# Investigating Adhesion Proteins by Single Cell Force Spectroscopy

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**Abstract** The ability to sense and measure adhesion forces by using force spectroscopy techniques has opened new perspectives in the field of mechanobiology. Single-cell force spectroscopy enables to directly measure interactive forces of single living cell with extracellular environment (i.e., cell, proteins and tissue) with extremely high resolution (single-protein level). Cell adhesion processes rely on the interaction of adhesion proteins with their environment. Cells sense and recognize the specific forces that are generated by the interaction with the environment, and transduce them into biochemical signals by which the cells evolve, move and grow. Single-cell force spectroscopy is the ideal tool to measure these forces and investigate the cellular response from its origin.

## 1 Introduction

The imaging and mechanical characterization of biological systems at a molecular level has become possible with the invention of atomic force microscope (AFM). The AFM initially was applied mainly in hard sciences, such as physics and engineering; however, soon it became clear that it would have a revolutionary impact also in the field of biochemistry and cell biology, since it allowed the imaging and manipulation of biological samples, in physiological condition, at the nanoscale [1]. This technique is able to sense and apply a wide range of forces

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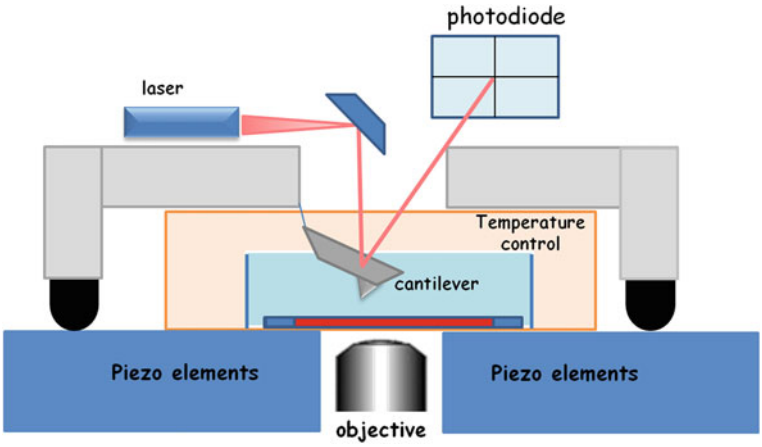



**Fig. 1** Sketch of the experimental setup of AFM including conical probe at the end of a cantilever. A laser is reflected to back of the cantilever toward a photodiode that detects changes in cantilever deflection. Nanometer precision movements are regulated by piezo-elements that can be positioned below sample stage. Measurements can be carried out in liquid cell at controlled temperature suitable for cell culture. Frequently for biological studies AFM is coupled with an optical microscopy working in transmission mode

(1 pN–100 nN) that corresponds to the forces that dominate the biological phenomena from the molecular to the single cell scale. Moreover, the nanometer scale precision in positioning the tip relative to the surface and ability to operate in aqueous physiological conditions made the technique capable to follow biological process in situ. The core of this instrumentation is represented by a micromachined cantilever that may deflect upon interacting with the sample surface. Cantilever deflection is detected by a laser beam reflected from the free end of the cantilever into a photodiode (Fig. 1) with a sub-nm precision. Usually a sharp tip is located at the free end of the cantilever and is used to investigate the sample property with in-plane nm resolution. The tip is usually made of silicon or silicon nitride and has a radius of curvature of about 10 nm. However, other materials can be used for higher spatial resolution (carbon nanotubes) such as increased stiffness (diamond whiskers) and electrical conductivity (tungsten carbide). For biological applications, chemical functionalization can be applied to the tip to exploit specific interactions; for force mapping or force spectroscopy, cantilevers may be used flat without sharp tips or with micrometer-sized beads. In image mode, the initial application of AFM, the tip/cantilever is scanned across the sample surface, while the tip senses the local forces that are used for feedback control. In biological applications, these forces may have biological, chemical or physical origins. A piezoelectric scanner allows high-resolution 3D positioning (1 Å) of the tip. Measurements can be performed into a liquid cell where temperature can be controlled and can be combined with the other imaging techniques used in biology [2]. AFM was used for imaging fixed cells [3] living cells [4], protein structures [5]

down to individual proteins [6, 7]. An AFM can be also operated in different fashion, in which the tip is kept fixed at a given position and the deflection versus distance is recorded. Thanks to the elastic properties of the cantilever, a force-distance curve is recorded that provides useful information on the mechanical and dynamic properties of the sample. This approach, known as force-spectroscopy, allows to probe biological, chemical and physical interaction forces of individual molecules with pN resolution. The ability to work in force-spectroscopy mode opened up the possibility to analyze the kinetics and the interaction strength of single proteins even in living cells [1, 8]. Single-molecule force spectroscopy (SMFS) measures the adhesive interactions between biological molecules: the force required to break a single intermolecular bond was measured directly by separating a protein linked to the AFM tip from that fixed on the substrate. The bond rupture force measured at different loading rates produces information about kinetic barriers, binding constants, binding mechanisms and free energy [9]. Whereas in molecular recognition mapping (MRM) a probe molecule bound to the tip is made interact with its receptor, generally embedded in membrane living cells, exposing an active recognition site to the outer environment [10, 11] mapping the interaction forces reproduces the receptor distribution on the cell surface, while the intrinsic force dependence of the mechanism is used to separate the contribution of different kind of receptors. Another application of AFM in the field of the biomechanics is so-called single-cell force spectroscopy (SCFS), in which a single living cell is first attached to a tipless AFM cantilever and then is brought into contact with a substrate, with another cell grown of a solid substrate or with a tissue, providing information about the cell adhesion properties as a whole and on the molecular mechanism of cell adhesion [12–14].

Cell adhesion is a complex biological process that plays a central role in regulating numerous fundamental physiological and pathological cellular activities such as proliferation, migration, differentiation, metastasis, immunological response and communication [15]. The process is mediated by focal adhesion points located on and close to the cell membrane. They are assembled by a core made of a large family of multidomain transmembrane proteins and a network of cytoplasmatic proteins that ensure the mechanical link to the cell cytoskeleton and are responsible of the regulation of a number of signaling pathways that mediate the cell adhesion [16–18]. These membrane areas include integrins, cadherins, immunoglobulin superfamily and selectins. They have an extracellular domain able to bind either to components of extracellular matrix or other cells and an intercellular domain that interacts with cytoskeleton directly via cytoskeleton-associated proteins (i.e., vinculin, talin) or indirectly (i.e., Rho-family GTPase, protein kinase, paxillin), which are involved in the transduction of the signaling process and regulation of cytoskeleton reorganization [17] (see Fig. 2). Usually, cell adhesion is investigated by using various adhesion assays: washing assay [19], spinning disk [20, 21] and centrifugation assay [19] which have disclosed important features of the key components regulating adhesion mechanism. The role of adhesion proteins is also investigated by several biochemical assays as immunoblotting that offers information about the different expression of adhesion proteins [22, 23] or by high-

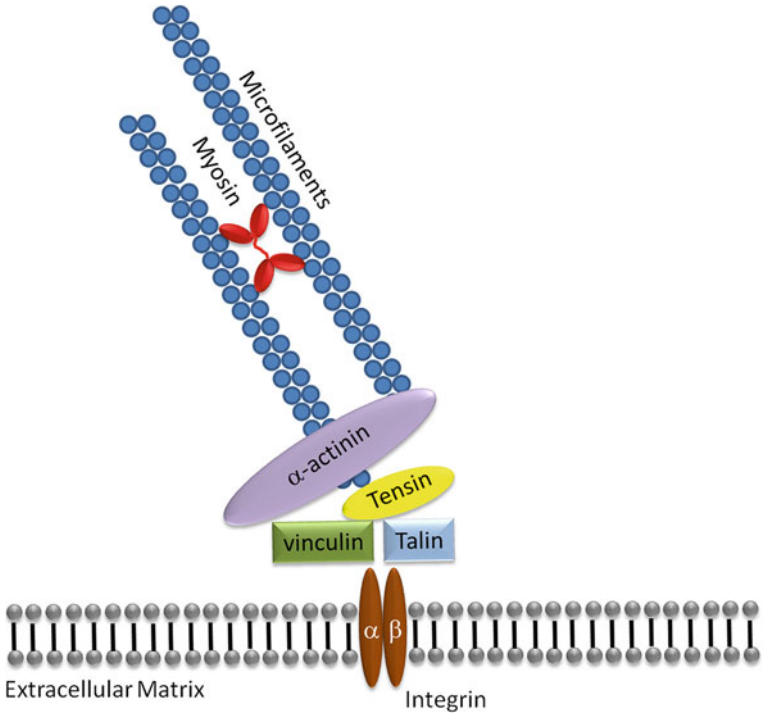



**Fig. 2** Simplified scheme of a focal adhesion site that depicts interaction of transmembrane adhesion proteins (integrin) with some of the cytoplasmatic proteins included in the transduction machinery that mediate the adhesion process

resolution fluorescence microscopy that can reveal their distribution and clustering at the cell membrane [24–26]. However, these methodologies have some important flaws: mechanical assays provide averaged information about cellular behavior while immunoblotting and immunofluorescence are useful to investigate expression and distribution of adhesion proteins but do not give a direct proof of binding; moreover, neither of them can be used to investigate the dynamics of the adhesion process.

The combination of AFM imaging with optical and fluorescence microscopy represents a very attractive tool for high-resolution study of cellular adhesion process [8, 27, 28]. An example of such a correlated fluorescence-AFM study is the high-resolution imaging of proteins localized within focal adhesion sites interacting with microfilaments of cytoskeleton. The high resolution of the AFM topographs overcomes that of the light microscope images and structural information about the 3D organization of microfilaments in focal adhesion areas can be provided without using complex protocol and in physiological environment [27]. More recently, a new cantilever holder which enables a side view of the cantilever tip-sample interaction was designed and commercialized by JPK Instrument (Fig. 3a). The

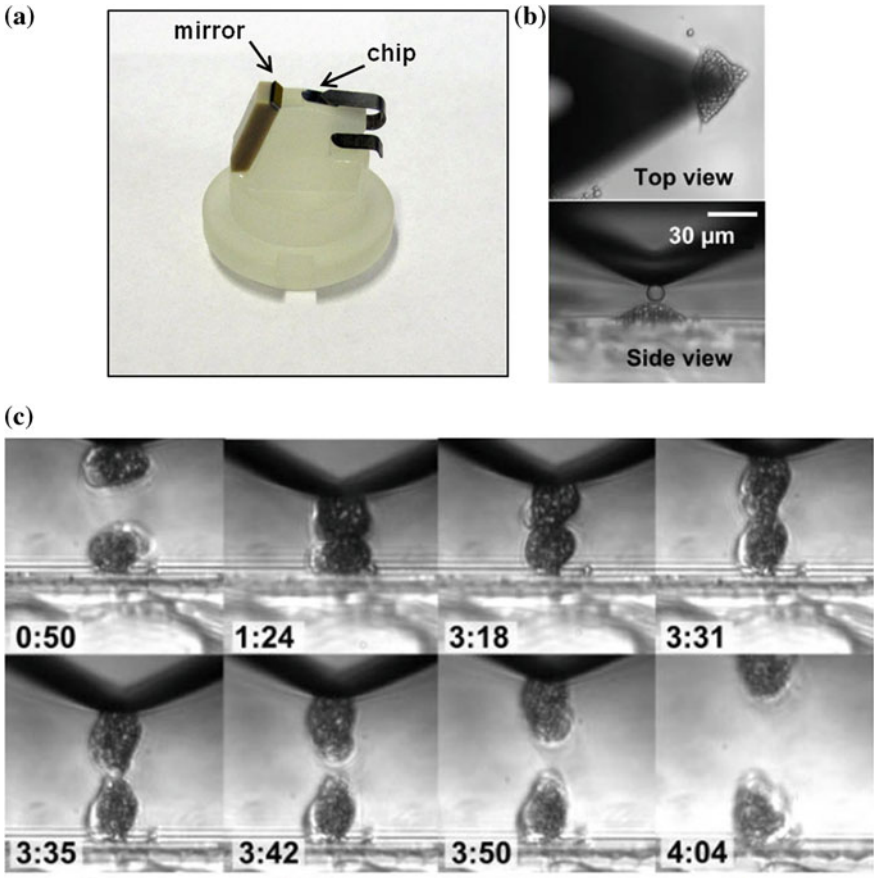



**Fig. 3** Side view of a SCFS measurement. **a** The special design of the cantilever with a 45° mirror makes possible to follow and visualize cell detachment with an optical or fluorescence microscopy. **b** *Top* and *side* view image show a colloidal nanoparticle in contact with a fixed neural crest cell of a Xenopus leavis. **c** An example of optically monitoring cell–cell detachment is shown. All images are data courtesy of Dr C. Franz KIT (Germany)

special design integrated with the transmission light techniques allows to fully monitor the cell membrane adherence points during the detachment process as shown in Fig. 3b–c.

Besides molecular resolution imaging of adhesion-proteins SCFS provides quantitative information of the cell adhesion behavior. Initially, the interaction strength of adhesion proteins at single-molecule level was widely investigated by SMFS. The dynamic of interaction process of cell adhesion proteins, including selectins [9], cadherins [29, 30] and integrins [7] disclosed new insight into the association and dissociation behavior of these proteins. However, these analyses were conducted on purified proteins or on isolated domains, removed from their

biological context: therefore, these investigations were unable to take into account the interactions with the cytoplasmatic molecules and the structures involved in the regulation of cell adhesion activity resulting in biologically nonrelevant information.

A better understanding of the biological mechanisms of cell adhesion can be attained by probing cell-adhesion protein behavior directly in living cells. In this framework, SCFS represents a very promising nanotechnological approach for dissecting localized signaling changes involving the adhesion proteins. This means that the cell signaling processes that strengthen adhesion bonds or the action of forces applied to cell-surface interface on intracellular components to trigger biochemical processes or the influence molecular interactions can be explored with nanometer scale resolution. Indeed, the interpretation of functional role of signaling adhesive components in the mechanosensing process requires a quantitative understanding of adhesion-proteins behavior. The mechanotransduction regulates gene expression and cell fate [31] and that it is now increasingly evident its relevant role in the cancer cell migration and diffusion [32]. In this chapter, we focus our attention on the investigation of adhesion proteins embedded into the membrane of a living cell at single molecule level by using SCFS, and we discuss the capabilities of the technique and the relevance of the findings obtained in correlation with their biological function.

## 2 Force-Spectroscopy Measurements and Experimental Details

In the case of SMFS and MRM, a conical/pyramidal tip positioned at the end of the cantilever is chemically functionalized with an adhesion binding molecule and is brought into contact with the adhesion receptor (SMFS) or living cell (MRM) immobilised on a supporting substrate. Thanks to the sharp tip of the AFM probe only one or few adhesion proteins interact with the ligand in each experiment. After a certain contact time, the cantilever is withdrawn at a constant speed or, correcting for the cantilever deflection, at a constant loading rate. The force acting on the ligand-receptor connection increases with time, thus reducing the connection lifetime until the interaction bonds break. The detachment force is measured by detecting the deflection of the cantilever by the photodiode which is converted in force using the cantilever elastic constant. A typical single molecule force-distance spectrum obtained by pulling a ligand-bound tip from its receptor counterpart on the cell membrane is displayed in Fig. 4 The result of such MRM experiments is a bidimensional map of unbinding events performed over the cell surface that gives a clear visualization of the receptor spatial distribution [33]. While SMFS measurements and analysis allow the estimation of binding affinity, rate constants and structural data of the binding pocket of ligand-receptor at single-molecule level of adhesion proteins [29, 34].

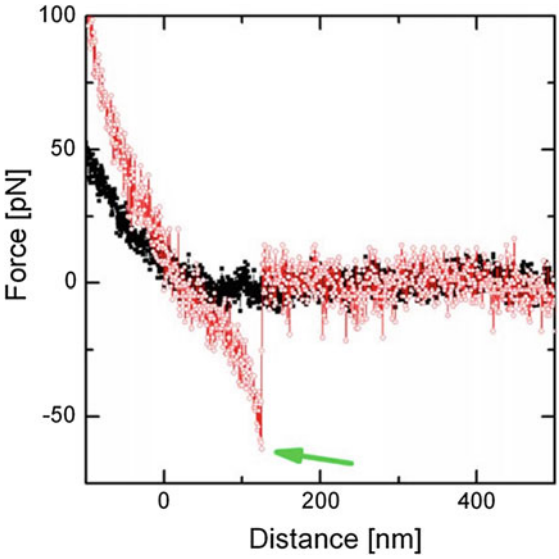



**Fig. 4** An example of force-distance *curve* obtained when pulling a receptor ligand (leuprorelin acetate) bound to the tip from its receptor counterpart embedded into the cell membrane of prostate cancer cells until the binding between ligand and receptor is broken. When receptor-ligand binding interactions are not detected (*black square*) and when interactions are detected (*red circle*). The unbinding event (or *rupture point*) is indicated by the *arrow* (Reprinted with permission from Lama et al. [33] copyright 2013 PlosOne)

In SCFS, a tipless cantilever is used for attaching a living cell. Even in this case, the stable binding of the cell is assured by functionalization of the cantilever. The choice of the protein for functionalization is very important to attain a firm cell binding without influencing cell state during the measurements. Usually proteins that are components of the extracellular matrix (i.e., concavalin-A, laminin, fibronectin, collagen, etc.) which have high affinity for receptors present on the cell membrane are used for functionalization.

Before attaching cell to the cantilever, the sensitivity and spring constant of the cantilever have to be calibrated. The deflection of an AFM cantilever is measured by the position of the reflected laser beam on the photodetector. Accordingly, the units of measurement are volts and to convert the units into newtons it is necessary to determine the cantilever sensitivity (i.e., the relationship between the output of the photodiode in volts and the deflection of the cantilever in nm) and the spring constant (which converts deflection in nm to force in nN). First, the sensitivity is determined from a force-distance (F-D) curve recorded by pressing the cantilever on a stiff surface. When cantilever and surface are in contact, the deflection of the cantilever is proportional to the vertical movement of the AFM piezo element. Most AFM softwares offer an option to measure the thermal noise of the cantilever and apply the equipartition theorem to calculate the cantilever spring constant [35].

Afterward, cells are introduced in the AFM fluid cell chamber and the cantilever is gently pushed with a force load of less than 1 nN for several seconds onto a cell selected by optical microscope in an area of the substrate coated with protein having very low affinity for integrin binding, bovine serum albumin (BSA) is commonly used [Fig. 5a (i, ii)]. The cell bound to the cantilever is separated from the support and allowed to establish firm adhesion for several minutes [Fig. 5a(iii)]. The

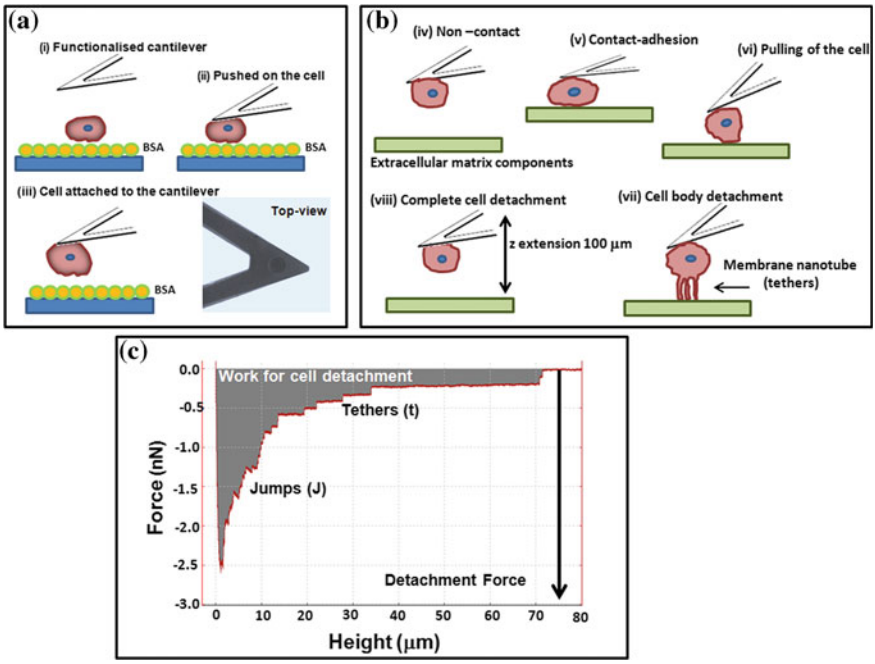



**Fig. 5** Scheme of cell capture by tipless cantilever (**a**). The functionalized cantilever is positioned over a cell in suspension at close proximity to the surface (i). Then the cantilever is gently pushed for a few seconds onto the cell (ii). After this, the cantilever-bound cell is separated by the support (iii) besides a phase-contrast microscopy image of cell firmly immobilized on the cantilever. Sketch illustrating a single SCFS measurement (**b**). The cell attached to the cantilever is approached to the substrate to establish cell-surface interaction (iv, v). After a predefined contact time, the cell is retracted and the cantilever bends because of the adhesive strength between the cell and the substrate. Once the force of the cantilever exceeds the strength of the interactions between cell and substrate, the cell starts to detach (vi) forming membrane nanotube (vii) up to a complete detachment (viii). **c** The resulting F-D retraction curves from the SCFS measurement provides quantitative measure of different cell adhesion stages

sequence of these events can be followed by an optical microscope that allows also to control the cell state during SCFS measurements. A phase-contrast image of a cell bound to a tipless cantilever is shown in Fig. 5a. The sketch in Fig. 5b shows the cycle of a SCFS measurement. The cell attached to the cantilever is approached either with a specific substrate or with a target cell until a predefined repulsive contact force is established Fig. 5b (iv). This contact force is held constant for a given time to allow establishment of cell adhesion [Fig. 5b (v)]. Contact time may range from milliseconds to several minutes. Different adhesion regime can be investigated by setting different contact times so that the signaling cascade pathway that leads to cell adhesion could be obtained. Upon cantilever retraction, the cantilever force exceeds the strength of the interactions between cell and substrate and the cell starts to detach (vi) forming membrane nanotube (vii). During this

procedure, the force as a function of the distance of the cantilever is recorded until contact with the cells is broken [Fig. 5b (viii)]. The result of this cycle is a F-D curve where the retraction curve represents a full characterization of the cell adhesion as shown in Fig. 5c. Care must be taken in order to avoid that the cell detaches from the cantilever. The complete cell separation from substrate requires a longer $z$ range extension. The CellHesionTM module (JPK Instruments, Berlin, Germany) is a technical solution for this problem. The module contains a sample stage that is fitted with piezo-elements that have a 100 μm range for moving the sample in the $z$ direction.

It is worth to mention that SCFS measurements can be performed also in presence of blocking agents (i.e., antibody or small RGB peptide) able to block the activity of the adhesion proteins on cell membrane [36–38]. Moreover, since measurements are performed on living cells, it can be possible to use blocking agents that can diffuse into the cell membrane and interact with specific intracellular intermediate of the transduction machinery [36]. This allows to molecularly associate interaction forces with proteins that mediate the signal between adhesion proteins and cytoskeleton. Even in this case, the coupling of SCFS measurements with high-resolution fluorescence microscopy can provide considerable advantages. The use of GFP-labeled adhesion proteins conjugated with fluorescent dye would reveal more detailed information on the adhesion characteristics of the cell in different conditions.

## 3 Analysis of Force-Distance Curve

The analysis of the F-D retraction curve points out three main features (see Fig. 5c): the peak minimum value (F detachment) that is the measurement of the maximum force exerted to detach the cell from substrate (i.e., adhesion force); a train of saw-like peaks following the detachment force involving receptors that remain anchored to cell cytoskeleton and unbind as force increases (named jumps); finally long plateau where receptors anchoring is lost and membrane nanotubes are pulled out of the cell (tethers). The area below the retraction curve represents the mechanical work done by the cantilever during the whole detachment processes or, in other words, the total adhesion energy. More detailed information about adhesion mechanism can be obtained by discriminating jumps from tethers. Both jumps and tethers correspond to small discrete rupture events, which result from unbinding of single receptor-ligand pairs that remain anchored to the cytoskeleton (jumps) or to the membrane (tethers) [8, 39]. Those that remain bound to the membrane lead to the formation of membrane nanotubes (tethers) that can be of potential physiological relevance, since this kind of membrane structures are generally related to cellular attachment, migration and communication [40].

The analysis of jumps and tethers features can reveal energetic and kinetic properties of adhesion proteins embedded into a living cell (Fig. 6) [8]. When the cantilever starts pulling the cell out of contact, if the anchor strength to the

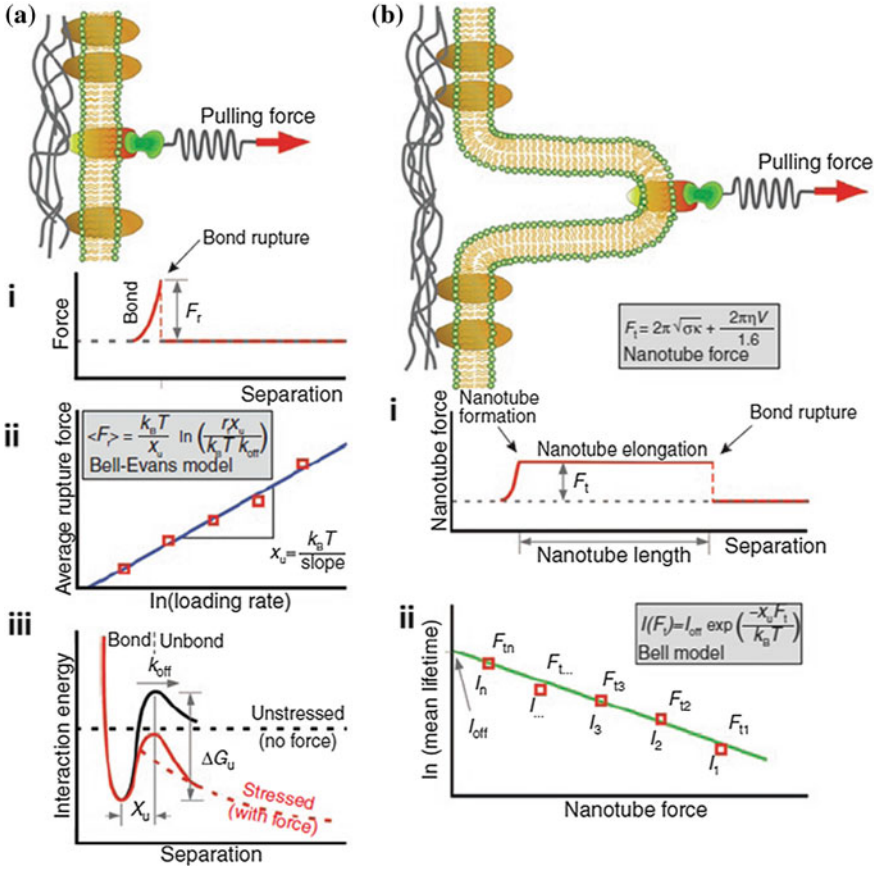



**Fig. 6** Energetic and kinetic properties of force bonds at cell surfaces for different adhesion proteins. **a** Cytoskeleton-bound adhesion proteins. **a** (i) The receptor-ligand bond is mechanically stressed until it ruptures at a force (Fr). **a** (ii) According to the Bell-Evans model, the average rupture force <Fr> of the bond linearly increases with the loading rate (rf). **a** (iii) Interpretation of how an externally applied force reduces the unbinding barrier and increases the unbinding rate of the probed bonds. **b** Membrane-bound adhesion protein presents at the tip of a membrane nanotube. The force required to extend a nanotube (Ft) depends on some factors: extension speed (V), isotropic tension (σ), bending rigidity (κ) and viscosity (η) of the cell membrane. **b** (i) The force to extend the nanotube remains constant at constant extension speed. The extension speed and length of the nanotube can be used to calculate the bond's lifetime. **b** (ii) The measurements of bond's lifetime for different extension speeds analyzed by the Bell model can provide information about the dynamics of these bonds (Reprinted with permission from [8] copyright 2009 Nature Publishing Group)

cytoskeleton is greater than that of adhesion, the transmembrane adhesion proteins will likely unbind first [Fig. 6a (i)]. In such a case, the force vs. distance curve shows a typical elastic character and the Bell-Evans model [Fig. 6a (ii)] [41] may be applied for data analysis. According to this model, the force at which a single

bond unbinds increases as the rate at which the force on the bonds is applied (loading rate, rf) increases. The loading rate is controllable, as it depends on the speed at which the AFM cantilever is retracted. For most receptor-ligand bonds, their rupture force increases linearly with the logarithm of the loading rate [Fig. 6a (ii)]. By measuring the most probable rupture force over a range of loading rates, the unbinding rate (koff), the distance to the transition state (xu) and the free energy (ΔGu) of the bond can be estimated [Fig. 6a (iii)]. In the opposite case, when the link of the focal adhesion points with the cytoskeleton is weaker, the transmembrane adhesion proteins are pulled away from the cytoskeleton, and remain localized at the tip of a membrane nanotube (Fig. 6b). The measured forces (Ft) have a typical viscous character depend on plasma membrane properties and increase with extension speed (V). As nanotube length has very little effect on extension force, cell membranes establish constant force clamps that can be used to measure the lifetime (ln) of receptor-ligand bonds under force [Fig. 6b (i)]. The measure of the mean lifetime of a bond at different forces (i.e., extension speeds) allows the lifetime at equilibrium (loff) and distance to the transition state (xu) to be determined [Fig. 6b (ii)] [42]. By analyzing nanotube extension forces, plasma membrane properties such as the extent of anchoring to the cytoskeleton and viscosity can also be characterized. It is worth to stress here that in both cases the observed force jumps refer to the transmembrane adhesion protein-ligand bond rupture, while the forces between the focal adhesion structures and the cytoskeleton is not investigated by SCFS.

## 4 The Advantages of SCFS in the Study of Single Adhesion Proteins

Besides biochemical and structural description of adhesion proteins, quantitative data about interaction forces involved in the binding with their surrounding environment are fundamental to understand the mechanisms that guide and regulate cell adhesion in physiological conditions and diseases. Numerous of SCFS capabilities can strongly improve the understanding of adhesion mechanisms. In the following list they are briefly summarized.

- Quantification of the interacting forces of adhesion proteins with their natural ligands in living cells [43];
- short-term investigation of adhesion steps and understanding of the activity of factors affecting adhesion formation [44];
- the ability to resolve interactions of individual proteins and examine their individual contribution instead of describing an average behavior [45];
- coupling with genetic manipulation [36, 44, 46];
- the possibility of modulating interaction parameters as contact force, contact time, speed retraction of the cantilever and also biochemical factors (i.e., pH, ion concentration of functional relevance, external stimuli, etc.) allows

studying the role of individual adhesion proteins and intermediate proteins regulating cell adhesion within a single SCFS experiment [36, 47].

The technique also opens up the possibility to study the role of adhesion proteins in various conditions that have great interest in the biomedical field, as for example:

- evaluation of adhesion-blocking drugs [48];
- investigation of initial steps in the interaction of cells with artificial surfaces of medical interest, such as different culture supports or receptor mediated adhesion of particles for phagocytosis.

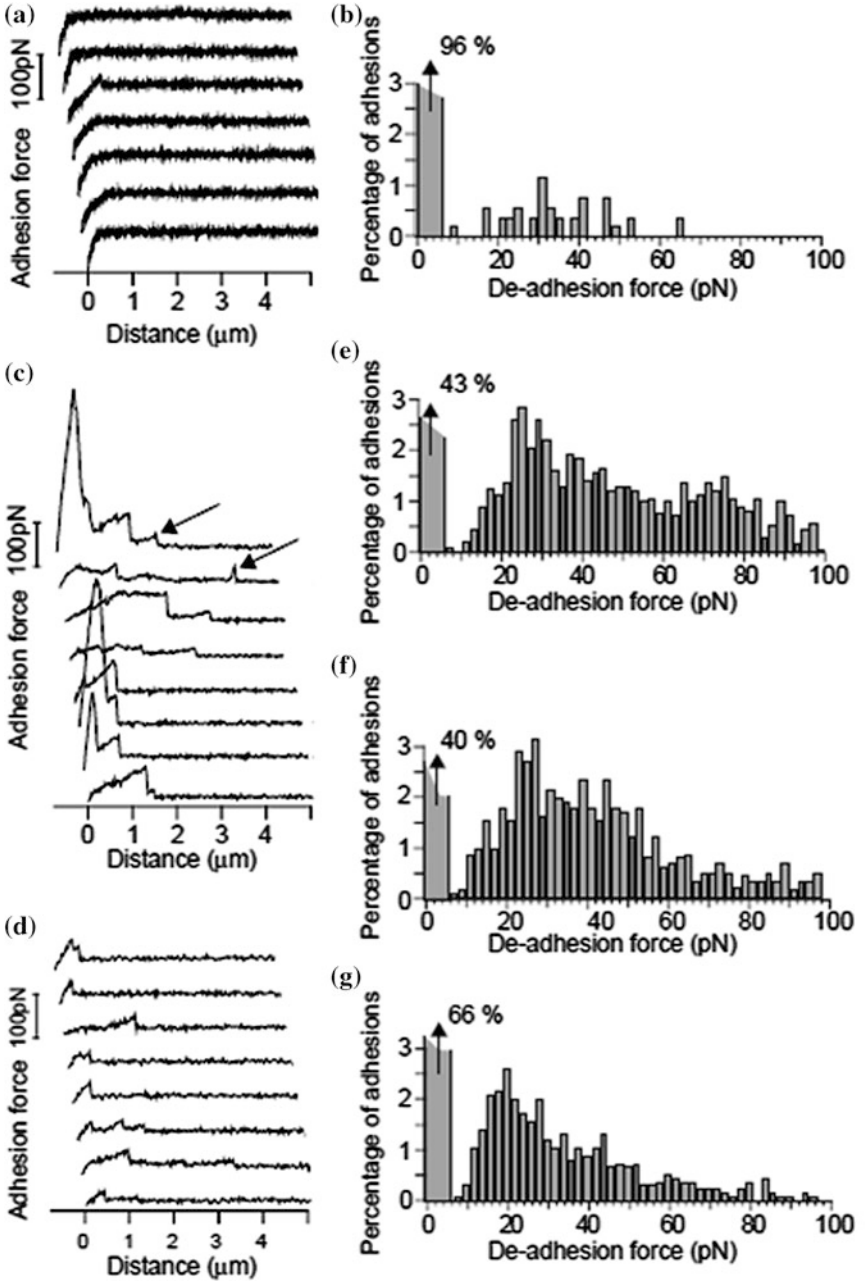
Through its numerous advantages, the technique has a main drawback in biological field. A single SCFS experiment is unfortunately confined to a reduced number of cells, and statistics is still a time-consuming procedure.

## 5 Quantifying Cell Adhesion at Single Molecule Scale

The advantages of SCFS have allowed of getting new insights about the complex protein machinery that regulates the cell adhesion down to single-protein resolution.

One of the first examples that demonstrate the capability of SCFS to measure adhesion force with molecular resolution is represented by the quantification of the adhesion strength of single glycoprotein contact site A (csA) expressed in aggregating cells of *Dictyostelium discoideum*. This protein is differently expressed depending on cellular aggregation stage (growth-phase and developing cells). The analysis of data obtained by performing SCFS measurements as function of contact force and time in two states of cellular development are shown in Fig. 7. During growth phase, a low number of steps in F-D curves were observed in agreement with the lower expression of the csA protein. Steps increase considerably for developing cells even at when contact force is reduced to decrease the contact area. By lowering the contact time, the number of steps is further reduced. By plotting the steps force values as histograms, a peak at $23 \pm 8$ pN can be observed in all graphs. This value is attributed to the force of single csA proteins, while the other peaks of the distribution seem to be related to multiples of the basic quantum of 23 pN. Genetic manipulation confirmed that this force value was associated with csA expression [45].

The role and the dynamics of the $\alpha 2\beta 1$ integrin in binding and spreading onto collagen type I matrix has been clarified by SCFS [49]. By comparing the adhesion strength of wild type-chinese hamster ovary cells (CHO) with that of (CHO)-A2 $\alpha 2\beta 1$-expressing cells as function of short contact time (5 s), it was found that the mean detachment force ($189 \pm 12$ pN) of CHO-A2 cell was almost 4 times higher than that of CHO-WT cells ($49 \pm 7$ pN). Moreover, measurements in absence of $Mg^{2+}$ definitively confirmed the principal role of $\alpha 2\beta 1$ integrin in the interaction


(a)
100pN
Adhesion force
0 1 2 3 4
Distance (μm)
(b)
96 %
Percentage of adhesions
0 1 2 3
0 20 40 60 80 100
De-adhesion force (pN)
(c)
100pN
Adhesion force
0 1 2 3 4
Distance (μm)
(e)
43 %
Percentage of adhesions
0 1 2 3
0 20 40 60 80 100
De-adhesion force (pN)
(d)
100pN
Adhesion force
0 1 2 3 4
Distance (μm)
(f)
40 %
Percentage of adhesions
0 1 2 3
0 20 40 60 80 100
De-adhesion force (pN)
(g)
66 %
Percentage of adhesions
0 1 2 3
0 20 40 60 80 100
De-adhesion force (pN)

◀**Fig. 7** Force spectra for stable adhesion of undeveloped and developed cells. **a** F-D curves for adhesions of growth-phase cells where cell–cell contacts were maintained for 0.2 s at 90 ± 10 pN. The resulting histogram of de-adhesion forces is reported in (**b**). Although high contact force (90 pN) is applied, only a small percentage of contacts resulted in measurable cell–cell adhesion. F-D curves for adhesions of discoideum cells at the developed stage (**c** and **d**) with cell–cell contacts for 2 s (**c**) or 0.2 s (**d**) at 35 ± 5 pN. Arrows indicate force steps for complete rupture. The resulting histograms of de-adhesion forces obtained at 35 ± 5 pN for different contact time 2 s (**e**) 1 s (**f**) 0.2 s (**g**) are shown. In all histograms, rupture events occurring at <7 pN are represented by the first bar (Reprinted with permission of [45] copyright 2000 Nature Publishing Group)

with collagen I. In fact, this metal binds to the domain of α2 activating the protein. Additionally in this work, adhesion measurements were performed as function of contact time and a minimum contact time was found for the activation of the integrin-based adhesion. Indeed, a tenfold increase of adhesion force was observed with contact time longer than 60. This effect was accompanied by a parallel increase of single rupture event jumps, which was explained in term of the onset of cooperative receptor binding. The authors proposed a two-step mechanism for the establishment of α2β1 integrin mediated adhesion: at short contact times single integrin–mediated binding events dominates the cell-substrate adhesion; for contact times longer than 120 s strong adhesive interactions involving receptor cooperativity and actomyosin contractility are observed.

The possibility to explore the adhesion over short range time reveals also new important details about the mechanism of activation pathway of integrins. The protein kinase C is an enzyme that can mediate the inside–out activation of integrins α2β1 and their interaction with cytoskeleton. Its activity can be induced by the presence of 12-O-tetradecanoyl-phorbol-13-acetate or inhibited by bisindolylmaleimide. SCFS measurements were performed immediately after activator incubation (10 min) show an increase in adhesion strength which results from α2 integrin activation due to the induction of kinase enzyme activity, thus excluding the genetic activation or protein expression in this condition and providing clear evidences for the role of the kinase activity in integrin activation [44].

The integrin cross talk between collagen-binding integrin α1β1 and fibronectin-binding integrin α5β1 in Hela cells was detected by a small variation in SCFS protocol. The Hela cells were bound to cantilever differently functionalized by using integrin binding proteins that can activate different cascade pathways. After 10 min incubation that allows the cascade to activate, the adhesion with collagen I and fibronectin (both proteins commonly present in the mammalian extracellular matrix) was measured. This approach combined with antibody blocking the integrin activity has allowed to selectively probe the role of α1β1 and α5β1 integrin receptors in the adhesion and to provide clear evidences about the cross talk of these adhesion proteins that is found to be unidirectional from integrin α1β1 to integrin α5β1. The cross talk seems to function through the regulation of integrin α5β1 endocytosis [47].

Multi-protein adherens junction that links cell–cell contact to the actin cytoskeleton and various signaling molecules play an important role also in adhesion

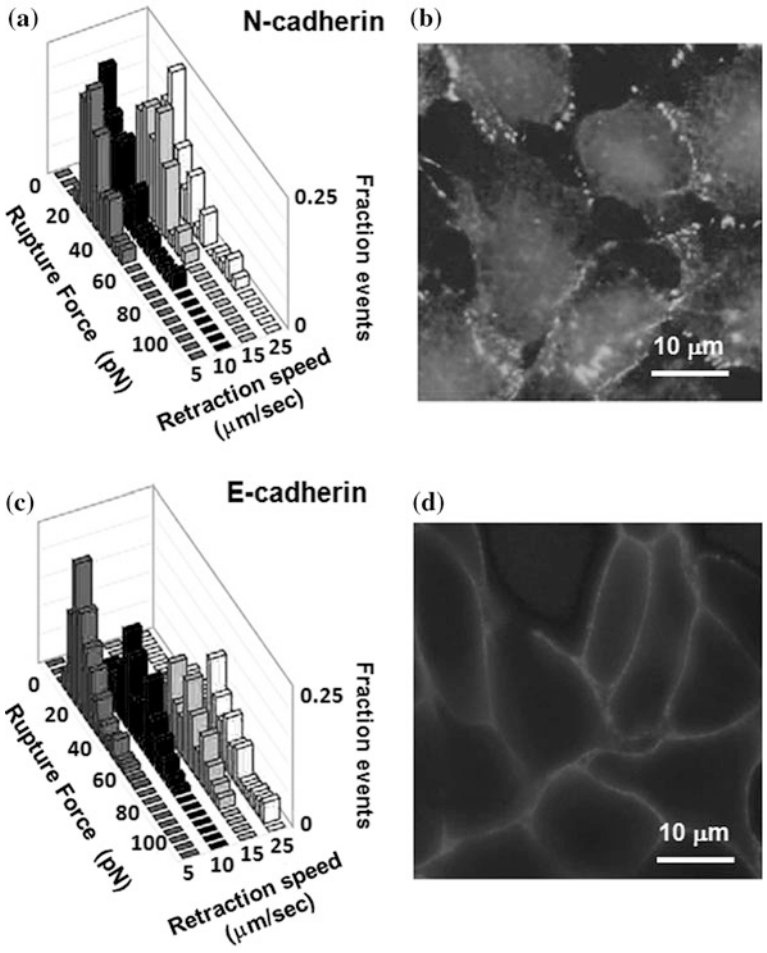



**Fig. 8** Rupture forces of a single cadherin–cadherin bond as a function of retraction speed. **a** Distribution of rupture forces to break a single E-cadherin–E-cadherin bond between apposing cells obtained by using different retraction speeds. **c** Distribution of rupture forces to break a single N-cadherin–N-cadherin bond at different retraction speeds. Immunofluorescence images facilitate the visualization of N-cadherin **b** E-cadherin **d** distribution in the CHO cells. Data courtesy of Prof. K. Konstantopoulos, The Johns Hopkins University (USA)

and intercellular communication. SCFS measurements demonstrate that type I N- and E-cadherins establish intercellular bonds that despite having a similar biological role, exhibit significantly different kinetic and micromechanical properties. This is highlighted by performing SCFS as function of detachment speed, which shows that the rupture forces of the two proteins are considerably different (30 pN for N-cadherins against 73 pN for E-cadherins) and also their dissociation rates ($0.98 \pm 0.46\ s^{-1}$ for N-cadherins against $1.09 \pm 0.35\ s^{-1}$for E-cadherins) strongly vary (see Fig. 8). The combination with immunofluorescence was very

useful to demonstrate the distribution of the receptors on the membrane. Additionally, the role of calcium ions in the mechanism of cadherin binding on cells was also confirmed in such experiment [36]. Analogously, SCFS provides convincing evidences that activation of the $Ca^{2+}$ receptors increases expression of the epithelial adhesion proteins E-cadherin and increases functional tethering between β-cells in pancreas [23].

The use of SCFS can be also determinant in biomedical field. The study of cell adhesion mechanism is fundamental to understand the ability of cancer cells to migrate and diffuse into tissues. The molecular mechanisms defining the different modes of cancer cell migration remains in most parts to be delineated [50]. A fundamental step in this study is the understanding of the behavior of adhesion proteins that mediate the interaction of cancer cells with extracellular matrix of cells within different tissue. In this way, cancer cells can invade tissue leading to metastasis diffusion. The quantification of adhesion strength can provide new insights into such mechanism, particularly because it is observed that the mechanical properties of the matrix could strongly influence the fate of the cells [32].

A representative example of this study is reported by Sariisik et al. [51]. They dealt with the problem of prostate cancer cells diffusion into bone tissue where these cells can form metastasis. The investigation of interaction strength of bone marrow-specific prostate cancer cell line (PC3) with the components of the bone tissue (collagen I and mesenchymal stem cells) was performed as shown in Fig. 9. In these measurements, SCFS techniques were combined with optical and fluorescence microscopy to select the different components of the substrate. This substrate organization allowed to probe the interaction of prostate cancer cells with both mesenchymal stem cells and collagen I simultaneously. The result findings provided quantitative data for the high affinity of prostate cancer for these bone components as compared to other nonspecific cell lines (LNCaP). These measurements coupled with semi-quantitative PCR data identified integrins α1β1 and α2β1 as possible responsible for this high-binding affinity. Analogously, higher binding affinity of breast cancer cells for mineralized extracellular matrices secreted by primary human osteoblasts was quantified and associated to β1 integrins which are critical for bone metastasis [52].

A good knowledge of cell interaction mechanism with the external environment is also a key requirement for numerous applications in biomedical field (i.e., tissue engineering, electrical and mechanical stimulation of cells, etc.). In such case, it is very important to identify the interaction mechanisms that facilitate cell proliferation, differentiation and migration on such materials. There are some very exemplificative examples of the SCFS application and how the technique enable the study, the involvement and the role of adhesion proteins in the interaction with various nanostructured materials. Such findings provided information about the adhesion behavior of cells with nanopattern substrates differently functionalized [53], the importance of nanopattern elasticity in guiding neural precursor adhesion and differentiation [54] and also implants formation [55].

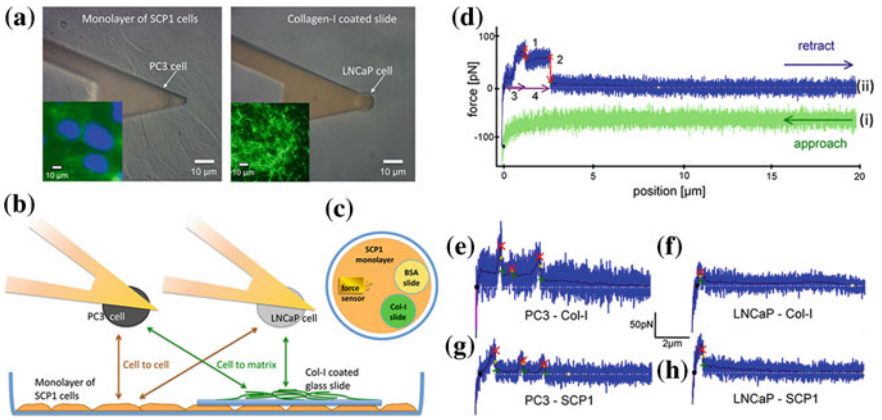



**Fig. 9** **a** Phase contrast images of a prostate cancer of two cell lines ($PC_3$ and LNCaP) attached to the cantilever indicated by the arrows above an SCP1 (mesenchymal stem cell) monolayer (*left*) and a Collagen-I-coated slide (*bone matrix*) (*right*). On the *lower left* corners immunofluorescence images are inserted illustrating cell and collagen distribution. Collagen I, labeled with AlexaFluor488 fluorescence dye appears in green and cell nuclei, stained with DAPI in blue. **b** Schematic illustration that depicts the SCFS experiment along with a schematic *top view* of the glass dish with a BSA-coated glass cover slip (as substrate for fishing prostate cancer cell) and a Collagen-I coated glass cover slip and the monolayer of mesenchymal stem cells (**c**). Representative force-distance curves are shown in (**d**) in *green* the approach of the prostate cancer cell to the substrate (i) and in *blue* the retraction (ii). The *black line* is the *smoothed curve* and the *red crosses* indicate detected de-adhesion steps. A *force curve* obtained from a PC3-cell interacting with Collagen I is used to illustrate the adhesion force evaluation (**d**): *Red arrow* (1) step height of the first de-adhesion event in the retraction *curve*; step height of the second de-adhesion event after a force plateau of 0.9 µm in length (2); step position of the first de-adhesion event (3); step position of the second de-adhesion event (4). Typical retraction curves from each of the four different experiments are shown: (**e**) PC3 on Col-I, (**f**) PC3 on SCP1 monolayer, (**g**) LNCaP on Col-I and (**h**) LNCaP on SCP1 monolayer (Reprinted with permission from modified [51] copyright 2013 PlosOne)

## 6 Future Perspectives

SCFS is a powerful technique recently introduced in the realm of biology, biophysics and medical science. Initially designed for characterize cell adhesion interaction, it was used to shed light on several different open issues, ranging from molecular biology, to biophysics, tissue engineering and biomedicine. Recently, application slightly different from the original ideas was proposed. It is worth to mention here the experiment described by Stewart et al. [56] in which the hydrostatic pressure exerted inside a cell during the mitosis circle is measured using a SCFS set up in constant height clamp configuration. In future, SCFS set up will be applied to investigate other biological issues, wherever forces are relevant. The most important limitation of these techniques is the relatively low throughput, due to the sequential process adopted in measurement collection. However, due to the increased interest in the technique and the possibility to combine it with other

techniques, we expect that semi-automatized instruments will be soon delivered by AFM manufacturer, in which cells are automatically identified by optical microscopy and image analysis and arrays of cantilevers are used in parallel, as already realized, at laboratory prototype level [57].

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