵⁰. Alternatively, the deflection sensitivity was also calibrated in situ and non-invasively by imposing that the result of the thermal noise calibrated should be compatible with the previously characterized spring constant, according to the SNAP procedure⁵². Assuming that the elastic response of the cell can be described by Hertz contact mechanics⁵³, Young's modulus was determined.

In the case of a conical approximation of the probing tip, the relation between load force F and the resulting indentation δ is:

$$F = \frac{2 \cdot \tan(\alpha)}{\pi} \frac{E}{(1 - \nu^2)} \delta^2$$

where α is the open-angle of the probing cone, ν is the Poisson's ratio, and E is the effective Young's modulus. For a sphere indenting purely elastic material, the relation between load force F and the resulting indentation δ is:

$$F = \frac{3}{4} \frac{E}{(1 - \nu^2)} R^{\frac{1}{2}} \delta^{\frac{3}{2}}$$

Where R is the probe's radius. All calculations were carried out assuming that $\nu = 0.5$ (incompressible cells).

Characterizing mechanical properties of different cellular regions. To evaluate Young's modulus changes occurring at different regions of the cell, information on both a local height of the cell body and the value of the apparent Young's modulus was determined from each single force curve, and further used to provide the topographical and the Young's modulus maps²³ (see **Suppl. Note S4**). A finite-thickness correction was applied to extract accurate values of the Young's modulus from the force curves⁵⁴ coming from experiments with colloidal probes because the rigid substrate underneath the cells can cause an apparent increase of the Young's modulus that depends on the local height of the sample, therefore masking fine mechanical modifications that take place inside the cells body. The correction was not applied to pyramidal sharp tips since the finite-thickness effect becomes relevant with big contact area (see **Suppl. Note S5**).

Force curves were linearized to identify the presence of multiple elastic regimes inside the cell (typically attributed to an upper layer made of the actin cortex, and to an inner layer including the region around the cell nucleus). Two linear regions were typically identified in the linearized curves: a first region corresponding to the 5-15% relative indentation range (to the local values of cells height), the shallow indentation range, and a second inner region, corresponding to the 20-40% relative indentation range, the deep indentation range. Then, the Hertz model with applied finite thickness corrections was applied to the force curves in the two indentation ranges, obtaining two maps, representing the rigidity of the surface and inner part of the cell, respectively (**Fig. 5, Suppl. Table 2**).

To characterize the elastic properties of different parts of the cell's body we created masks to select force curves belonging only to specific regions of interest (nuclear and peripheral region with varying indentation). Nuclei are usually located in the tallest cell region. Since the nucleus elasticity could be hidden by that of the stiff actin cortex, we decided to build the mask by multiplying point by point the topographic and the elasticity maps. We found this procedure more reliable than identifying the nuclei simply as belonging to the tallest regions in the topographic maps. The masks allowing to segregate the force curves belonging to the nuclear regions were built exploiting both the elastic and the topographical maps **as shown in Fig. 5**.

Statistical analysis for the determination of Young's modulus for given cellular regions. The Young's modulus of each cellular region typically follows a lognormal distribution, which appears as Gaussian distributions in semilog10 scale. A Gaussian fit in semilog10 scale provides the peak value E' and the geometric standard deviation σ_g^{10} , from which the median E_{med} and the standard deviation of the median σ_{med} can be calculated⁵⁹:

$$E_{med} = 10^{E'}$$

$$\sigma_{med} = \sqrt{\frac{\pi}{2}} E_{med} \frac{\sigma_g^{10}}{\sqrt{N}}$$

where N is the number of force curves recorded for each cell cluster. The total error for the modulus median σ_{clust} associated to each cluster was calculated by summing in quadrature σ_{med} and a calculated instrumental error $\sigma_{inst} = 3\%^{37}$:

$$\sigma_{clust} = \sqrt{\sigma_{med}^2 + \sigma_{inst}^2}$$

Then, the median mean E_{mean} of the moduli median, measured for different clusters at a given condition for the different cellular regions, was evaluated as: $E_{mean} = \frac{\sum_i^n E_{med,i}}{n}$ where i is the number of clusters investigated. The total error σ_E associated to E_{mean} , representative for a specific condition and cellular region, was calculated by summing in quadrature the propagated error of the medians σ_S and the standard deviation of the mean σ_{mean} :

$$\sigma_E = \sqrt{\sigma_S^2 + \sigma_{mean}^2}$$

where

$$\sigma_S = \frac{1}{n} \sqrt{\sum_i \sigma_{med,i}^2} \text{ and } \sigma_{mean} = \sqrt{\frac{\sum_i (E_{med,i} - E_{mean})^2}{n}}$$

and n is the number of clusters investigated.

Immunofluorescence staining. To assess the impact of microtubule-targeting drugs on the organization of the microtubule network in DU145 cells fluorescent microscopy was employed. Cells were seeded in density 3000 cells/cm² on glass coverslips and allowed to stabilize at substrate overnight. Afterward, the culture medium was exchanged to a fresh medium containing final concentrations of microtubule-targeting drugs. After 72 hours, the culture medium was removed and cells were rinsed 3 times with Dulbecco's Phosphate Buffered Saline (DPBS, Sigma-Aldrich).

Next, cells were fixed with 3.7% formaldehyde solution and washed with DPBS to remove remaining formaldehyde. Permeabilization of cells membranes was realized by the treatment of fixed cells with 0.2% Triton X-100 solution, followed by washing with DPBS. Actin filaments were stained with phalloidin conjugated with Alexa Fluor 488 dye (0.33 μ M) for 45 minutes. Afterward, cells were incubated overnight with anti- β -tubulin antibody conjugated with fluorescent dye Cy3 (1:200) (Sigma-Aldrich), followed by nuclei counterstaining using Hoechst 33342 (0.2 μ g/mL).

Fluorescent and confocal microscopy. Epi-fluorescent images were acquired using a fluorescent microscope (Olympus IX53) equipped with fluorescence camera XC10 (1.4 mln pixels). The focal plane was set close to the cell surface. High-resolution 2D and 3D images recorded at the focal planes close to the glass surface and in the apical sections (where AFM probed mechanical properties of cells) were acquired by Leica TCS SP8 X confocal laser-scanning microscope (Leica Microsystems CMS GmbH, Germany) using 63x HC PL APO CS2 1.40 NA oil immersion objective. Image acquisition was performed bidirectionally along X-axis at 200 Hz scan speed and image format 1024x1024 (pixel size 72 nm), zoom 2.5, and line average 2. Three-dimensional images were reconstructed using Leica Application Suite X software (3D Movie Editor, Leica Microsystems CMS GmbH, Germany) or ImageJ 1.51n (Wayne Rasband, National Institute of Health, USA) on default parameters (except non-linear intensity adjustment – gamma parameter was 0.6).

Calcein efflux monitoring. Cells were seeded into 6-well plates at a density of 60 000 cells/cm² and treated with proper concentrations of MTDs for 24 h. After incubation with 0.75 μ M calcein-AM (Sigma-Aldrich) for 30 min, the cells were washed with a warm PBS buffer and incubated in a fresh medium for another 3h. After harvesting with trypsin/EDTA solution, the cells were centrifuged and re-suspended in FluoroBrite® DMEM (supplemented with 10% FBS and 1% GlutaMAX; Gibco) and acquired with ImageStreamX MkII system and ISX software (Luminex Corp.) to determine the efficiency of dye efflux. Data processing was performed with IDEAS 6.2 software. The analysis was done on “*in-focus*” images of single cells. In-focus single cells were classified based on their bright-field area and high bright-field aspect ratio (width to height ratio). At least 10 000 images were collected for data analysis.

Statistics. For cell biology methods (proliferation and MTS assay, cytometry, morphometric analysis) data are presented as a mean $\pm$ standard deviation (s.d) (**Fig. 1b-e, Fig. 2e-f**) calculated from three independent experiments. Data describing the morphometric properties of cells (**Suppl. Fig. S1 and Suppl. Fig. S2**) are expressed as a mean $\pm$ s.d. calculated for n denoting the number of cells measured (specified in the figure legends). Original Young's modulus values were calculated as a mean $\pm$ s.d considering the number of cells or clusters measured (**Suppl. Tables S1-2**). Relative Young's modulus changes presented in **Figs. 4, 5, Suppl. Figs. 2-3**) were accompanied by an error calculated using the error propagation method. In all cases, statistical significance was estimated by unpaired t-Student test at the level of 0.05. The following notation was applied: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information file. Raw data underlying the presented figures are available from the corresponding authors upon reasonable request.

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Supplementary results

Stiffening of prostate cancer cells driven by actin filaments microtubules crosstalk confers resistance to microtubule-targeting drugs

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Content of the supplementary information:

- ***Supplementary Note 1: Proliferation rate of DU145 cells treated with MTDs***
- ***Supplementary Note 2: Morphology of DU145 cell nucleus is affected by MTDs (Supplementary Figure S1; Supplementary Figure S1)***
- ***Supplementary Note 3: Time-dependent changes in mechanical properties of DU145 cells (Supplementary Table S1; Supplementary Figure S3)***
- ***Supplementary Note 4: Mapping the mechanical properties of cells***
- ***Supplementary Note 5. Finite-thickness correction (Supplementary Figure S4)***
- ***Supplementary Note 6: 3D organization of MTs visualized using confocal microscopy***
- ***Supplementary Note 7: Actin organization inside DU145 cells (Supplementary Figure S5, Supplementary Figure S6)***

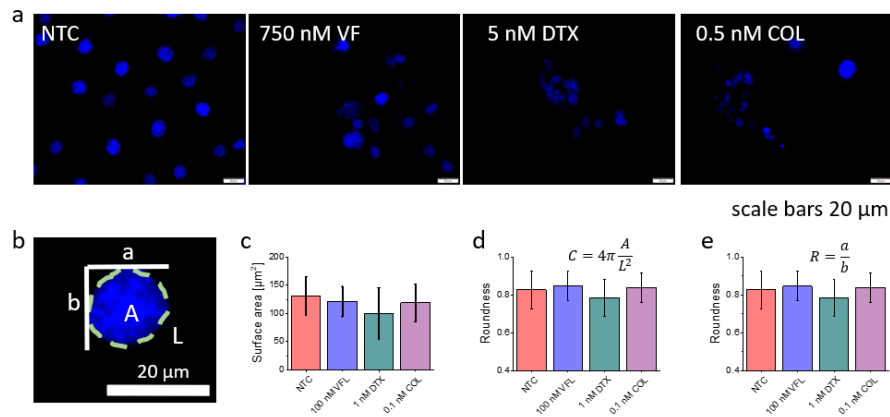
Supplementary Note 1: Proliferation rate of DU145 cells treated with MTDs

To estimate how fast the number of cells increases, a linear regression was applied to quantify roughly the rate of proliferation (**Fig. 1**). Although for NTC cells an exponential-like growth can be observed, the linear fit was chosen to avoid an exponential fit to three points. As expected, NTC cells had the highest proliferation rate, which was of 4300 ± 1100 cells per hour (Pearson's coefficient = 0.939). For low doses of drugs, i.e. 100 nM VFL, 1 nM DTX and 0.1 nM COL, obtained rates were 3000 ± 270 cells per hours (Pearson's coefficient = 0.992), 2270 ± 220 cells per hour (Pearson's coefficient = 0.991) and 3340 ± 740 cells per hour (Pearson's coefficient 0.954), respectively.

Supplementary Note 2: Morphology of DU145 cell nucleus is affected by MTDs

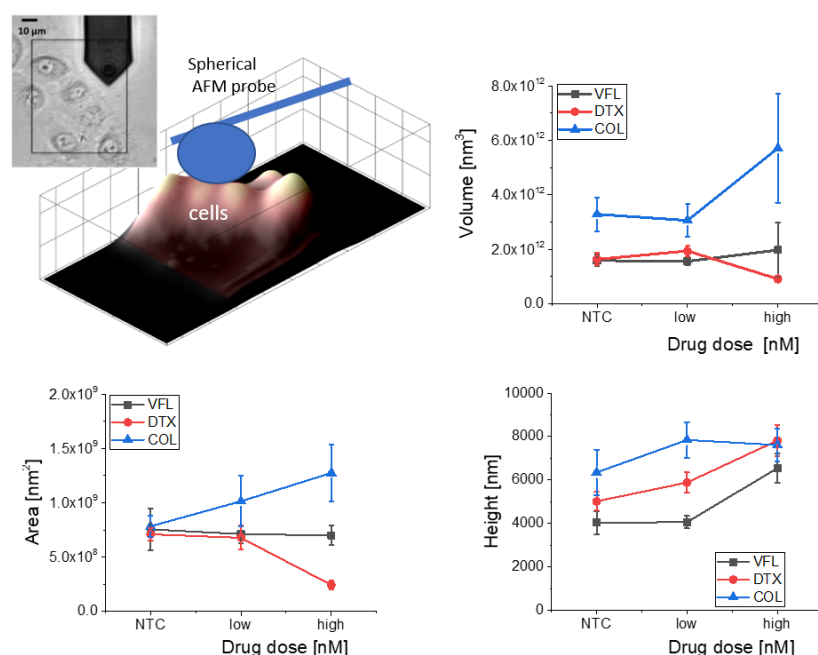
To investigate nuclear morphology fluorescence microscopic images were analyzed in ImageJ with package Fiji and Cookbook with the function Particle Analysis. At least 5 images for each group for the following drugs concentrations: 100 nM and 750 nM for VFL, 1 nM and 5 nM for DTX, 0.1 nM and 0.5 nM for COL and control without addition of drug.

Although observed changes in microtubule organization correspond roughly with the results of cell viability, we wanted to verify the morphology of the cell nucleus (**Suppl. Fig. S1**). At large drug doses, regardless of the drug applied, cell nuclei were heavily irregular and defragmented as one can see in the Hoechst stained fluorescent images (**Suppl. Fig. S1a**).



Suppl. Figure S1. Morphology of the cell nucleus is strongly affected by microtubule-targeted drugs. **a**, High concentrations of VFL, DTX, and CL induce fragmentation of cell nuclei, especially for DTX and COL. In low drug concentration, nucleus morphology is preserved (**b**), however, DTX seems to affect nucleus changes, as shown in morphometric analysis of surface area (**c**), circularity (**d**), and roundness (**e**) for untreated and treated prostate DU145 cells. Data are presented as a mean ± standard deviation calculated from $n = 79$ -105 cells per condition.

To evaluate nuclear changes occurring at low doses, three morphometric parameters were calculated, namely, nuclear surface area, circularity, and roundness (**Suppl. Fig. S2**). Obtained results showed large variations in the nucleus shape, in particular for DTX treated cells, for which circularity and roundness values dropped, too. For cells treated with COL and VFL cell nuclei seem to be less affected by the MTDs action.



Suppl. Figure S2. Cells change their morphology in response to MTDs. AFM-based morphometric parameters of DU145 clusters showing alterations in clusters volume, surface area, and height induced by MTDs. Data are presented as a mean $\pm$ σ_E ; $n = 8-12$ per condition.

The obtained results show significant and opposing alterations of the cell volume and surface area for DTX (decrease) and COL (increase), especially for the high doses. For high VFL concentration, a significant increase in total volume in comparison to NTC is also observed. Such observation is leading us to the conclusion that the swelling of cells treated with a high concentration of drugs is characteristic of microtubule-disrupting agents. The height of the analyzed cell clusters shows an increasing trend for all MTDs, although significant observations were unique for high drug concentrations. Interestingly, in the low-dose (100 nM) VFL case, for which pronounced deformability changes were recorded, volume, surface area, and height of cells remained at the same level of the NTC cells. Thus indicating cytoskeleton contribution in mechanical features changes of 100 nM VFL treated cells, rather than changes in their dimensions.

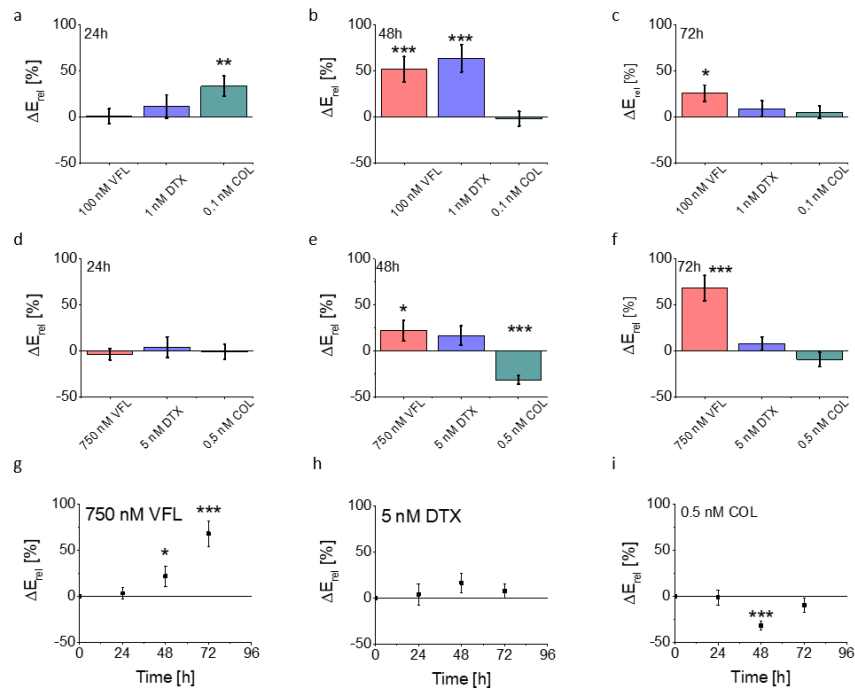
Supplementary Note 3: Time-dependent changes in mechanical properties of DU145 cells

To measure the mechanical properties of DU145 cells, cells were seeded on glass coverslips ($\varnothing = 25$ mm) immersed in a Petri dish with internal $\varnothing = 34$ mm (TPP) at the density of 50 000/ml. Measurements were carried out using atomic force microscopes: model Xe120 (Park Systems) and working in a force spectroscopy mode. A rectangular cantilever ORC8 (Bruker) with a nominal spring constant of 0.05 N/m, the opening angle of $36 \pm 2^\circ$, and a radius of 15 nm were used. Cantilever spring constants were calibrated by the use of the thermal noise method¹. For measurements, coverslips with cells were mounted in a transparent liquid cell and kept at room temperature in standard culture medium. Room temperature was not significantly harmful for Du145 during experiments which lasted up to 2 hours². Keeping a temperature below 37°C reduces the cell motility, which was beneficial for the elasticity measurements. 25 force curves were acquired within an elasticity map of 5×5 points with a pixel size of $1 \mu\text{m}^2$, load force ~ 10 nN, and tip velocity of $8 \mu\text{m/s}$. Each experiment was conducted in triplicate.

The relative Young's modulus variations are, calculated as $\Delta E_{rel} = (E_{drug} - E_{NTC})/E_{NTC}$, are reported in **Suppl. Table S1 and Suppl. Fig. S2**. In our analysis, the maximum indentation was 500 nm. The final Young's modulus was calculated as the mean $\pm$ standard error (s.e.) from $n = 80$ -96 cells measured for each group separately (**Suppl. Table S1**).

Suppl. Table S1. Young's modulus obtained for MTDs treated Du145 cells (s.e. – standard error, n – number of cells measured; p -value was calculated using two tails unpair Student t -test). The relative Young's modulus variations are reported in both Fig. 4 and Suppl. Fig. S2.

<i>time</i> <i>MTDs</i> <i>treatment</i>	24h $E \pm s.e. (n) [kPa]$	48h $E \pm s.e. (n) [kPa]$	72h $E \pm s.e. (n) [kPa]$
<i>Vinflunine (VFL)</i>			
<i>NTC</i>	$3.1 \pm 0.2 (94)$	$2.2 \pm 0.1 (93)$	$1.5 \pm 0.1 (96)$
<i>100 nM VFL</i>	$3.1 \pm 0.3 (94)$ $p = 0.9512$	$3.3 \pm 0.3 (93)$ $p = 0.0001$	$1.9 \pm 0.1 (93)$ $p = 0.0016$
<i>750 nM VFL</i>	$3.0 \pm 0.2 (76)$ $p = 0.7147$	$2.7 \pm 0.2 (88)$ $p = 0.0412$	$2.5 \pm 0.2 (91)$ $p = 0.0001$
<i>Docetaxel (DTX)</i>			
<i>NTC</i>	$4.2 \pm 0.3 (88)$	$2.9 \pm 0.2 (88)$	$2.6 \pm 0.1 (89)$
<i>1 nM DTX</i>	$4.7 \pm 0.4 (86)$ $p = 0.3610$	$4.8 \pm 0.4 (83)$ $p = 0.0001$	$2.8 \pm 0.2 (89)$ $p = 0.2977$
<i>5 nM DTX</i>	$4.4 \pm 0.4 (83)$ $p = 0.7280$	$3.4 \pm 0.3 (86)$ $p = 0.1085$	$2.8 \pm 0.1 (87)$ $p = 0.2971$
<i>Colchicine (COL)</i>			
<i>NTC</i>	$3.3 \pm 0.17 (90)$	$3.0 \pm 0.15 (91)$	$2.2 \pm 0.1 (90)$
<i>0.1 nM COL</i>	$4.4 \pm 0.3 (88)$ $p = 0.0012$	$2.9 \pm 0.2 (86)$ $p = 0.7853$	$2.3 \pm 0.1 (87)$ $p = 0.4623$
<i>0.5 nM COL</i>	$3.3 \pm 0.2 (87)$ $p = 0.9140$	$2.0 \pm 0.1 (88)$ $p = 0.0001$	$2.0 \pm 0.2 (88)$ $p = 0.2520$



Suppl. Figure S3. Relative change ΔE_{rel} of the Young's modulus measured at the same time-point for low (a-c) and high (d-f) MTDs doses. (g-i) Time-dependent ΔE_{rel} changes showing cell stiffening or softening dependently of the MT drug, concentration, and time applied. Error bars were calculated using the error propagation method. Statistical significance was estimated by unpaired t-Student test at the level of 0.05 (* $p < 0.05$; ** $p < 0.01$).

Supplementary Note 4: Mapping the mechanical properties of cells: combined topographical and mechanical mapping

For acquiring maps of mechanical properties, DU145 cells were plated in the standard experimental conditions on $\varnothing = 40$ mm glass-bottom dishes for cell culture (Willco Wells, Amsterdam, Netherlands). Cells were maintained in DMEM without phenol red but supplemented with 10% heat-inactivated FBS and 5 nM L-glutamate. Glass coverslips with cells were mounted in transparent liquid cell and kept at room temperature in standard culture medium.

Combined topographical and mechanical imaging was performed using a Bioscope Catalyst AFM (Bruker). During the AFM measurements, the temperature of the medium was maintained at 30°C using a perfusion stage incubator and a temperature controller (Lakeshore 3301, Ohio, USA). The choice of the temperature below 37°C was made to decrease the cellular metabolism, which slows down also the cell motility, making it possible to acquire large maps without disturbance due to strong movements of the cells during the measurements (acquisition of one map takes 30-40 min).

We used custom monolithic borosilicate glass probes consisting in spherical glass beads with radii R in the range 4500–5500 nm, attached to ultra-soft silicon, tipless cantilevers (Micromash HQ:CSC38/tipless/no Al) with elastic constant $k = 0.02\text{--}0.03$ N/m, measured using the thermal noise calibration³. Probes were fabricated and calibrated, in terms of tip radius, according to an established custom protocol⁴.

The topographical/mechanical maps were obtained, as described elsewhere⁵, from sets of force curves, collected in Point and Shoot (P&S) mode, selecting the regions of interest from optical images, exploiting the accurate alignment of the optical and AFM images obtained using the Miro software module integrated in the AFM software. Each set of force curves consisted of an $N \times M$ array of curves spatially separated by approximately 1.5 μm , each force curve containing 4096 points, with ramp length $L = 6$ μm , maximum load $F_{\text{max}} = 10$ nN, ramp frequency $f = 1$ Hz. Typical maximum indentation was 2-3 μm , larger than in the case of sharp pyramidal probes (max indentation 500 nm).

Suppl. Table S2. Young's modulus obtained for MTDs treated DU145 cells showing the mean value of the median E extracted per each cluster; error represents the final error σ_E that take into account the three main errors: the instrumental error σ_{inst} , the error associated to one single cluster median value σ_{med} and the deviation of the all clusters σ_{mean} , n – number of cell clusters measured; p -value was calculated using the two-tails unpair Student t -test.

	VFL	DTX	COL
whole-cell	$E \pm \sigma_E [Pa]$	$E \pm \sigma_E [Pa]$	$E \pm \sigma_E [Pa]$
NTC	375.1 ± 23.2 (8)	429.0 ± 35.9 (11)	376.6 ± 37.6 (9)
low	444.61 ± 24.4 (8) $p = 0.0058$	407.9 ± 34.3 (12) $p = 0.6759$	360.6 ± 79.6 (8) $p = 0.8528$
high	447.1 ± 29.8 (7) $p = 0.0755$	336.6 ± 25.5 (12) $p = 0.0456$	436.8 ± 132.1 (7) $p = 0.6321$
nuclear region (5-15%)			
NTC	346.6 ± 19.8 (8)	410.8 ± 32.7 (11)	422.9 ± 43.6 (9)
low	466.5 ± 36.9 (8) $p = 0.0125$	388.3 ± 41.0 (12) $p = 0.6760$	470.5 ± 99.2 (8) $p = 0.6536$
high	539.0 ± 44.9 (7) $p = 0.0012$	394.4 ± 33.6 (12) $p = 0.7296$	628.4 ± 167.9 (7) $p = 0.2054$
nuclear region (20-40%)			
NTC	381.9 ± 13.2 (8)	378.7 ± 24.3 (11)	429.0 ± 38.2 (9)
low	488.9 ± 22.7 (8) $p = 0.011$	341.8 ± 25.5 (12) $p = 0.3093$	486.1 ± 60.6 (8) $p = 0.4271$
high	537.6 ± 44.8 (7) $p = 0.0037$	340.0 ± 16.5 (12) $p = 0.1957$	642 ± 107 (7) $p = 0.0584$
cell periphery			
NTC	397.3 ± 29.9 (8)	401.4 ± 27.6 (11)	376.8 ± 35.9 (9)
low	437.5 ± 24.2 (8) $p = 0.3139$	403.4 ± 23.1 (12) $p = 0.9544$	388.5 ± 79.3 (8) $p = 0.8905$
high	398.3 ± 41.7 (7) $p = 0.9841$	321.1 ± 24.8 (12) $p = 0.0416$	536 ± 152 (7) $p = 0.2702$

Supplementary Note 5. Finite-thickness correction

To overcome issues linked with the influence of the underlying stiff support on the mechanical properties of cells, finite thickness correction was applied to the obtained data shown in **Suppl. Fig. S4**.

The finite-thickness correction is needed when the requirement that the sample thickness is much larger than the maximum indentation ($h \gg \delta$) is not satisfied. When indenting cells, which are very soft, with maximum indentation δ well beyond 1 μm , the finite-thickness effect must be accounted for. In this condition, the influence of the rigid substrate (the bottom of the glass dish from Wilco Weels®) makes the cell elastic response stiffer, i.e. the measured YM is larger. This effect increases when the sample becomes even thinner, like in the peripheral regions of the cell body. As a rule of thumb, the condition $10-15 \delta < h$ should be satisfied to neglect finite-thickness effects, although the incidence of finite thickness depends also on the radius of the indenter (see below). It turns out actually that this effect is stronger for large colloidal probes, while it is usually negligible for sharp AFM tips.

We implemented the analytic correction developed by Dimitriadis *et al.*⁶ for spherical probes, as described by Puricelli *et al.*⁵. Since cells adhere to the substrates using highly dynamical focal adhesion complexes, following the advice of Gavara and Chadwick⁷, we used a correction factor to the Hertz equation, which represents an average between the two conditions of perfectly adherent and non-adherent cells:

$$F = \frac{9}{16} E R^{\frac{1}{2}} \delta^{\frac{3}{2}} [1 + 1.009\chi + 1.032\chi^2 + 0.578\chi^3 + 0.051\chi^4]$$

This is the effective equation for the correction of the finite-thickness effect. Defining $\Delta(\chi) \equiv \Delta(\chi(R, \delta, h))$, with

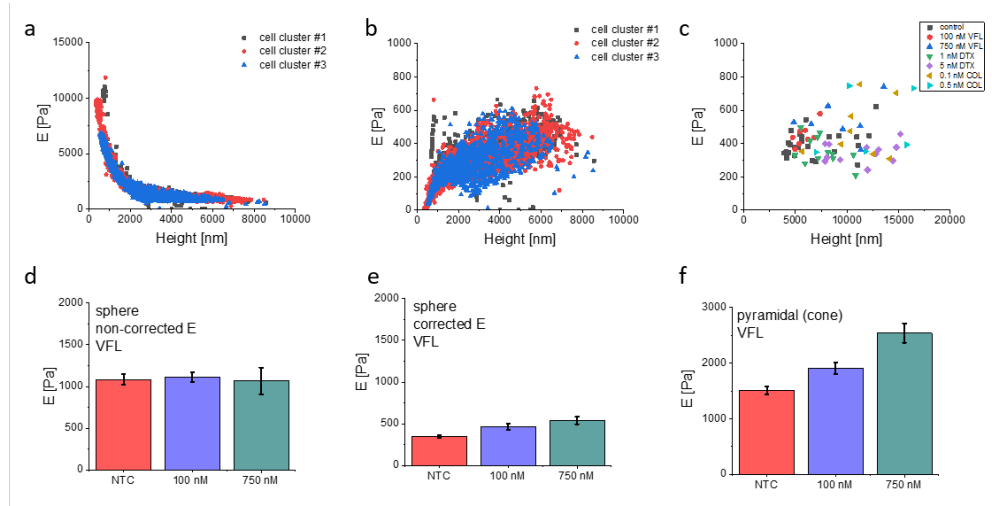
$$\Delta = 1 + 1.009\chi + 1.032\chi^2 + 0.578\chi^3 + 0.051\chi^4,$$

the finite-thickness corrected Hertz Model can be written as:

$$\frac{F}{\Delta(\chi)} = \frac{9}{16} E R^{\frac{1}{2}} \delta^{\frac{3}{2}}$$

The variable $\chi = \frac{\sqrt{R\delta}}{h}$ combines the three critical lengths of the system, the radius of the probe R , the indentation δ and the height of the sample h . Considering that for the Hertz model the contact area between the probe and the sample is $a = \sqrt{R\delta}$, we have $\chi = \frac{a}{h}$, meaning that the finite-thickness effect does not depend directly on the ratio of the vertical lengths δ and h , but rather on the ratio of the horizontal dimension of the contact area, i.e. the contact radius a , to the sample height h . As a consequence, for a given indentation δ , the finite-thickness effects will be significantly stronger for the colloidal probes, compared to the sharp ones.

An example of the effects of the finite-thickness correction is shown in **Suppl. Figure S4**. The value of the Young's modulus extracted from each force curve is plotted as a function of the local cell height, for three representative different clusters of cells (control conditions), measured in a single day. Data are shown with (**Suppl. Fig. S4a**), or without (**Suppl. Fig. S4b**), the finite-thickness correction.



Suppl. Figure S4. Finite thickness correction for colloidal probes based on Dimitriadis et al.⁶ Data are presented as Young's modulus extracted from single force curves, coming from three clusters of cells of the control condition, using (a) standard Hertz model and (b) the finite - thickness corrected Hertz model. (c) Young's modulus median value of single cell clusters (E_{md} , see Materials and Methods). (d-e) median Young's modulus $\pm \sigma_E$; $n = 3$ clusters per condition. (f) mean Young's modulus $\pm$ standard error; $n = 76-94$ cells per condition.

To verify that the results of the mechanical analysis are not affected by the change in the height of the cells inside the clusters (for example, the thickening of cells could determine a loss of sensitivity to the inner region of the cells, for the same maximum indentation, or to a redistribution of organelles in the cytoplasmic region), we plotted the median of Young's modulus from the inner cells body (nuclear region, deep indentation) of each cluster versus the mean cluster height (**Suppl. Fig. S4c**). There is no evidence of any correlation between the height of the cluster or the cell and Young's modulus, which led us to exclude that our analysis is significantly biased by the morphological changes of the cells. It is clear from the comparison of **Suppl. Fig. S4a and S4b** that the measured Young's modulus increases as the cell height decreases, leading to severe overestimation of the intrinsic (effective) value of a cell modulus. Noticeably, the substrate-induced also in the highest points. In our experimental conditions, only with cell heights of approximately 20-30 μm would the finite-thickness effect negligible.

The effect of the rigid substrate underneath the cells is to hide the fine mechanical modifications that take place inside the cells body, upon the action of the drug, resulting in a loss of sensitivity. Since we have shown that the morphological properties, and in particular the height of the cells, change upon the treatment with the drugs, the application of the finite-thickness correction is important to compare results obtained at different drug concentrations. This is shown in **Suppl. Fig. S4d-f**, where the results obtained with VFL with and without the finite-thickness correction are shown. Without the correction, apart from a rigid shift to higher Young's modulus, the differences between the three conditions are lost; with the application of the correction, instead, the differences are evident and the same trend is observed as with the pyramidal probes.

Supplementary Note 6: 3D organization of MTs visualized using confocal microscopy

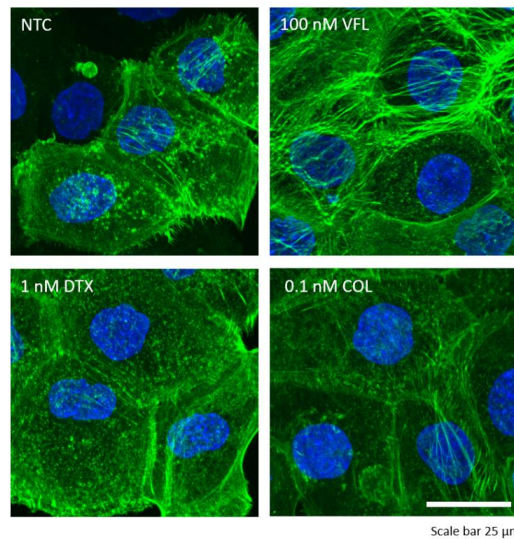
To visualize the 3D organization of MTs, movies were prepared by combining all recorded z-stacks for DU145 cells treated with low MTDs doses. List of movies:

- DU145_MTs_NTC.mp4
- DU145_MTs_VFL.mp4

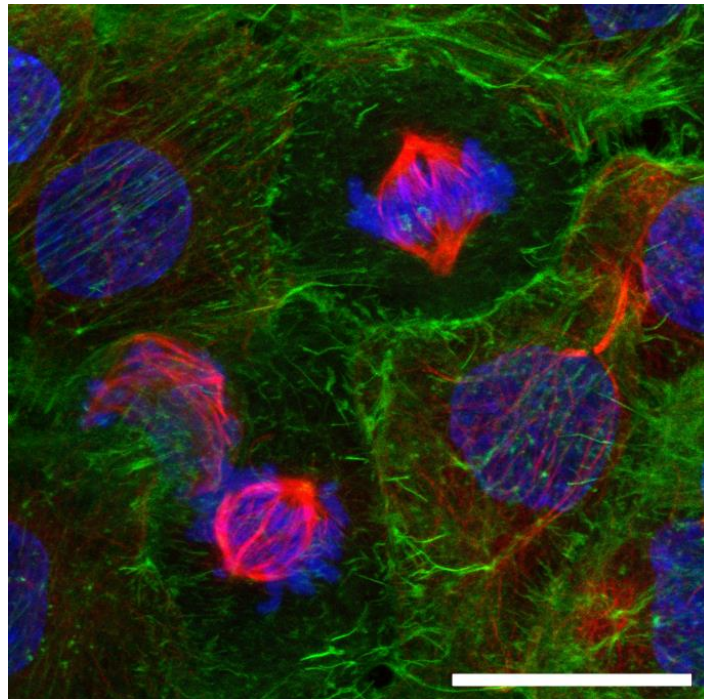
- DU145_MTs_DTX.mp4
- DU145_MTs_COL.mp4

Supplementary Note 7: Actin organization inside DU145 cells

Maximum fluorescence intensity (maximum intensity projections of the Z-stacks of microscope images) obtained from phalloidin conjugated with Alexa Fluor 488 showing the organization of actin filaments in non-treated (NTC) and MTD-treated prostate DU145 cancer cells (**Suppl. Fig. S5**).



Suppl. Figure S5. Organization of actin filaments in non-treated (NTC) and MTDs-treated prostate DU145 cancer cells visualized using confocal microscopy.



Suppl. Figure S6. Example of proliferating DU145 cells during the mitosis process after 72h of treatment with VFL. Maximal projection image from the fluorescent confocal microscope. Scale bar = 25 μm .

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6.1 Additional discussion

As mentioned in the Supplementary Information (Section note 5), the accurate measurement of the cell mechanics requires the fine mapping of the whole cell plus the surrounding substrate. The reason behind this approach is the need to obtain a pointwise information about the cell's height to eventually apply the finite-thickness correction⁶⁵. Moreover, different portions of the cells may have different properties. The achievement of such high-resolution maps is usually challenging and time-consuming:

1. To reduce the hydrodynamic drag^{130,131}, and also to avoid viscoelastic effects, the ramping frequency should be kept low (around 1 Hz), with a resulting acquisition time of the order of 40-60 min.
2. A fine sampling implicates that a considerable amount of time would be necessarily spent on the substrate that is not a region of interest.
3. The resulting acquisition time is compatible with the movement of the cells in the range of few microns. Indeed, motile cells can actually move significantly during the time frame of a measurement which might invalidate the result. This can be limited by working at lower temperatures (slowing down their metabolism), although the risk in stressing the cells is high, with a consequent alteration of their natural behaviour.

A more useful approach would consist in spending as little time as possible on the substrates, acquiring few FCs in order to characterize the orientation substrate baseline in the 3-dimensional space, then focus the attention only on the regions of interest on the cells (like just the cell nucleus or the lamellipodia). A preliminary development of such protocols will be discussed in detail in the Appendix A.

Chapter 7

Mechanics of the extracellular environment in peritoneum metastases*

As discussed in the Introduction, the field of mechanobiology is based on the study of reciprocal physical interactions of single cells and the ECM composing the tissues. Indeed, while it is evident that the ECM composition, morphology and stiffness influence the cell fate, it is also true the ECM itself is a product of the cellular metabolism, and that cells can actually modify the local environment they are living in. Cells can change indeed the ECM chemical composition, influence the enzymatic activity or structurally reorganize its components, thus giving rise to a feedback loop by which cells and the ECM transform each other.

A direct consequence of this interplay is that the progression of cancer is strongly dependent on ECM deregulation, which in turn promotes abnormal cell behaviour and metastases.

It has been widely demonstrated that the extracellular matrix in tumoral tissue is stiffer than its healthy counterpart^{3,132,133}, but in an interesting work Nebuloni et al.¹⁵ have studied the progression of human colorectal carcinoma (CRC) and have shown that ECM modifications precede the tumour invasion. They discovered that the perilesional ECM possesses intermediate mechanical properties between the ones coming from healthy and tumoral tissues, thus demonstrating that even cancer cells need to modify their surrounding microenvironment in order to invade it.

Moving towards advanced stages of CRC progression, among the first organs colonized by CRC metastases is the peritoneum¹³⁴. In the following work we have

* An advanced draft is under preparation and will be soon uploaded (as a pre-print) on BiorXiv database. The list of authors that contributed to this project are listed in the following.

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tested the mechanical and morphological properties of human peritoneal ECM, healthy tissue and already invaded by metastases.

In the past, peritoneal metastases (PM) were considered a terminal condition, amenable only to palliative treatments, and about 70% of treated patients still experience peritoneal relapse¹³⁵. It is therefore desirable to develop new pre-clinical cellular models able to faithfully recapitulate the PM pathology to identify more effective therapeutic strategies. PM development consists of a series of steps that begin with cell detachment from the primary tumour. These cells bind to the peritoneum as a result of molecular interactions with elements of the peritoneal microenvironment, and they invade the sub-peritoneal layer where angiogenesis promotes their growth. The process is known as peritoneal metastatic cascade¹³⁶. Fine-tuned interactions between the biochemical factors released by the tumour and biomechanical events, such as the remodelling of the extracellular matrix (ECM), govern the cascade and allow formation of the metastatic niche^{137,138}. Development of the metastatic niche facilitates organotrophic metastasis through the direct promotion of cancer stem cells survival, exploiting a tissue-specific microenvironment more suitable for the attachment of exfoliated neoplastic cells¹³⁹.

However, the biology behind these interactions is poorly understood, particularly because experimental models that demonstrate organ specificity are lacking. The advent of collagen-based matrices and Matrigel that reproduce a tissue-like environment has allowed three-dimensional (3D) cultures resemble the *in vivo* environment. However, the composition of these substrates differs from the tissue microenvironment encountered by cells during metastatic spread¹⁴⁰.

Most data on metastatic spread have been obtained using cancer cell lines or patient-derived xenografts models, which do not fully reflect the physiopathology of their tumour of origin¹⁴¹.

Tumour-derived organoids (TDO) are an intermediate model between cell lines and xenografts; they grow three-dimensionally and retain cell–cell and cell–matrix interactions, which more closely reflect the characteristics of the original tumor¹⁴². Organoids can be established in a relatively short time and are easy to manipulate¹⁴²; in addition, they maintain the genetic status of the original tissues and can be used to identify new therapeutic targets¹⁴³. However, TDO lack native tissue structures, including ECM, and thus do not mimic important features of PM. One possible approach to engineer PM *in vitro* could be to grow PM-derived organoids on decellularized ECMs that constitute the scaffold of the PM.

Here, a protocol for the purification of tissue-derived ECM is successfully applied and tested, aimed at the complete removal of cells and cellular debris. The protocol follows a decellularizing protocol developed by Genovese et al¹⁴⁴ in which the decellularized ECMs maintain their own initial biochemical properties and 3-dimensional structures, allowing to study ECM features decoupled from cell interference.

Interestingly the metastatic ECMs display a structural organization characterized by the loss of fibrillar-like structures accompanied by a significant stiffening of the sample. In particular, when seeded with CRC malignant cells, healthy ECMs

shows a noticeable ability in constraining cell infiltration and downregulating the proliferation rate, compared to the metastatic ECMs where the malignant cells proliferate and assumes the same behaviour as during *in vivo* metastases.

The aim of this projects was thus to check the possibility to exploit the already modified metastatic ECM as scaffold for the reproduction of metastatic like culture *in vitro*.

Indeed, even though cancer cells have extreme resistance to disparate environments and are relatively easy to culture in standard conditions, the petri dishes do not resemble the natural environment for the cancer, leaving the cells in an unnatural state. This new system instead would give the chance to follow the spontaneous evolution of a metastatic tissue in its natural environment *in vitro*, allowing to test the real efficiency of anticancer drugs without any risk for the patient providing in general a reproducible and realistic model for the study for peritoneal metastasis.

7.1 Material and Methods

7.1.1 Human tissues

Peritoneal tissue was collected from six patients who underwent resection for metastatic grade IV CRC, at the Unit of Peritoneal Malignancies of our Institution. The patients had been diagnosed according to the WHO classification¹⁴⁵. The study was approved by the review board of our Institution (134/13; I249/19). Each patient provided written informed consent for the resected tissue, left over after diagnostic procedures, to be used for research purposes. The study was conducted in accordance with the Declaration of Helsinki, 2009.

Metastatic lesions and apparently normal tissue (> 10 cm from the metastatic lesion) were collected. Part of the metastatic tissue (1 cm in diameter) was placed in ice-cold PBS (Thermo Fisher Scientific) with gentamicin (50 ng/ml, Thermo Fisher Scientific) and amphotericin B (50 ng/ml, Thermo Fisher Scientific) for the development of PM-derived organoids, while a second piece was frozen in liquid nitrogen for molecular and histopathological analyses. The remaining tissue was used to develop 3D decellularized matrices (3D-dECMs). Normal tissue was partly used to develop 3D decellularized.

7.1.2 Development of peritoneal metastasis-derived organoids

Fresh PM tissues were processed following the protocol by Fuji et al.¹⁴³ (Figure 30A). Briefly, the tissue was cut into small pieces, washed with ice-cold PBS at least ten times, and subsequently digested with 500 U/ml collagenase type II (Sigma Aldrich) in DMEM-F12 for 60 min at 37 °C with vigorous pipetting every 15 min. The remaining fragments were additionally digested with 1 mg/ml trypsin,

5mM EDTA (Thermo Fisher Scientific) at 37 °C for 20 min. The supernatant was collected and centrifuged at 300 g for 5 min at 4 °C. The cell pellet was suspended with Matrigel (growth factor reduced, Corning) and dispensed into 24-well culture plates (50 µl/well). After Matrigel polymerization, the cells were overlaid with 500 µl of basal culture medium consisting of Advanced DMEM-F12 (Thermo Fisher Scientific). Medium was refreshed every three days.

Organoids were split every 1-2 weeks as follows: they were mechanically removed from the Matrigel by pipetting, incubated in Cell Recovery Solution (Corning) for 1 hour at 4 °C, washed two times with ice-cold PBS and seeded as before. Aliquots of each organoid culture were frozen or prepared for IHC analyses as follows: samples were fixed in 10 % formalin at room temperature for 10 min and embedded into 200 µl Bio-Agar (Bio Optica). The samples were then cooled at -20 °C until solidification. For each sample, sections of 3 µm were obtained.

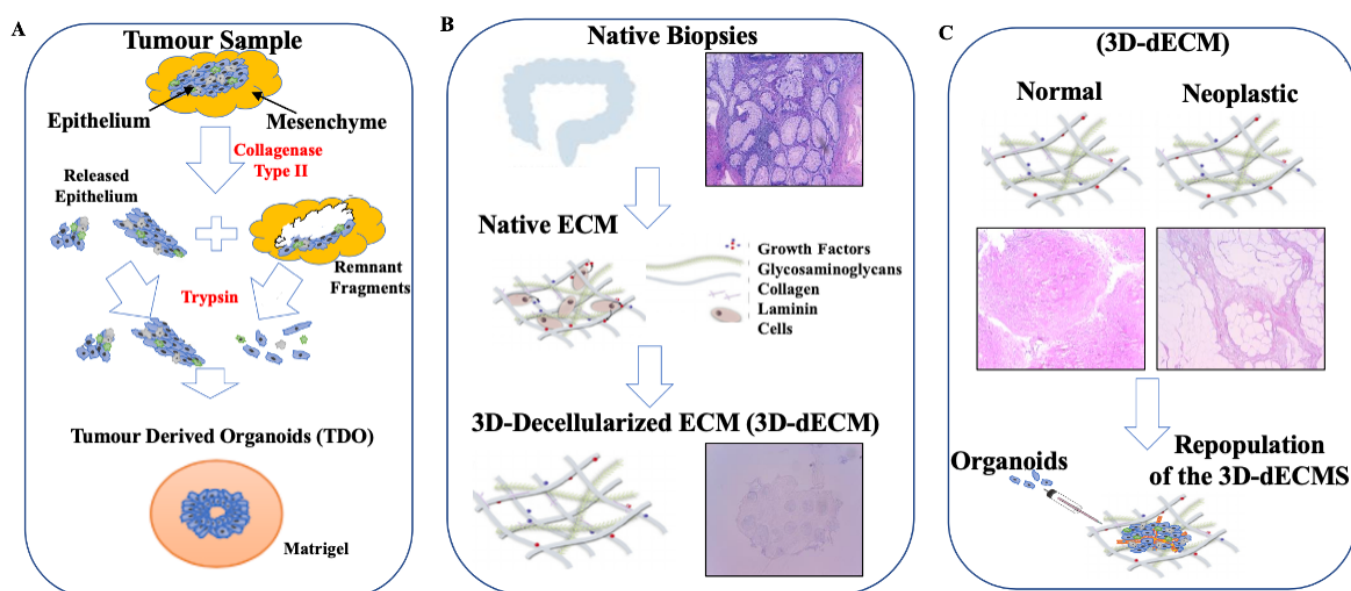


Figure 30. Diagram of optimized established method.

A) Schematic representation of the protocol used for developing CRC PM-derived organoids. **B)** Schematic representation of the protocol used for obtaining the peritoneal-derived 3D-dECMs. **C)** Experimental design used to develop the ex vivo 3D engineered PM lesions.

7.1.3 Preparation of 3D-decellularized extracellular matrices

3D-decellularized extracellular matrices (3D-dECMs) were derived from both PM and the corresponding normal peritoneum (Figure 30B). Three to 10 different surgical specimens derived from different PM patients were used for each experiment. The decellularization procedure used followed the protocol described in Genovese et al.¹⁴⁴. Briefly, both PM and normal peritoneum samples of 60-100 mg wet weight were kept in ice-cold PBS for 1 h before processing.

The specimens were divided into two fragments and one was kept untreated for a comparison of the characteristics of the 3D-dECM. For decellularization, the fragments were washed with ice-cold PBS supplemented with 50 ng/ml gentamicin and 50 ng/ml amphotericin B and then treated with solutions containing detergents and enzymatic agents. After decellularization, 3D-dECMs were washed with ice-cold PBS and either transferred into chilled freezing solution (90 % DMEM-F12, 10 % DMSO) and frozen for storage, or fixed for IHC and immunofluorescence analyses.

7.1.4 *Ex-vivo* engineered peritoneal metastasis lesion

Engineered PM lesions were obtained from three organoid cultures. TDO were removed from the Matrigel as described above and dissociated in single cells with Trypsin-EDTA by petting vigorously for 10 min. 3D-dECMs obtained from both normal peritoneal tissue and PM were incubated overnight at 37 °C in DMEM-F12 supplemented with 10 % FBS (Euroclone) and 50 ng/ml gentamicin plus amphotericin B. To reduce intra-sample variability, the 3D-dECMs were cut into fragments of comparable size. TDO were suspended in 1 ml of culture medium and seeded on the top of 50 mg of 3D-dECMs. Repopulated matrices were placed in a 24-well plate (Corning) containing DMEM-F12 supplemented with 10 % FBS and 50 ng/ml gentamicin plus amphotericin B and incubated for 2 h at 37 °C. Each well was filled with 2 ml of medium, which was changed every 2 days (Figure 30C).

7.1.5 Topographical analysis of the decellularized matrices

The topographical evaluation of the 3D-dECMs was performed by atomic force microscopy (AFM) technology on samples derived from healthy peritoneum and PM donors. The experiments were conducted on samples from three different PM patients. Prior of the AFM analysis, the slides were left 30 minutes at room temperature in order to dissolve the optimal cutting temperature (OCT) compound and then carefully washed with ultrapure water and 3D-dECM samples were then filled with PBS buffer. Measurements were carried out at room temperature. Surface topography was performed in air using NanoWizard3 AFM (JPK, Germany) coupled to an Olympus BX61 inverted microscope and equipped with tapping mode silicon ACTG AFM probes (APPNANO). The 50 µm tissue slices were mounted onto polarized glass slide (Thermo Fisher) and were left 30-minute RT in order to dissolve the OCT and then carefully washed with ultrapure water. The topography of each tissue was determined using at least 10 areas (5x5 µm) of the sample with 512 × 512 two-directional lines scanned at 3 µm/Hz.

7.1.6 Nanoindentation measurements by atomic force microscopy

AFM analysis was carried out on 3D-dECMs derived from healthy peritoneum and PM of three patients. 3D-dECMs were embedded in OCT to fully cover the tissue sample and then frozen with nitrogen-cooled 2-propanol for 10 seconds; slides of 100 μm thickness was cut with a microtome (Leica) and then attached to positively charged poly-lysine coated glass coverslips (Thermo Fisher Scientific) exploiting the electrostatic interaction.

Nano-mechanical tests were performed in liquid on samples covered by a PBS droplet confined by a circular ridge of hydrophobic two-component silicone paste (Leica). A Bioscope Catalyst AFM from Bruker was used, resting on an active anti-vibration base (DVIA-T45, Daeil Systems), and closed into an acoustic enclosure (Schaefer). The measurements were performed at constant temperature of 30 °C.

Custom monolithic borosilicate glass probes consisting in spherical glass beads with radii R in the range 7.5–9.5 μm , attached to force-modulation silicon, tipless cantilevers (Nanosensor, TL-FM) with nominal spring constant $k = 2.8 \text{ N/m}$ were used. Probes were fabricated and calibrated, in terms of tip radius, according to an established custom protocol⁶⁷, while the spring constant was measured using the thermal noise calibration^{68,69} and corrected for the contribution of the added mass of the sphere^{55,72}. The deflection sensitivity was calibrated *in situ* and non-invasively before every experiment by using the previously characterised spring constant as a reference, according to the SNAP procedure described in Ref⁵⁰.

The mechanical properties of the 3D-dECMs were obtained fitting the Hertz model to sets of force vs distance curves (FCs), as described elsewhere⁴⁵. FCs were collected in Point and Shoot (P&S) mode, selecting the regions of interest from optical images, exploiting the accurate alignment of the optical and AFM images obtained using the Miro software module integrated in the AFM software. Each set of FCs consisted in an $15 \times 15 = 225$ array of curves spatially separated by approximately 10 μm , each FC containing 8192 points, with ramp length $L = 8 \mu\text{m}$, maximum load $F_{\text{max}} = 150 \text{ nN}$, ramp frequency $f = 1 \text{ Hz}$. Typical maximum indentation was 3–4 μm .

Mechanical tests were carried out on ECM samples obtained from three different patients. For each patient, two conditions were tested: healthy 3D-dECM (apparently normal tissue > 10 cm from the metastatic lesion) and neoplastic 3D-dECM. For each condition, three samples were characterised. In each sample three P&S of 225 FCs each were recorded in macroscopically separated locations, resulting in ~2000 FCs per patient's condition.

7.1.7 Stem cells maintenance, proliferation, and apoptosis assays

Growing cells derived from the disaggregation of TDO were detected on FFPE sections stained with mouse anti-human Ki-67 monoclonal antibody (clone MIB-1) plus DAPI; their growth rate was expressed as the percentage of Ki-67⁺ cells out of the total number of cells present in regions without dead cells. Stem cells

were detected on FFPE sections stained with mouse anti-human LGR5 monoclonal antibody (clone OTI2A2) plus DAPI; their density was expressed as the percentage of LGR5⁺ cells positive out of the total number of cells present in regions without dead cells. The percentage of Ki-67⁺ LGR5⁺ cells was obtained by counting three independent fields (40 X magnification by means of ImageJ software).

Apoptotic cells were detected on FFPE sections stained with rabbit anti-human cCASPASE-3 monoclonal antibody (clone 9661) plus DAPI staining; Apoptotic cells rate was obtained as percentage of cCASPASE-3⁺ cells out of the total number of cells present in the field. The percentage of ki-67⁺, LGR5⁺ and cCASPASE-3⁺ cells was obtained by counting three independent fields (40 X magnification by means of ImageJ software). All the experiments were performed in triplicate.

7.2 Results

7.2.1 3D-dECMs: Morphological features and mechanical properties

Confocal reflection and polarized light microscopy techniques gave us information on the surface pattern of the decellularized tissues at the micro scale. The differences between normal and neoplastic-derived tissues obtained from three different PM patients were related both to the tissue texture and to the distribution and integrity of the single collagen fibre (Figures 31A). The 3D-dECM of normal tissues showed higher density and loose collagen fibres, while the neoplastic 3D-dECM assumed a particular reticular organization.

The AFM topographical analysis highlighted an even distribution of collagen: the normal-derived matrices had an organization of very thin fibres, characterized by homogeneous and cross-linked reticular structures; while in the neoplastic-derived ones we observed a corrugated surface pattern, with a loss of the original fibrillar organization (Figure 31A).

Figure 31B shows the cumulative boxplots built with all data coming from the same condition (normal or neoplastic) and patient. Each young module (YM) value in the boxplots was obtained from a single FC. Peritoneal 3D-dECM showed great variability in mechanical properties, and inhomogeneities were present in both healthy and neoplastic ECMs, although the neoplastic 3D-dECM was markedly stiffer than the normal 3D-dECM. The distributions show that data are considerably scattered, and a significant overlap is present between the two conditions. The ECM is evidently a complex and diverse system that does not entirely transform from one condition to another, but which remains locally heterogeneous on the scale of several typical cellular lengths, i.e. 10-100 μm .

In Figure 31C aggregated mechanical values are reported¹⁵: the stiffening is statistically significant for all the three patients; this is confirmed by the results normalized to the mean YMs value of the healthy samples (Figure 31D).

Of note, the patient with the softer healthy 3D-dECM (Pt1) has the strongest relative stiffening in the tumour tissue, with an increase in YM of more than 300%. In Figure 31E we tried to correlate the big difference in absolute values of the YM of different patients with some specific patients' conditions. We found that the rigidity of the healthy ECM clearly increases with the age of the patient, e.g. the YM value of the tumoral ECM of Pt1 is comparable to the YM of healthy ECM of Pt3; the relative stiffening instead seems to be related to the aggressiveness of the cancer. The interesting results of these preliminary correlative assays prompt for other experiments with more donors to confirm the observed trends.

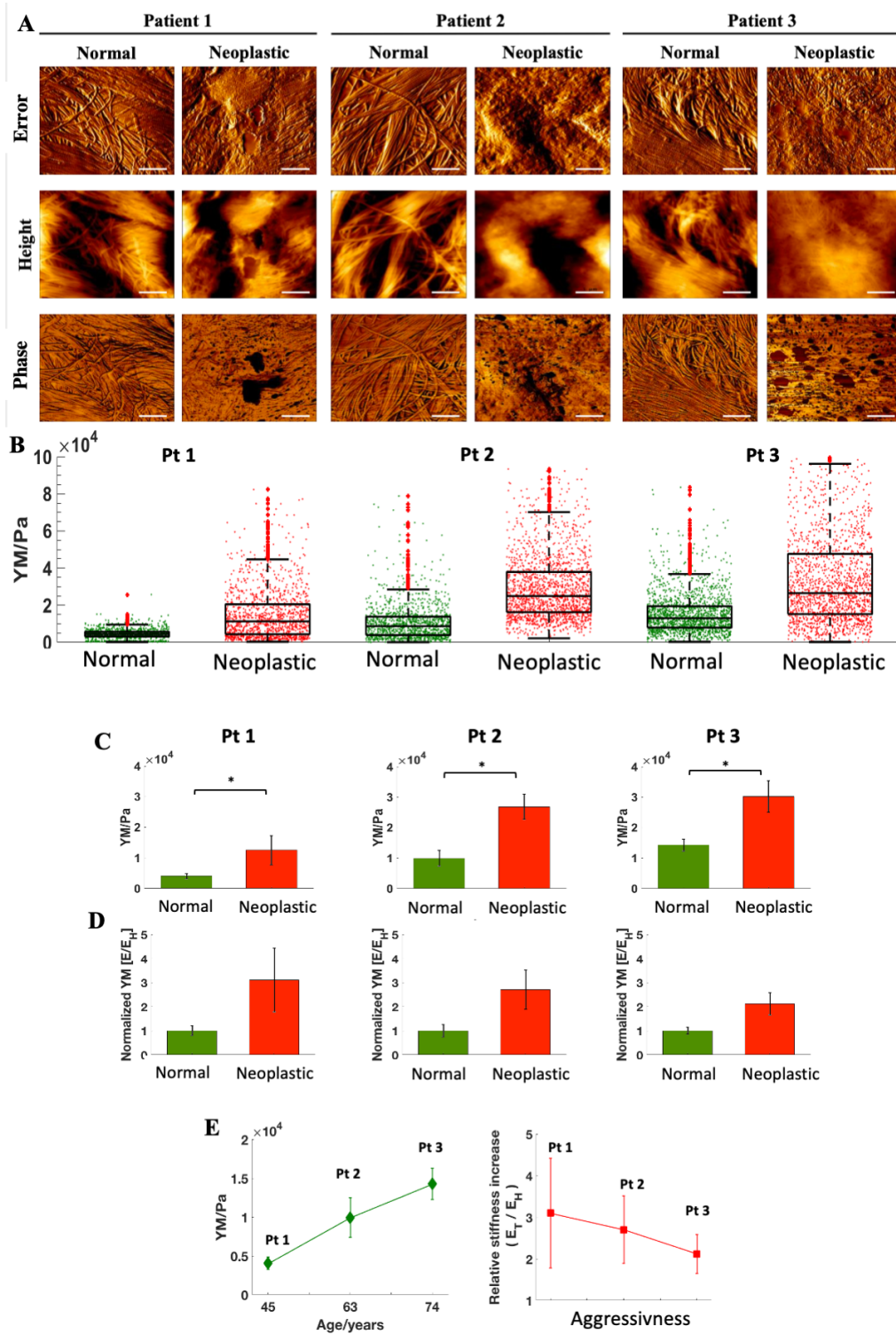


Figure 31. Results of the AFM investigation on ECMs samples. (A) Changes in peritoneal-derived 3D-dECMs topography. Height, phase and peak force error images of both normal and neoplastic-derived decellularized matrices (scale bar: 1 μ m), images obtained by A. Oldani (IFOM, The FIRC Institute of Molecular Oncology, Milan, Italy). (B) Dots represents the YM values obtained from each FCs grouped in boxplots, for each patient (Pt) for two different conditions: Normal and Neoplastic, with the quartile group, outliers and median values. (C) Result of the statistical analysis for each patient¹⁵: the bars represent the mean of the median values extracted by each location while the error-bars represent the standard deviation of the mean integrated with propagated instrumental error and normal deviation in each location. (D) Results of the statistical analysis normalized to the mean healthy YM value. (E) Observed trends of the mechanical properties of the ECM correlated with patients' information: age and aggressiveness of the CRC.

7.2.2 3D decellularized scaffolds support the proliferation of organoids

The growth rate of live cells was estimated by Ki-67 immunofluorescence (Figure 32). 3D-dECMs from neoplastic peritoneum had a significantly higher percentage of Ki-67⁺ cells on days five and 12 ($*** p < 0.001$), indicating faster growth early in culture (Figures 32A). No differences were observed after 21 days. LGR5⁺ staining highlighted that the stem cell pool was maintained in both normal and neoplastic 3D-dECMs after five and 12 days of seeding (Figures 32B), but after 21 days it was significantly lower in 3D-dECMs from normal peritoneum ($** p < 0.01$ Figure 32B).

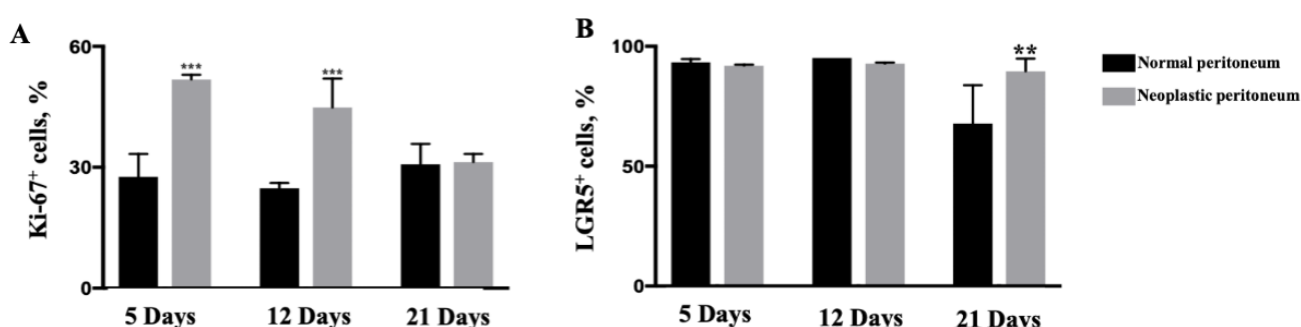


Figure 32. A) Proliferation rate of PM-derived organoids measured as the percentage of ki-67⁺ cells out of the total number of cells in the regions free of dead cells. Five fields per experiment (40x magnification) were counted. Data are median and SD for surgical specimens of three patients. One-way ANOVA ($***p < 0.001$). (B) LGR5⁺ PM-derived organoids, measured as the percentage of LGR5⁺ cells on the total number of cells present in the regions free of dead cells. Data are median and SD for surgical specimens of three patients. One-way ANOVA ($**p < 0.01$). Data obtained by S. Bricchi (Laboratory of Molecular Pathology, Department of Pathology, Fondazione IRCCS Istituto Nazionale dei Tumori, via Venezian 1, 20133 Milan, Italy).

7.2.3 Ex vivo engineered PM lesions reproduce the patient's PM

To compare the similarity of the engineered model with the original patients' lesions, we evaluated the presence of colorectal markers on TDO, on tissue derived from the PM of origin and on the repopulated 3D-dECMs. Repopulated 3D-dECMs summarize the main characteristics and the morphology of their tumour of origin (Figure 33), presenting the typical histological features observed in PM patients, such as: i) signet ring cells, ii) bizarre mitotic figures, iii) necrotic debris, iv) pleomorphic cell size and shape and v) multinucleated cells. Interestingly, signet ring cells have been reported only once in an *ex vivo* model system¹⁴⁶, highlighting that the *ex vivo* engineered model of the PM we generated is highly representative of the *in vivo* lesions.

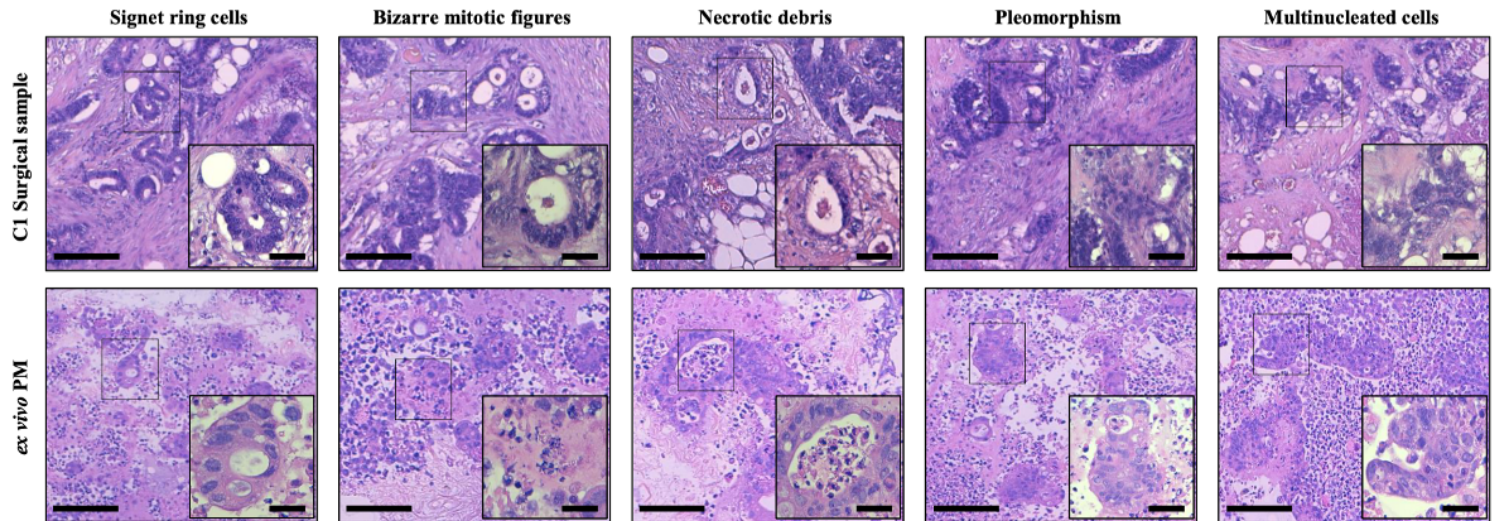


Figure 33. Histological comparison between peritoneal metastasis and neoplastic-derived 3D-dECM repopulated with PM-derived organoids. The *ex vivo* engineered PM lesion present histologic features typical of PMs of gastrointestinal origin (scale bar: 20 μ m). *Data obtained by S. Brich* (Laboratory of Molecular Pathology, Department of Pathology, Fondazione IRCCS Istituto Nazionale dei Tumori, via Venezian 1, 20133 Milan, Italy).

7.3 Discussion

The number of *in vitro* models available to study PM disease is limited by the challenges in reproducing the main cell-cell and cell-matrix interactions of the metastatic niche^{140,141}. Here we describe a PM-specific engineered model that combines the main features of TDO within the surrounding microenvironment, enabling a precise recapitulation of the PM niche under physiologically relevant conditions. The creation of organoids from peritoneal metastasis of colorectal cancer is unique and highly relevant for most of the colorectal cancer patients suffering for a widespread peritoneal disease. The 3D-dECM models reproduces the complex tissue architecture and cell-matrix interactions of the native environment of PM, as evidenced by its full colonization by TDO. Moreover, the 3D-dECM derived from the neoplastic peritoneum allows the development of a tissue microenvironment that preserves the stem cell pool of TDO, enhancing their proliferation and favouring a repopulation pattern similar to that usually observed in *in vivo* lesions.

Our findings are in keeping with previous works showing that cancer-derived ECM sustains the proliferation and invasion of CRC cell lines, thus highlighting that ECM can be used as a scaffold for the growth of tumour cells. Scaffolds derived from neoplastic peritoneum showed greater stiffness and cross-linking of collagen fibres than those derived from normal peritoneum, most likely because they contained less collagen. These findings confirm the results of a study investigating the biochemical and physical diversity of ECM in CRC that shows that the perilesional ECM presents increased crosslinking, stiffness and fibrils organisation¹⁴⁷. As reported in other studies, the rigidity of the healthy tissues

clearly increases with the age of the patient¹⁴⁸. Regarding neoplastic-derived tissues the relative stiffening seems to be related to the aggressiveness of the cancer, which was greater in the younger patient (Fig. 31). This confirms previous works showing that the tumour stiffness favours its metastatic spread¹⁴⁹.

The mechanical properties of the ECM could be involved in the direct reprogramming of the epithelial cells conferring them neoplastic features via the interactions between YAP/TAZ and specific oncogene aberrations, such as RAS signalling¹⁵⁰. Indeed, expression of specific genes fundamental for tissue architecture and stiffness¹⁵¹.

Results obtained through AFM microscopy experiments show that the neoplastic scaffold undergoes to further complex structural modifications that enhance the adhesion and the proliferation of TDO.

The engineered model we propose, which presents the 3D cellular organization of the TDO and the appropriate ECM supporting microenvironment observed *in vivo*, could therefore be a drug screening tool closer to patients than the classical monoculture 2D models, still used, or even 3D-cultures¹⁵².

In conclusion, our 3D model could be used as a pre-clinical platform to study the role of tumour ECM in the development of the PM niche. The model represents a physiological tool to identify key molecular and biomechanical players in the metastatic development and may allow to select in a biologically relevant setting new therapeutic strategies that could help in define new prognostic targets, also providing a new tool to boost the bench-to-bed-side process for improving patient care.

7.4 Additional discussion

Apart from the demonstrated feasibility of the proposed model, several aspects of the system remain open and further investigations are needed for a better understanding of the fine mechanisms through which CRC cancer cells invade the peritoneum.

- The macroscopic nature of the ECM and its topographic heterogeneity suggest that too high resolved sampling would not give significantly spatially averaged information about its mechanics. This aspect is not valid only along the lateral (XY-plane) dimensions, but also along the indentation axis Z. Thus, a large indentation is recommended, well beyond the typical 1-3 μm achieved with the AFM.

The problem of the indentation is first of theoretical nature, i.e. the correct application of the Hertz model requires that the indentation δ is much smaller than the radius R of the spherical indenter; this condition could be overcome using larger CPs with the previously discussed drawbacks (like the correct measurement of a reliable deflection sensitivity, the fine sampling of the deflection signal and the application of the specific corrections for the chosen geometrical conformation, see Chapter 3), or implementing contact mechanics models (like

the Sneddon model⁴⁹) that do not need the $\delta \ll R$ restriction. An implementation of the approximated Sneddon model⁶⁶ is discussed and tested in the Appendix B.

- It is evident from the analysis in the results and from previous works¹⁵ that CRC malignant cells are less able to proliferate on the healthy ECM. So, the metastatic progression should involve a co-evolution of the tumour and its microenvironment, with a modification of the ECM intrinsic properties that happens before the metastatic cell's attachment. New findings suggest that the host cells of the target tissue play a key role in influencing the metastatic behaviour¹⁵³ and membrane vesicles derived both from tumour and host cells have been recognised as new candidates in the promotion of ECM modifications¹⁵⁴. The characterization of the mechanical fingerprint of tissues undergoing this cancer-like modification would help reducing the peritoneal relapse.

- While in the case of colon ECM the stiffening was accompanied by a progressive degree of anisotropy and fibres linearization¹⁵, on the peritoneum instead the physical origin of the tissue stiffening is still unclear. Image 31A highlights that the fibril structure in the neoplastic ECM seems to be covered by a thin layer with the appearance of holes and niches, which is likely the product of the metabolism of malignant cells covering the “older” healthy ECM but a deeper morphological characterization is required to better understanding this phenomenon.

- Figure 31E points out that, beside the marked variation of the ECM mechanical properties, also an evident correlation between healthy ECM Young's Modulus and the age of the patient, and a more subtle correlation between the relative increase of stiffness in the neoplastic tissue and the “aggressiveness” of the tumour, are observed.

Despite the interesting correlations found, the samples tested come from just three patient and a statistically relevant population needs to be investigated.

The creation of a detailed database that correlates the mean Young's modulus of the peritoneal ECM (healthy and neoplastic) with relevant information of the patient (such as age, sex, tumour aggressiveness, tumour stage, previous clinical treatment) would provide a knowledge of the range of the possible expected YM from specific conditions. Such a database could be used as a reference for other laboratories pushing forward the standardization of the nano/micromechanical approaches and possibly provide a chance to obtain an additional diagnostic parameter to analyse besides the standard biopsy techniques.

Chapter 8

Conclusions

It is now established that the biological specimens (like cells, ECMs and eventually the whole tissue) display physical properties strictly correlated to their physio/pathological state, which manifest themselves at multiple levels, and influence each other. Despite the versatility of the AFM, in terms of working environments and accessible length-scales, a multidisciplinary approach, based on the integration of diverse expertise and the collaboration among scientists from different fields, is indispensable for the achievement of reliable and useful results. Nevertheless, AFM can offer a strong support to many other experimental techniques.

In the following the major results of my PhD course are summarized

- I. The possibility to fabricate probes with the appropriate geometrical and mechanical properties allows investigating mechanobiological processes at several different scales. Nevertheless, a correct calibration of CPs is not always straightforward and attention must be paid to the fact if the relative dimensions of the cantilever and the attached sphere become comparable. We have seen how the added mass could modify the thermal oscillation dynamics of the probe, leading to an underestimation of the intrinsic spring constant. We provide a new correction parameter β that takes into account the above-mentioned modifications, and we proposed a calibration protocol thought to minimize the sources of possible issues (Chapter 3).
- II. The colloidal probes provide also a reliable scaffold for various modifications/functionalizations. Exploiting the supersonic cluster beam deposition technique, I fabricated CPs with controlled morphological surface properties at the nanoscale. Those nanotopographical colloidal probes (nt-CPs) were used to simulate the morphological properties of the cellular microenvironment, furnishing an insight view of the early cellular adhesive response (Chapter 4).
- III. Selecting the microenvironmental topographical stimulus on the probe, it is then possible “to play” with the cellular modifications, e.g. inhibiting or manipulating specific cellular components or capabilities, or using cells in which the expression of certain proteins of interest have been *knocked-out*. In this manner, complex molecular cascade reaction can be dissected and key components in the mechanotransductive pathways be identified. In light of this prospect, we decided to investigate the role of the glycocalyx, a pericellular sugar brush, in the complex molecular processes

that permit the cell to recognize the topography of the surrounding. In particular, we found that the glycocalyx is an important component that influences the integrin adhesion spot formation and eventually the molecular clutch engagement and force loading (Chapter 5).

- IV. The fine application of nano-mechanical tests on prostate cancer cells provided the opportunity to dissect the mechanical behaviour of separated cellular regions. This procedure allowed us to characterize the cellular reaction to three different microtubule-targeting-drugs, observing and identifying a possible mechanism for the ability of cancer cell to resist to anticancer-drugs (Chapter 6).
- V. The connection between ECM and cancer metastases development has been acknowledged by the mechano-biology community, and attempts to exploit this connection have already been proposed¹⁵⁵ (Chapter 7). Here, we investigate the physical properties of human peritoneal metastatic ECM, in comparison to the healthy one. Then we cultivated *in vitro* peritoneum metastases in their natural environment, directly observing the influence of the microenvironmental mechanical and morphological properties on CRC metastatic growth.

Perspectives

9.1 Application of nt-CPs

The AFM force spectroscopy approach with nt-CPs has been applied on the PC12 cell line. The reason behind this choice was the need to first test the new approach on a well-known system and to further deepen the understanding of the behaviour of these cells in relation to the biocompatible ns-ZrO₂ materials^{30–32,56}. Now, that the protocol is implemented, it would be interesting to apply it on cellular systems with higher biological and biomedical relevance, such as primary neurons, mouse embryonic fibroblasts (MEF) or cells with different pathophysiological conditions, e.g. healthy vs cancer cells, or cancer cells at different progression stages).

9.2 Development of new protocols for the functionalization of CPs in order to create ECM-CPs

The functionalization of CPs with nanostructured thin films provided a reliable scaffold to reproduce the characteristic three-dimensional nanotopography of the ECM. However, the morphology is not the only information the ECM provides. It is well known that different integrin subtypes link to different ECM ligands and, as already discussed, ECM from different tissues, healthy or tumoral, express different components with different relative abundancies and structural organisation.

One idea is to move towards the recreation of ECM physico-chemical properties directly on the probe contact surface, via subsequent functionalization with single ECM elements, such as fibronectin, laminin, collagens, or RGD peptides (which is a motif recognised by certain integrins). Indeed, it has already been demonstrated that CPs can be functionalized with single ECM components^{156,157}. A further functionalization would be the direct attachment of micrometric ECM patches, coming from different tissues in different conditions, on the CPs. This research would help to characterize the adhesive affinity between cancer/metastatic cells and specific tissues, helping to gain an understanding for the reason why certain cancer types metastases preferentially to specific organs.

9.3 Characterization of the glycocalyx in different metastatic stages

As described in Chapter 5, the glycocalyx layer has a fundamental role in the interaction with the microenvironment and forms a protective barrier against the entry of chemicals and substances harmful to cells^{97,98}. Metastatic cells have the ability to invade different tissues and move through the vessels, exposing themselves to different environments. It has been suggested that cancer cells possess peculiar glycocalyx structure and composition different from their healthy counterpart⁹⁹. Through methods discussed in Chapter 5, AFM imaging, AFM nanoindentation and targeted enzymatic treatments, it would be possible to inspect the glycocalyx structure and composition of metastatic cells as a function of their invasive capabilities. This would help in dissecting its role in metastatic cancer cell survival strategies and may open the possibility for new drug targeting approaches.

9.4 Following the direct modification of ECM physical properties upon interactions with cancer cells

As discussed in Chapter 7, the ECM in the tumoral/metastatic tissues possess morphological and mechanical properties that are very different from the healthy ones^{15,18,158,159}. The molecular mechanisms behind the modification of ECM properties (cell-related metabolism and biochemical processes) and the time scale of these modifications are still poorly investigated and understood. Starting from the results obtained by Nebuloni et al.¹⁵ and those shown in Chapter 7, a possible experiment would be to exploit the developed decellularization protocol and reverse the tissue components; i.e. plating tumoral cells on the healthy ECM and the healthy cells on tumoral ECMs. Then the mechanical and biochemical properties of cells and ECM would be followed and characterized over time in order to understand the effects of the mutual interactions and identify the mechanism through which these modifications take place.

Appendices

Appendix A

Optimisation of the acquisition time for mechanical and topographical maps of living cells

The simultaneous acquisition of both mechanical and topographical maps allows to spatially resolve the cell mechanics and identify regions of interest and eventually compare the morphological modification with the possible cytoskeletal rearrangement detected. Furthermore, a 3-dimensional map of the cell provide precise information about the cell height, area and volume, and can be exploited to apply the finite-thickness correction^{45,65} needed for the evaluation of the real cell Young's modulus.

As outlined in the additional discussion of Chapter 6, the acquisition of detailed map of cells could cost a consistent amount of time, with the considerable risk that the cell undergoes cytoskeletal reorganization during the acquisition time. Another drawback is the upper limit in the number of cells that could be examined in a single session, forcing to extend the measurements on several different days and so different cell cultures, providing results that are potentially less compatible between among them.

In the following I will present a new protocol that was thought to maintain or even increase the desired sampling resolution over the region of interest of the cell body and, at the same time, to reduce significantly the time spent on the characterization of the substrate region, allowing to measure with accurateness the cell's height in every point sampled.

The standard acquisition protocol, described in detail by Puricelli et al.⁴⁵, consists in the identification of the interested cell trough the optical microscope (see Figure 34a), the definition of a grid of points over the region of interest, which includes both the cell and the substrate, in the optical image and then, exploiting the accurate alignment of the optical and AFM images, the acquisition of FCs per each point defined on the grid.

Once the acquisition is finished and the data analysis is performed as follow:

- FCs are rescaled on the tip sample distance.
- The baseline is removed using a suitable noise threshold.
- The first 20% of the contact part of each FC is fitted by the Hertzian model and the fit is back-extrapolated in order to reach the $F = 0$ N applied force. The contact point is identified by the intersection of the back-extrapolated fit with the $F = 0$ N axis.
- The deformation map is calculated with the maximum indentation measured (in reference to the contact point) per each FCs.
- The uncompressed topographic map is calculated by adding the deformation map to the compressed topographic map, then subtracting the average height of the substrate.
- Eventually with the evaluated YM per each FC the mechanical map is reconstructed.

The results of the described protocol are shown in Figure 34b-c.

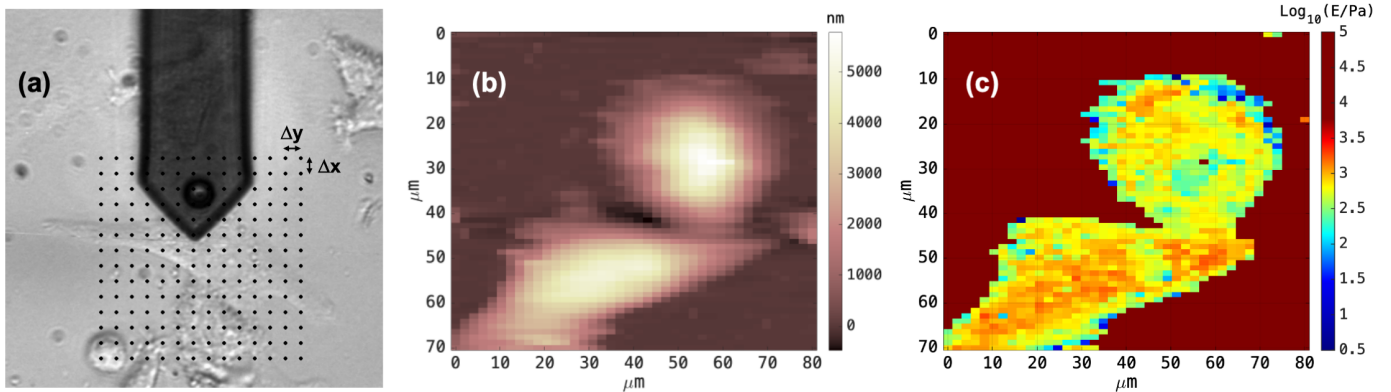


Figure 34. (a) Optical image of two cells with the grid of FCs defined with the Miro software. (b) Topographical map of the two cells reconstructed. (c) Mechanical map.

It is possible to observe that a large portion of the whole set of FCs is used to characterize the substrate; unfortunately, this time is necessarily needed to achieve a good characterization of the substrate for the correct evaluation of the cell height. As discussed, the fine characterization of the cell's height is strictly necessary for the application of the finite-thickness correction. Indeed, the cells lay over a substrate that is (usually) much stiffer than cells and resulting effects is an overestimation of the effective YM and a loss of sensitivity in the fine mechanical modification (for details see pg 143-145).

The new developed protocol is now described in details:

- a) Instead of acquiring FCs along a rectangular grid spamming the cell and the substrate, just few points on the substrates are taken in regions around the cell (three are the inferior limit to define a baseline plane in the 3-D space), while a dense grid of points is created on the cell body.
- b) Starting from the acquired set of points on the substrate in the 3-D space $S = \{x_j^S, y_j^S, z_j^S\}_{j=1, \dots, N_S}$, the coefficient (a, b, c, d) of a plane in 3-Dimensional space, $ax + by + cz + d = 0$, are evaluated by fitting the points set S .
- c) For each point acquired on the cell ($C = \{x_i^C, y_i^C, z_i^C\}_{i=1, \dots, N_C}$, where $N_C \gg N_S$), of the i -th force curve taken, the corresponding substrate height is evaluated trough the plane equation $z_i^{subs} = -\frac{a}{c}x_i^C - \frac{b}{c}y_i^C - \frac{d}{c}$.
- d) The pointwise uncompressed cell height is evaluated as $h_i = z_i^C - z_i^{subs} + ind_i$.

The results of the described protocol are shown in Figure 35.

A test of the reliability of the new algorithm is shown in Figure 36, where the plane defined from the previous grid approach (Figure 36a, black dots) is compared to the one (Figure 36b) evaluated from nine random FCs of the substrate (red crosses). It is possible to observe that even if the reference sample is limited a very good agreement between the real substrate and the evaluated one is evident. Clearly the FCs on the substrate should be distributed appropriately around the cells, and a reasonably large number of FCs should be collected in order to effectively average out the noise.

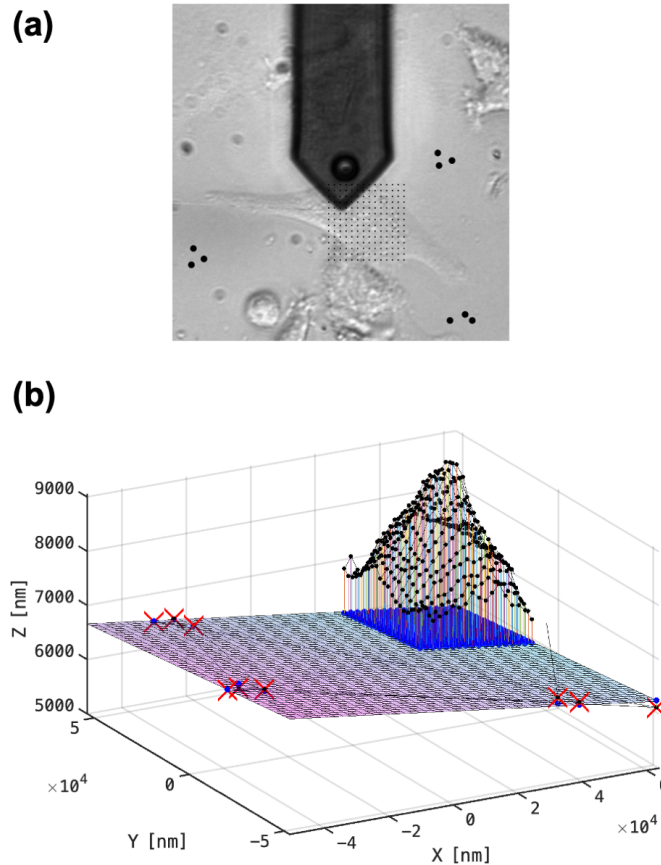


Figure 35. (a) Optical image of a cell with the new set of FCs drawn on the cell nuclear (small dots) region and the reference FCs on the substrates (big dots). (b) Reconstructed cell nuclear region topography (h_i , black dots) in reference to the calculated substrate plane, the height of the substrate beneath the cell are shown (z_i^{subs} , blue dots).

A part from the fastening, the new acquisition process permits to focus only on specific portions of the cell. As an example, we tested the protocol on Mouse Embryonic Fibroblast (MEF) performing mechanical test with the two approaches (old and new) only on the nuclear region of the fibroblasts. Fibroblast are typical cells of the connective tissue, and possess a dimension of the cytoplasm that could cover even a hundred of microns. It is therefore useful to focus the analysis only on the target location within the cell body.

With the standard protocol an acquisition of a single cell map could easily take 40-50 minutes while with the new one the acquisition time was reduced to around 7-10 minutes. The results of this preliminary test are shown in Figure 36c. The mean YM values obtained from the two approaches are compatible.

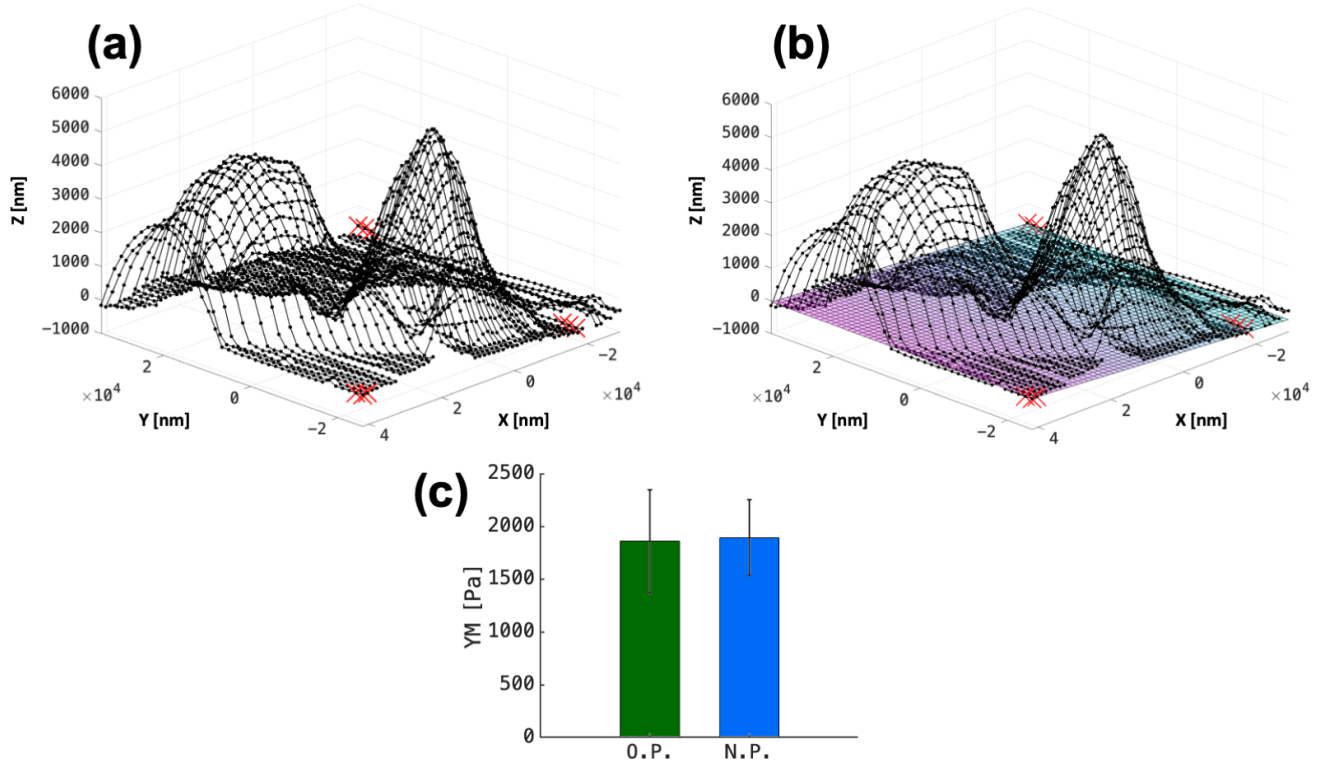


Figure 36. (a) Old acquisition protocol: 3-Dimensional map from the absolute position of the FCs (black dots) with nine random-selected FCs of the substrates (red crosses). (b) Reconstructed substrate plane (coloured plane) from the nine FCs (red crosses). (c) Comparison of the Young's modulus measured with the Old Protocol (O.P.) and the New Protocol (N.P.).

Appendix B

Implementation of the approximated Sneddon Model

The contact mechanics models, under the hypothesis of negligible or null adhesion, can be cast in a simple generalized equation linking the applied force F and the resulting indentation δ of the sample¹⁶⁰

$$F = \lambda \delta^\beta \quad (1)$$

In Eq. 1 the parameter λ contains the relevant mechanical information, like the sample's Young's modulus and the Poisson ratio, as well as the indenter properties, as the radius of curvature R or the tip angle α . The exponent β instead depends on the indenter geometry (see table 2)⁴⁵.

γ	β	Probe shape
$\frac{4}{3} \frac{E}{(1-\nu^2)} \sqrt{R}$	$3/2$	Sphere or paraboloid of radius R
$\frac{2}{\pi} \frac{E \tan \alpha}{(1-\nu^2)}$	2	Conical tip with angle 2α
$0.7453 \frac{E \tan \alpha}{(1-\nu^2)}$	2	Four-sided pyramidal tip with angle 2α
$0.7071 \frac{E \tan \alpha}{(1-\nu^2)}$	2	Blunted four-sided pyramidal tip with angle 2α
$2 \frac{E R}{(1-\nu^2)}$	1	Flat-ended cylindrical tip with radius R

Table 2. Summary of the most common AFM tips' geometry and the corresponding parameters.

Among the variety of different probe-shaped implemented models, the Sneddon model⁴⁹ for the spherical indenter, developed in the framework of classical theory of elasticity, does not have the above mentioned constrain of $\delta \ll R$. The Sneddon model for the indentation δ of an elastic flat surface deformed by a spherical indenter is the following:

$$F = \frac{E}{(1-\nu^2)} \left[(a^2 + R^2) \ln \left(\frac{R+a}{R-a} \right) - 2aR \right] \quad (2)$$

$$\delta = \frac{1}{2} a \ln \left(\frac{R+a}{R-a} \right) \quad (3)$$

The drawback of such equation is the lack of an analytical form, which makes the solution hard to implement. To overcome this issue, Muller et al⁶⁶ approximated it; the proposed equation combines the Hertz model with a polynomial correction factor based on a truncated power series approximation:

$$F = \frac{4}{3} \frac{E}{(1-\nu^2)} \sqrt{R} \delta^{3/2} \left[1 - \frac{1}{10} \frac{\delta}{R} - \frac{1}{840} \left(\frac{\delta}{R} \right)^2 + \frac{11}{15120} \left(\frac{\delta}{R} \right)^3 + \frac{1357}{6652800} \left(\frac{\delta}{R} \right)^4 \right] \quad (4)$$

Calling

$$\xi = \frac{\delta}{R} \quad (5)$$

It is possible to rewrite the polynomial correction as

$$\Gamma(\xi) = \left[1 - \frac{1}{10} \xi - \frac{1}{840} \xi^2 + \frac{11}{15120} \xi^3 + \frac{1357}{6652800} \xi^4 \right] \quad (6)$$

It is interesting to notice that the polynomial correction of Eq. 6 is quite similar to those of the finite-thickness correction provided by Dimitriadis et al⁶⁵

$$\Delta(\chi) = [1 + 1.009\chi + 1.032\chi^2 + 0.578\chi^3 + 0.051\chi^4] \quad (7)$$

With the non-banal differences that $\chi = \sqrt{R\delta}/h$ where h is cell height and $\sqrt{R\delta}$ is the contact radius.

In Figure 37 the behaviour of the function $\Gamma(\xi)$ is shown. The inset represents the real *physical* upper indentation limit for an AFM $\delta = 2R$ (indeed is unlikely to indent more than the microsphere diameter of a CP, in fact $\delta = R$ represent already a upper reasonable upper limit), In this region the trend is almost linear with a correction that could reach the 20%

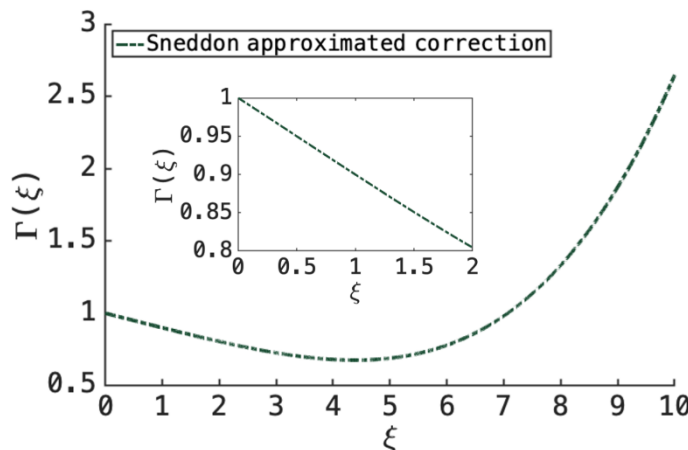


Figure 37. Trend of the approximated Sneddon correction as a function of the ratio $\xi = \frac{\delta}{R}$.

Now the Hertz equation model can be written in term of a reduced force $F' = \frac{F}{\Gamma(\xi)}$ as:

$$\frac{F}{\Gamma(\xi)} = \frac{4}{3} \frac{E}{(1 - \nu^2)} \sqrt{R} \delta^{3/2} \quad (8)$$

Following the approach of Puricelli et al⁴⁵, the above equation can be linearized as:

$$F^* = \left(\frac{4}{3} \frac{E}{(1 - \nu^2)} \sqrt{R} \right)^{2/3} \delta. \quad (9)$$

F^* represents an effective pseudo-force, a variable with the dimensions of a force elevated to the power of 2/3, given by

$$F^* = \left(\frac{F}{\Gamma} \right)^{2/3} \quad (10)$$

Linearization of the above equation allows extracting the value of the Young's modulus from experimental data by means of a simple linear fit, which can be implemented using matrix algebra to be applied to many FCs at once.

To test the effectiveness of such correction, I measured the YM of a soft hydrogel (Petrisoft, nominal YM= 25 KPa, Matrigel, USA), using three different CPs with curvature radius ranging from few hundred nanometres to tens of microns (see Table 3). Samples were pre-hydrated before the measurement using MilliQ water, for 30 minutes.

The AFM-based nanomechanical characterization of the samples was implemented as follow. Measurements were made in fluid at room temperature, using a Bioscope Catalyst (Bruker). Monolithic colloidal probes were fabricated and calibrated, in terms of tip radius, according to an established custom protocol⁵⁵. Sets of force vs distance curves (FCs), were collected selecting macroscopically separated regions from optical images, exploiting the accurate alignment of the optical and AFM images obtained using the Miro software (Bruker). Each set of FCs consisted in an NxM array of curves spatially separated by approximately 1.5 μm , each FC containing 4096 points, with ramp length $L = 8 \mu\text{m}$, ramp frequency $f = 0.7 \text{ Hz}$. The mean indentations achieved with the three probes are quite similar [2-4] μm , thanks to the increasing force constant of the cantilever of larger probes.

Probe type	Probe name	Tip Radius [nm]	Force Constant [N/m]
CM	P1	720	0.76
FM	P2	8116	2.86
TM	P3	32730	50.49

Table 3. Properties of the CPs fabricated for this study. CM, FM, TM, describe the tipless cantilever used (Contact Mode, Force Modulation, Tapping Mode)

To show the effect of the correction factor $\Gamma(\xi)$, a linearized force-indentation curve, taken with a colloidal probe P1 is plotted in Figure 38 where before and after the correction.

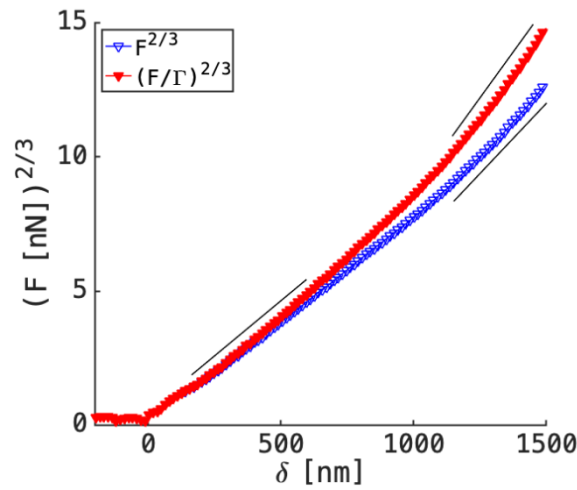


Figure 38. Linearized force Vs indentation curves with the two linear regimes highlighted (black lines): (blue) without the Sneddon correction, (red) with the Sneddon correction.

Looking at the two curves it could be notice the presence of two linear regimes (i.e. where the sample is responding Hertzian-like): the first that covers the first 700-800 nm of indentation and the second between 900-1000nm and 1400 nm. It is interesting to notice how the correction start to be relevant when $\delta > 0.5 R$ approximately.

The results of the YMs extracted from the two linear regimes (showed in Figure 38) evaluated from the measurements taken from all the CPs used in the experiment, before and after the correction are shown in Figure 39.

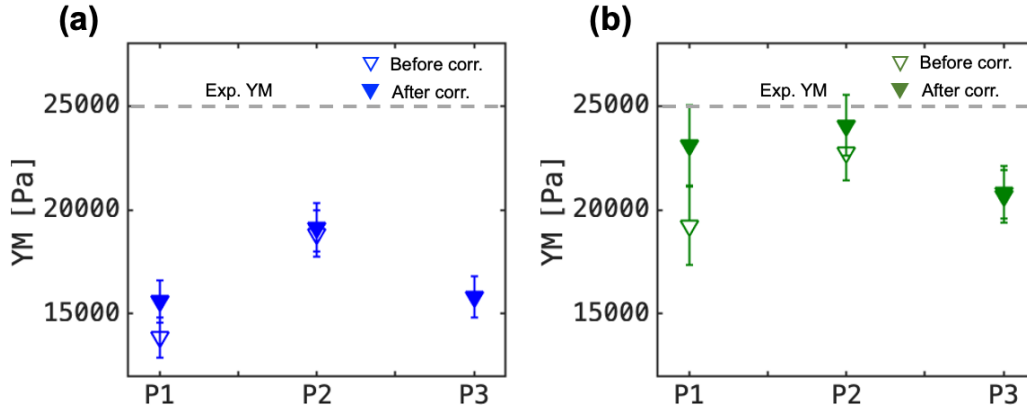


Figure 39. Histograms of the evaluated Young's Moduli, evaluated at the two linear regimes, with a gaussian fit: (a) without the Sneddon correction, (b) with the Sneddon correction.

As a consequence of the behaviour of the correction factor $\Gamma(\xi)$ represented in Figure 37-38, the first linear regime is poorly affected by the correction (a part from P1, where already in the first regime we have the condition $\delta \cong R$). The correction become more evident for the second-deep linear regime (P3 excluded, where $\delta \ll R$ in both the regimes) providing a YM mean value compatible with the expected one. The underestimation of the YM evaluated with P3 could be the effect of inaccuracies in the force constant calibration procedure, as highlighted in Ref⁵⁵, due to the peculiar configuration of such a large colloidal probe (a TM cantilever with length $L=125 \mu\text{m}$ with a sphere of diameter $D=65 \mu\text{m}$ is likely affected by a rigidification effect, as described in the additional discussion of Chapter 3). Eventually, the two linear regimes result better separated and even the differences inside of the same regimes are smaller after the correction.

As a final remark, it would be interesting to combine the large-deformation (Eq. 6) and the finite-thickness (Eq. 7). Indeed, also working with thin samples (like cells) the condition $\delta \ll R$ is not always satisfied.

From a theoretical point of view the pseudo-force written in Eq. 10 could be re-defined as:

$$F^{**} = F^{2/3} \left(\frac{1}{\Delta(\chi)} \right)^{2/3} \left(\frac{1}{\Gamma(\xi)} \right)^{2/3} = \left(\frac{F}{\Delta(\chi)\Gamma(\xi)} \right)^{2/3}$$

Where the finite-thickness correction provides the linearized force-deformation relation on a semi-infinite sample, and the large-deformation then correct for any possible $\delta \cong R$ related artefacts.

Appendix C

Table of the correction factor β for the calibration of spring constant of large colloidal probes

As widely discussed in Chapter 3, if the mass of the attached microsphere is comparable to that of the cantilever, the resulting oscillation dynamics would be modified. As consequence the β correction factor in the thermal noise formula needs to be modified accordingly. Here, a table with the values of the correction factor $\beta \equiv \beta(\tilde{m}, \tilde{r})$ is reported in order to facilitate the applicability of such modified model.

Furthermore, we report the presence of this online calibration tool (developed by Prof. Ludovic Bellon) for the calculation of $\beta \equiv \beta(\tilde{m}, \tilde{r})$:

“http://perso.ens-lyon.fr/ludovic.bellon/AFM_colloidal_probe_online_calibration/”

$\tilde{r}$																			
	0,01	0,02	0,03	0,04	0,05	0,1	0,15	0,2	0,25	0,3	0,4	0,5	0,6	0,7	0,8	0,9	1		
$\tilde{m}$	0	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170	0,8170		
	0,001	0,8181	0,8181	0,8181	0,8181	0,8181	0,8182	0,8183	0,8184	0,8186	0,8188	0,8193	0,8201	0,8209	0,8219	0,8231	0,8245		
	0,002	0,8189	0,8189	0,8189	0,8189	0,8189	0,8191	0,8192	0,8195	0,8199	0,8203	0,8214	0,8228	0,8245	0,8266	0,8289	0,8315		
	0,005	0,8213	0,8213	0,8213	0,8213	0,8214	0,8217	0,8221	0,8228	0,8237	0,8248	0,8275	0,8309	0,8351	0,8401	0,8457	0,8521		
	0,01	0,8251	0,8251	0,8252	0,8252	0,8253	0,8259	0,8268	0,8281	0,8298	0,8319	0,8372	0,8439	0,8520	0,8614	0,8722	0,8842		
	0,02	0,8324	0,8324	0,8325	0,8326	0,8327	0,8338	0,8356	0,8381	0,8414	0,8453	0,8552	0,8678	0,8829	0,9002	0,9195	0,9405		
	0,05	0,8510	0,8511	0,8513	0,8515	0,8518	0,8542	0,8582	0,8637	0,8708	0,8793	0,9005	0,9266	0,9567	0,9896	1,0241	1,0588		
	0,1	0,8746	0,8747	0,8750	0,8754	0,8759	0,8799	0,8865	0,8957	0,9072	0,9210	0,9544	0,9937	1,0364	1,0794	1,1205	1,1577		
	0,2	0,9050	0,9053	0,9057	0,9062	0,9070	0,9129	0,9227	0,9361	0,9528	0,9723	1,0177	1,0675	1,1168	1,1615	1,1994	1,2301		
	0,3	0,9238	0,9240	0,9245	0,9252	0,9260	0,9331	0,9447	0,9605	0,9798	1,0022	1,0528	1,1057	1,1551	1,1972	1,2308	1,2566		
	0,4	0,9364	0,9367	0,9372	0,9380	0,9389	0,9467	0,9595	0,9766	0,9976	1,0216	1,0748	1,1286	1,1769	1,2165	1,2471	1,2697		
	0,5	0,9454	0,9458	0,9463	0,9471	0,9481	0,9565	0,9700	0,9881	1,0101	1,0351	1,0897	1,1437	1,1908	1,2285	1,2569	1,2775		
	0,6	0,9523	0,9526	0,9532	0,9540	0,9551	0,9638	0,9778	0,9967	1,0194	1,0451	1,1005	1,1543	1,2004	1,2366	1,2634	1,2826		
	0,7	0,9576	0,9579	0,9585	0,9594	0,9605	0,9694	0,9839	1,0033	1,0266	1,0527	1,1087	1,1622	1,2074	1,2424	1,2680	1,2861		
	0,8	0,9618	0,9622	0,9628	0,9637	0,9648	0,9740	0,9888	1,0086	1,0323	1,0587	1,1151	1,1683	1,2128	1,2467	1,2714	1,2888		
	0,9	0,9653	0,9657	0,9663	0,9672	0,9684	0,9777	0,9928	1,0128	1,0369	1,0636	1,1202	1,1732	1,2169	1,2501	1,2740	1,2908		
	1	0,9682	0,9686	0,9693	0,9702	0,9713	0,9808	0,9961	1,0164	1,0407	1,0677	1,1244	1,1771	1,2203	1,2529	1,2761	1,2924		
	1,1	0,9707	0,9711	0,9717	0,9726	0,9738	0,9834	0,9989	1,0194	1,0439	1,0710	1,1279	1,1803	1,2231	1,2551	1,2779	1,2937		
	1,2	0,9728	0,9732	0,9738	0,9748	0,9760	0,9857	1,0013	1,0220	1,0466	1,0739	1,1308	1,1831	1,2254	1,2569	1,2793	1,2948		
	1,3	0,9746	0,9750	0,9757	0,9766	0,9778	0,9876	1,0034	1,0242	1,0490	1,0764	1,1333	1,1854	1,2274	1,2585	1,2805	1,2957		
	1,4	0,9762	0,9766	0,9773	0,9782	0,9794	0,9893	1,0052	1,0262	1,0511	1,0786	1,1355	1,1874	1,2291	1,2598	1,2815	1,2964		
	1,5	0,9776	0,9780	0,9787	0,9797	0,9809	0,9908	1,0068	1,0279	1,0529	1,0805	1,1375	1,1892	1,2305	1,2610	1,2824	1,2971		
	1,6	0,9789	0,9793	0,9800	0,9809	0,9821	0,9921	1,0082	1,0294	1,0545	1,0821	1,1392	1,1907	1,2318	1,2620	1,2831	1,2977		
	1,7	0,9800	0,9804	0,9811	0,9821	0,9833	0,9933	1,0095	1,0307	1,0559	1,0836	1,1407	1,1921	1,2330	1,2629	1,2838	1,2982		
	1,8	0,9810	0,9814	0,9821	0,9831	0,9843	0,9944	1,0106	1,0320	1,0572	1,0850	1,1420	1,1933	1,2340	1,2637	1,2844	1,2986		
	1,9	0,9819	0,9823	0,9830	0,9840	0,9852	0,9954	1,0116	1,0331	1,0584	1,0862	1,1432	1,1944	1,2349	1,2644	1,2850	1,2990		
	2	0,9828	0,9832	0,9839	0,9848	0,9861	0,9963	1,0126	1,0341	1,0595	1,0873	1,1443	1,1954	1,2357	1,2650	1,2854	1,2994		
	2,1	0,9835	0,9839	0,9846	0,9856	0,9868	0,9971	1,0134	1,0350	1,0604	1,0883	1,1453	1,1963	1,2364	1,2656	1,2859	1,2997		
	2,2	0,9842	0,9846	0,9853	0,9863	0,9875	0,9978	1,0142	1,0358	1,0613	1,0892	1,1463	1,1971	1,2371	1,2661	1,2863	1,3000		
	2,3	0,9848	0,9853	0,9860	0,9869	0,9882	0,9985	1,0149	1,0366	1,0621	1,0900	1,1471	1,1979	1,2377	1,2666	1,2866	1,3003		
	2,4	0,9854	0,9859	0,9866	0,9875	0,9888	0,9991	1,0156	1,0373	1,0629	1,0908	1,1479	1,1986	1,2383	1,2670	1,2870	1,3005		
	2,5	0,9860	0,9864	0,9871	0,9881	0,9894	0,9997	1,0162	1,0379	1,0635	1,0915	1,1486	1,1992	1,2388	1,2674	1,2873	1,3008		
	2,6	0,9865	0,9869	0,9876	0,9886	0,9899	1,0002	1,0168	1,0385	1,0642	1,0922	1,1492	1,1998	1,2393	1,2678	1,2875	1,3010		
	2,7	0,9870	0,9874	0,9881	0,9891	0,9903	1,0007	1,0173	1,0391	1,0648	1,0928	1,1498	1,2003	1,2397	1,2682	1,2878	1,3012		
	2,8	0,9874	0,9878	0,9885	0,9895	0,9908	1,0012	1,0178	1,0396	1,0653	1,0934	1,1504	1,2008	1,2401	1,2685	1,2880	1,3013		
	2,9	0,9878	0,9882	0,9889	0,9899	0,9912	1,0016	1,0183	1,0401	1,0659	1,0939	1,1509	1,2013	1,2405	1,2688	1,2883	1,3015		
	3	0,9882	0,9886	0,9893	0,9903	0,9916	1,0020	1,0187	1,0406	1,0663	1,0944	1,1514	1,2017	1,2409	1,2690	1,2885	1,3016		
	3,1	0,9886	0,9890	0,9897	0,9907	0,9920	1,0024	1,0191	1,0410	1,0668	1,0949	1,1519	1,2021	1,2412	1,2693	1,2887	1,3018		
	3,2	0,9889	0,9893	0,9900	0,9910	0,9923	1,0028	1,0195	1,0414	1,0672	1,0953	1,1523	1,2025	1,2415	1,2695	1,2888	1,3019		

$\tilde{\gamma}$																	
	0,01	0,02	0,03	0,04	0,05	0,1	0,15	0,2	0,25	0,3	0,4	0,5	0,6	0,7	0,8	0,9	1
3,3	0,9892	0,9897	0,9904	0,9914	0,9926	1,0031	1,0199	1,0418	1,0676	1,0958	1,1527	1,2029	1,2418	1,2698	1,2890	1,3021	1,3109
3,4	0,9895	0,9900	0,9907	0,9917	0,9930	1,0034	1,0202	1,0422	1,0680	1,0961	1,1531	1,2032	1,2421	1,2700	1,2892	1,3022	1,3110
3,5	0,9898	0,9902	0,9910	0,9920	0,9932	1,0037	1,0205	1,0425	1,0684	1,0965	1,1535	1,2036	1,2424	1,2702	1,2893	1,3023	1,3111
3,6	0,9901	0,9905	0,9912	0,9922	0,9935	1,0040	1,0208	1,0428	1,0687	1,0969	1,1538	1,2039	1,2426	1,2704	1,2895	1,3024	1,3112
3,7	0,9903	0,9908	0,9915	0,9925	0,9938	1,0043	1,0211	1,0432	1,0690	1,0972	1,1542	1,2042	1,2428	1,2706	1,2896	1,3025	1,3112
3,8	0,9906	0,9910	0,9917	0,9927	0,9940	1,0046	1,0214	1,0434	1,0693	1,0975	1,1545	1,2044	1,2431	1,2707	1,2897	1,3026	1,3113
3,9	0,9908	0,9913	0,9920	0,9930	0,9943	1,0048	1,0216	1,0437	1,0696	1,0978	1,1548	1,2047	1,2433	1,2709	1,2898	1,3027	1,3114
4	0,9910	0,9915	0,9922	0,9932	0,9945	1,0050	1,0219	1,0440	1,0699	1,0981	1,1550	1,2049	1,2435	1,2710	1,2900	1,3028	1,3114
4,1	0,9913	0,9917	0,9924	0,9934	0,9947	1,0053	1,0221	1,0442	1,0702	1,0984	1,1553	1,2052	1,2437	1,2712	1,2901	1,3028	1,3115
4,2	0,9915	0,9919	0,9926	0,9936	0,9949	1,0055	1,0224	1,0445	1,0704	1,0986	1,1556	1,2054	1,2439	1,2713	1,2902	1,3029	1,3115
4,3	0,9917	0,9921	0,9928	0,9938	0,9951	1,0057	1,0226	1,0447	1,0707	1,0989	1,1558	1,2056	1,2440	1,2715	1,2903	1,3030	1,3116
4,4	0,9918	0,9923	0,9930	0,9940	0,9953	1,0059	1,0228	1,0449	1,0709	1,0991	1,1560	1,2058	1,2442	1,2716	1,2904	1,3031	1,3116
4,5	0,9920	0,9925	0,9932	0,9942	0,9955	1,0061	1,0230	1,0451	1,0711	1,0993	1,1563	1,2060	1,2443	1,2717	1,2904	1,3031	1,3117
4,6	0,9922	0,9926	0,9933	0,9944	0,9957	1,0063	1,0232	1,0453	1,0713	1,0996	1,1565	1,2062	1,2445	1,2718	1,2905	1,3032	1,3117
4,7	0,9923	0,9928	0,9935	0,9945	0,9958	1,0064	1,0234	1,0455	1,0715	1,0998	1,1567	1,2064	1,2446	1,2719	1,2906	1,3032	1,3118
4,8	0,9925	0,9929	0,9937	0,9947	0,9960	1,0066	1,0235	1,0457	1,0717	1,1000	1,1569	1,2065	1,2448	1,2720	1,2907	1,3033	1,3118
4,9	0,9927	0,9931	0,9938	0,9948	0,9961	1,0067	1,0237	1,0459	1,0719	1,1002	1,1571	1,2067	1,2449	1,2721	1,2908	1,3034	1,3119
5	0,9928	0,9932	0,9940	0,9950	0,9963	1,0069	1,0239	1,0461	1,0721	1,1004	1,1572	1,2069	1,2450	1,2722	1,2908	1,3034	1,3119
5,5	0,9934	0,9939	0,9946	0,9956	0,9969	1,0076	1,0246	1,0468	1,0729	1,1012	1,1580	1,2076	1,2456	1,2727	1,2912	1,3036	1,3121
6	0,9940	0,9944	0,9952	0,9962	0,9975	1,0082	1,0252	1,0475	1,0736	1,1019	1,1587	1,2082	1,2461	1,2730	1,2914	1,3038	1,3122
6,5	0,9944	0,9949	0,9956	0,9966	0,9979	1,0086	1,0257	1,0480	1,0741	1,1025	1,1593	1,2087	1,2465	1,2733	1,2917	1,3040	1,3124
7	0,9948	0,9953	0,9960	0,9970	0,9983	1,0091	1,0261	1,0485	1,0746	1,1030	1,1598	1,2091	1,2468	1,2736	1,2919	1,3042	1,3125
7,5	0,9952	0,9956	0,9964	0,9974	0,9987	1,0094	1,0265	1,0489	1,0751	1,1034	1,1602	1,2094	1,2471	1,2738	1,2920	1,3043	1,3126
8	0,9955	0,9959	0,9967	0,9977	0,9990	1,0097	1,0269	1,0493	1,0754	1,1038	1,1606	1,2098	1,2474	1,2740	1,2922	1,3044	1,3126
8,5	0,9958	0,9962	0,9969	0,9980	0,9993	1,0100	1,0272	1,0496	1,0758	1,1041	1,1609	1,2101	1,2476	1,2742	1,2923	1,3045	1,3127
9	0,9960	0,9964	0,9972	0,9982	0,9995	1,0103	1,0274	1,0499	1,0761	1,1044	1,1612	1,2103	1,2478	1,2743	1,2924	1,3046	1,3128
9,5	0,9962	0,9967	0,9974	0,9984	0,9997	1,0105	1,0277	1,0501	1,0763	1,1047	1,1615	1,2105	1,2480	1,2745	1,2925	1,3046	1,3128
10	0,9964	0,9968	0,9976	0,9986	0,9999	1,0107	1,0279	1,0503	1,0766	1,1049	1,1617	1,2107	1,2482	1,2746	1,2926	1,3047	1,3129
10,5	0,9966	0,9970	0,9978	0,9988	1,0001	1,0109	1,0281	1,0505	1,0768	1,1052	1,1619	1,2109	1,2483	1,2747	1,2927	1,3048	1,3129
11	0,9967	0,9972	0,9979	0,9990	1,0003	1,0111	1,0283	1,0507	1,0770	1,1054	1,1621	1,2111	1,2484	1,2748	1,2928	1,3048	1,3130
11,5	0,9969	0,9973	0,9981	0,9991	1,0004	1,0112	1,0284	1,0509	1,0772	1,1055	1,1623	1,2113	1,2486	1,2749	1,2928	1,3049	1,3130
12	0,9970	0,9975	0,9982	0,9992	1,0006	1,0114	1,0286	1,0511	1,0773	1,1057	1,1624	1,2114	1,2487	1,2750	1,2929	1,3049	1,3130
12,5	0,9971	0,9976	0,9983	0,9994	1,0007	1,0115	1,0287	1,0512	1,0775	1,1059	1,1626	1,2115	1,2488	1,2751	1,2930	1,3050	1,3131
13	0,9973	0,9977	0,9984	0,9995	1,0008	1,0116	1,0288	1,0513	1,0776	1,1060	1,1627	1,2116	1,2489	1,2752	1,2930	1,3050	1,3131
13,5	0,9974	0,9978	0,9985	0,9996	1,0009	1,0117	1,0290	1,0515	1,0777	1,1061	1,1629	1,2118	1,2490	1,2752	1,2931	1,3050	1,3131
14	0,9975	0,9979	0,9986	0,9997	1,0010	1,0118	1,0291	1,0516	1,0779	1,1063	1,1630	1,2119	1,2490	1,2753	1,2931	1,3051	1,3131
14,5	0,9976	0,9980	0,9987	0,9998	1,0011	1,0119	1,0292	1,0517	1,0780	1,1064	1,1631	1,2120	1,2491	1,2753	1,2932	1,3051	1,3132
15	0,9976	0,9981	0,9988	0,9999	1,0012	1,0120	1,0293	1,0518	1,0781	1,1065	1,1632	1,2120	1,2492	1,2754	1,2932	1,3051	1,3132

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Appendix D

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