Single-cell force spectroscopy

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Summary

The controlled adhesion of cells to each other and to the extracellular matrix is crucial for tissue development and maintenance. Numerous assays have been developed to quantify cell adhesion. Among these, the use of atomic force microscopy (AFM) for single-cell force spectroscopy (SCFS) has recently been established. This assay permits the adhesion of living cells to be studied in near-physiological conditions. This implementation of AFM allows unrivaled spatial and temporal control of cells, as well as highly quantitative force actuation and force measurement that is sufficiently sensitive to characterize the interaction of single molecules. Therefore, not only overall cell adhesion but also the properties of single adhesion-receptor–ligand interactions can be studied. Here we describe current implementations and applications of SCFS, as well as potential pitfalls, and outline how developments will provide insight into the forces, energetics and kinetics of cell-adhesion processes.

Key words: Atomic force microscopy, Cell adhesion, Cellular interaction, Dynamic force spectroscopy, Extracellular matrix, Molecular interaction, Single molecule

Introduction

A broad spectrum of biological processes requires controlled cell adhesion, including embryonic development, assembly of tissues and the nervous system, cellular communication, inflammation and wound healing, tumor metastasis, cell culturing, and viral and bacterial infection. Although much is known about cell adhesion, many questions remain unanswered owing to its multiple facets and complexity. Cell adhesion is commonly defined as the binding of a cell to a substrate, which can be another cell, a surface or an organic matrix. The process is regulated by specific cell-adhesion molecules (CAMs), which are typically transmembrane receptors that comprise an intracellular domain that interacts with cytoplasmic proteins, including the cytoskeleton, and an extracellular domain that specifically binds to adhesion partners (Kemler, 1992). Binding is commonly heterotypic, but it can be homotypic, such as that involving cadherins. The major classes of CAMs in mammals include cadherins, selectins, integrins and Ig-CAMs (cell-adhesion molecules of the immunoglobulin superfamily). Molecular and genetic approaches have identified the adhesion proteins and their ligand specificities, and have determined the processes in which they are involved. However, the molecular mechanisms by which CAMs work and how they regulate different types of adhesion are open debates (Morgan et al., 2007). For example, an extensive array of proteins is known to be involved in adhesive assemblies, i.e. focal adhesions [cell–extracellular-matrix (ECM) junctions], but the contributions of these proteins to the strength of adhesion are not quantitatively understood (Lo, 2006). To understand cell adhesion, therefore, the vast amount of qualitative data that is available must be augmented with quantitative data of the physics of adhesion.

Historically, the strength of the adhesion of a cell to a substrate has been studied using simple washing assays (Klebe, 1974)[1]. Surprisingly, given the lack of standardization, washing assays have proven to be versatile and useful in identifying CAMs, important ECM components and other proteins that are involved in various forms of cell adhesion. To estimate the force to which cells are subjected, various assays that are based on the regulated flow of media have been implemented, including spinning-disk (Garcia et al., 1997) and flow-chamber (Kaplanski et al., 1993) assays. Unfortunately, the shear force that is exerted on the cells in these assays depends on parameters such as cell size, cell shape and how the cell is attached to the substrate, and can therefore only be estimated. For a more controlled and quantitative approach to measurements of adhesion strength, single-cell methods are needed.

Three types of single-cell force spectroscopy (SCFS) assays have been developed to measure the strength of cell adhesion down to single-molecule levels. All three assays use optical microscopes to observe the cell while force measurements are made, but differ in how cells are manipulated and forces are determined. The oldest method uses micropipettes to grasp and hold cells. The detachment force is measured using a bio-membrane force probe[2], which can gauge force between 10^{-2} pN (pico-Newtons) and 100 pN (Evans et al., 1995). A second method uses a pipette to hold a cell while the strength of interactions between the cell and a functionalized bead are determined using a laser trap. The laser trap allows three-dimensional positioning of the bead with nanometer precision and force measurement from 10^{-2} pN to 200 pN (Litvinov et al., 2002). The third method uses a cell that is attached to a cantilever of an atomic force microscope (Fig. 1). By combining atomic force microscopy (AFM) and optical microscopy, cells can be positioned to assess cellular interactions at a given location on a functionalized surface, tissue or on another cell (Benoit et al., 2000). The deflection of the cantilever is used to measure interaction forces. Among SCFS

[1]In washing assays, poorly or non-adhering tissue-culture cells are washed from a surface by running a solute (usually medium) over them. The ratio of the number of bound cells to the number of cells that are initially present provides a measure of adhesion.

[2]The bio-membrane force probe is a pressurized red blood cell. The force is measured by determining the deflection of its membrane.

Journal of Cell Science

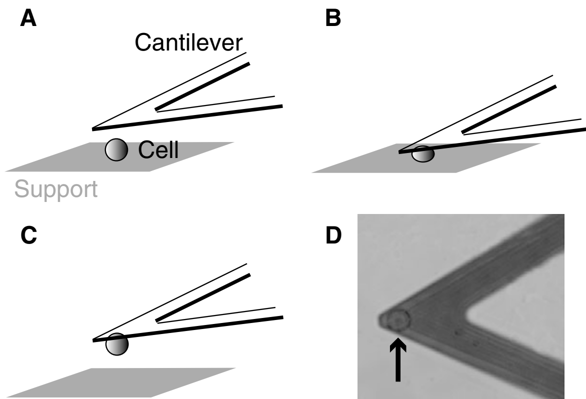

Fig. 1. Converting a cell into a probe. (A) The apex of a lectin-functionalized (often by binding concanavalin A) AFM cantilever is positioned above a cell. (B) The cantilever is gently pushed (generally with a force of <1 nN) for several seconds onto the cell. (C) The cantilever-bound cell is separated from the support and allowed to establish firm adhesion. (D) A phase-contrast image of a cell (arrow) bound to a tip-less cantilever.

approaches, the AFM-based technique allows for the widest practical force range, from 10 pN to 10^6 pN. This Commentary will be limited to the AFM-based method, which will henceforth be referred to as SCFS.

The capability of AFM to image cell topology or characterize cell-surface properties is outside the scope of this article, and readers are referred to other reviews (Radmacher, 2002; Dufrene, 2004). This Commentary will focus on the use of AFM to measure adhesion strength between a single cell and a substrate that is presented by a functionalized surface or by another cell. We will explain and demonstrate the capabilities of AFM and familiarize the reader with it benefits and limitations.

SCFS set-up and experimentation

Experimental set-up

The basic experimental AFM-type set-up for SCFS is straightforward. An atomic force microscope that is fitted with a fluid chamber allows measurements to be made in aqueous environments under controlled temperatures. Suspended cells are added to the fluid chamber and allowed to settle. Thereafter, a single cell is captured by gently pressing a functionalized AFM cantilever onto it (Fig. 1). This converts the living cell into a probe, which is brought into contact with functionalized surfaces or other cells at a set force and for a specific adhesion time. Subsequently, the cantilever is withdrawn at a constant speed, detaching the cell from its binding place. During this separation process, the cantilever deflection, which is proportional to the vertical force that exists between the cell and substrate, is recorded in a force-distance curve (Fig. 2). This curve provides the signature of the cell adhesion. The challenge, however, lies in interpreting this signature, because various specific as well as unspecific adhesion processes can occur simultaneously.

Interpreting the cell-adhesion signature

The de-adhesion of a cell from a substrate that is described by the force-distance retrace curve can be broken into three phases (Fig. 2). During the initial phase (Fig. 2Ba), the retraction of the cantilever inverts the force that is acting on the cell from pushing to pulling. As the overall pulling force increases, the force that is acting at individual cell-substrate adhesion points increases. If many receptors act together, the applied detachment force will be sufficiently high to mechanically deform the cell cortex. The binding strengths of the receptors, as well as their number and geometric placement, determines at what force the cell will start to detach. The largest adhesion force that is recorded, the detachment force (F_{detach}), represents the maximum strength of cell-substrate binding. Because detachment of the cell is a complicated process, the maximum adhesion strength represents only a useful general measure. The work that is required to detach the cell can also be used to describe the adhesion strength of the cell. It is calculated from the area that is enclosed by the retraction-force–distance curve (Fig. 2B). Here, it is important to consider that the detachment force is a composition of many different properties of the cell (Bershadsky et al., 2006). These include cell elasticity, cortex tension, membrane properties, cell geometry and receptor properties such as binding strength, cooperativity and placement.

After the cell starts to detach from the substrate, individual force steps can be observed during the second phase (Fig. 2Bb). During this phase, the receptor(s) either detaches from the substrate surface or is pulled away from the cell cortex at the tip of a membrane tether. While parts of the cell cortex are in contact with the substrate, either of these processes can occur. During the final phase of detachment (Fig. 2Bc), the cell body is no longer in contact with the substrate and, thus, attachment is mediated exclusively by tethers (Sun et al., 2005a). The force that is required to extend a tether depends on the lipid composition of the cellular membrane and on the mechanical properties of the cell cortex. Thus, the lifetime of a membrane tether is dependent on the receptor-ligand interaction at its tip, whereas the force that is required to maintain and extend a tether is not (Marcus et al., 2004). Once initiated, this force is largely independent of tether length (Hochmuth et al., 1996). In cell-cell adhesion experiments, retraction distances that approach 100 μm are required, owing to tethers, to fully separate cells (Benoit and Gaub, 2002; Puech et al., 2006; Thie et al., 1998). There is ongoing research aimed at trying to use the mathematically tractable tethers to analyze receptor anchoring (Schmitz et al., 2008). Once all of the tethers have detached from the substrate, another cycle of adhesion and detachment is started after a short cell-recovery time.

A common variation of this method is an inversion of the set-up. Here, the cantilever of the atomic force microscope is functionalized with ECM proteins and used to probe immobile tissue-culture cells (Lehenkari and Horton, 1999). The experimental set-up is flexible and varies with the biological system. Many different cells and extracellular adhesion substrates have been used. It is also possible to apply this approach to detect the molecular adhesion events of microbial surfaces (Dufrene, 2004). This set-up can also be used to map the probe-binding properties of cell surfaces. However, SCFS that uses the cell as a probe has certain advantages – most importantly, cell-cell interactions can be probed and there is more freedom in which substrates, such as ECM components, are presented to the cell.

Versatility of AFM-based SCFS

Many aspects of adhesion can be examined using AFM-based SCFS, ranging from cell-cell to single-molecule experiments, and there are no restrictions that govern which CAMs can be studied or which cells can be used. Initial experiments, which were performed a decade ago, studied cell-cell adhesion of *Escherichia*

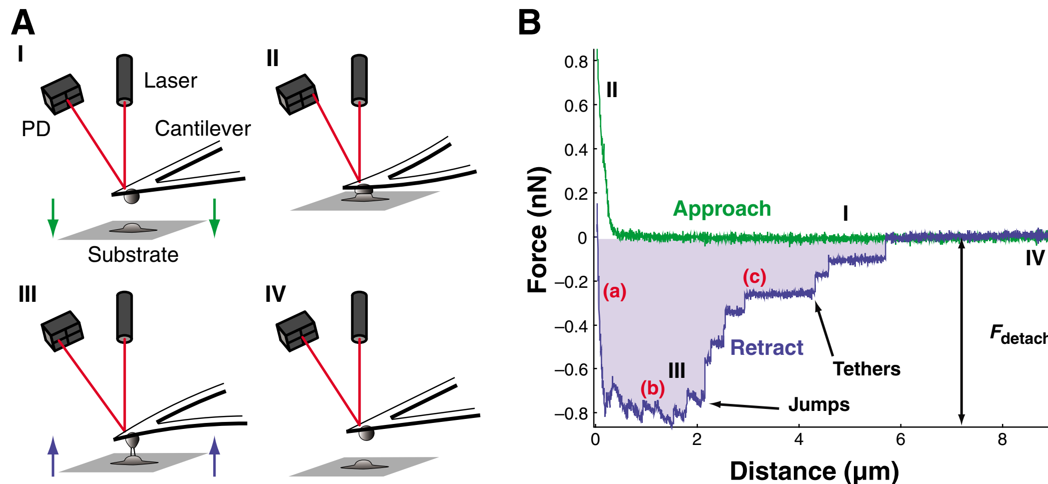

Fig. 2. Single-cell force spectroscopy. Depiction of a cell-adhesion measurement (A) for which characteristic approach (green) and retraction (blue) traces are shown (B). (A) In this technique, the cell and the substrate are brought into contact (AI). The substrate that is probed can be another cell, a functionalized surface or an organic matrix. The position on a photodiode (PD) of a laser beam (red line) that is reflected off the back of the cantilever measures the deflection of the cantilever and thus the force that acts on the cantilever. During the approach (denoted by green arrows), the cell (probe) is pressed onto the substrate until a pre-set force (usually <1 nN) is reached (AII). After a contact time ranging from 0 to 20 minutes, the cell is retracted from the substrate (marked by blue arrows), and a force-distance curve is recorded (B). This curve corresponds to a cell-adhesion signature. As the strain on the cell increases, bonds that have been formed between the substrate and the cell break sequentially (AIII) until the cell has completely separated from the surface (AIV). The maximum downward force exerted on the cantilever of the atomic force microscope is referred to as the detachment force (F_{detach}). During the separation of the cell from the surface, two types of molecular unbinding events can occur. In the first event, the receptor remains anchored in the cell cortex and unbinds as the force increases (denoted as jumps). The second type of unbinding event occurs when receptor anchoring is lost and membrane tethers are pulled out of the cell. In the unbinding-force–distance curve, long plateaus of constant force characterize tethers. The shaded area in B represents the measured work of cell detachment from the substrate. The lower-case letters (a, b and c) denote different phases of cell-substrate detachment (see text for details). Steps I-IV shown in A are also indicated in B.

coli and mammalian cells that were grown on cantilevers, but these studies were not at the single-cell level (Razatos et al., 1998; Thie et al., 1998). Shortly thereafter, measurements of the binding strength between RGD peptides (synthetic peptides that contain the RGD integrin-binding motif) and osteoclasts indicated the viability of using living cells and AFM to study single-molecule binding properties (Lehenkari and Horton, 1999). Since then, a wide variety of adhesive interactions have been studied using many types of cells. Table 1 lists the combination of single-molecule receptor-ligand interactions that have been studied using SCFS. The various cell types that have been used are also shown in the table.

Although the atomic force microscope is a high-precision force-measuring tool, it is versatile. Using piezoelectric actuators, the atomic force microscope probe can be positioned with sub-nanometer accuracy at relatively high speeds (>100 μm/second). With SCFS, cell-substrate contact times can range from milliseconds to tens of minutes. The imaging capability of the atomic force microscope can also be used to characterize the adhesion substrate at a spatial resolution that approaches 2 nm, which clearly exceeds that of light microscopy (Cisneros et al., 2006; Franz and Muller, 2005; Taubenberger et al., 2007). Thus, for example, AFM images of cells adhering to aligned collagen matrices revealed cell-induced rearrangements of individual collagen fibrils (Friedrichs et al., 2007a). In addition, AFM can be combined with most modern optical techniques, such as fluorescence-correlation spectroscopy, wide-field fluorescence, total internal-reflection fluorescence and confocal microscopy (Chiantia et al., 2007; Franz and Muller, 2005; Puech et al., 2006; Trache and Meininger, 2005). Commercial atomic force microscopes that can be integrated into standard and modern inverted and transmission optical microscopes are available[3]. In addition, the flexibility and ease of AFM-based SCFS measurements extends its use from quantitatively characterizing whole-cell adhesion down to single receptor-ligand interactions.

Examining different aspects of cell adhesion using SCFS

As one would expect, the strength of adhesion increases with the length of time a cell is allowed to adhere to a substrate or another cell. Initially, single receptor-ligand pairs anchor the cell. These quickly increase in number and undergo modifications to greatly increase the total strength of adhesion (Friedrichs et al., 2007b; Taubenberger et al., 2007; Thie et al., 1998). Thus, by simply varying the cell-substrate contact time during SCFS, both the adhesion properties of single molecules and whole cells can be quantified.

Observing the adhesion of single molecules

Since its inception (Binnig et al., 1986), AFM has been used to study molecular interactions (Butt, 1991; Ducker et al., 1991). However, it took 8 years until the first set-ups that could measure discrete interactions between single molecules were designed (Lee et al., 1994; Moy et al., 1994). Since then, this technique, termed single-molecule force microscopy (SMFS), has been applied to characterize the binding behavior of many different oligosaccharides, nucleic acids and proteins (Hansma et al., 2004; Kedrov et al., 2007; Zhuang and Rief, 2003). Receptor-ligand interactions are examined by measuring the unbinding (or rupture) forces between receptors (or ligands) that are attached to the stylus of the cantilever of the atomic force microscope and ligands (or

[3]For example, CellHesion and NanoWizardII BioAFM, JPK Instruments; BioScope II, Veeco; and MFP-3D, Asylum Research.

Journal of Cell Science

Table 1. Receptor-ligand interactions studied by SCFS using living cells as probes

Receptor	Ligand(s)	SCFS rupture force [pN]*	SMFS rupture force [pN]	Cell type	Reference
Integrin α2β1	Collagen I and IV	65 (collagen I)	Not determined	CHO	(Taubenberger et al., 2007)
Integrin α4β1	VCAM1	20	Not determined	U937	(Alon et al., 2005; Zhang et al., 2004)
Integrin α5β1	Fibronectin	60 (80), 35, 40	Not determined	Epithelial, K562	(Li et al., 2003; Sun et al., 2005b; Trache et al., 2005)
Integrin αLβ2 (LFA-1)	ICAM1	35, 40 (80), 70	Not determined	Jurkat and 3A9 HUVEC	(Thie et al., 1998; Zhang et al., 2002; Zhang et al., 2006)
Integrin αLβ2 (LFA-1)	ICAM2	40 (50)	Not determined	Jurkat	(Wojcikiewicz et al., 2006)
Integrin αVβ3	RGD peptide	42	Not determined	Bone	(Lehenkari and Horton, 1999)
E-cadherin	E-cadherin	73	25	CHO	(Panorchan et al., 2006b; du Roure et al., 2006)
N-cadherin	N-cadherin	30	Not determined	CHO	(Panorchan et al., 2006b)
VE-cadherin	†	50	45	HUVEC	(Baumgartner et al., 2000; Panorchan et al., 2006a)
PMN‡	E-selectin	140	Not determined	PMN	(Hanley et al., 2004)
PMN‡	L-selectin	80	Not determined	PMN	(Hanley et al., 2004)
PSGL-1 (SELPLG)	P-selectin	130	150	PMN and LS174T	(Fritz et al., 1998; Hanley et al., 2003)
NIH3T3 cell‡	Concavalin A	80	95	NIH3T3	(Baumgart and Offenhausser, 2003; Chen and Moy, 2000)
Surface-expressed mannose residues	Concavalin A	86	Not determined	NIH3T3	(Chen and Moy, 2000)
Saccharides from blood types A and O	Helix pomatia lectin	65	Not determined	Red blood cells	(Grandbois et al., 2000)
Galectin 3, galectin 9	Collagen I and laminin 3	Not determined	Not determined	MDCK	(Friedrichs et al., 2007b)
SGLT1 (SLC5A1)	Monosaccharides	51 (glucose)	Not determined	CHO	(Puntheeranurak et al., 2006; Puntheeranurak et al., 2007)
hbhA	Heparin	53	50	*Mycobacterium tuberculosis*	(Dupres et al., 2005)
csA	csA	20	Not determined	*Dictyostelium discoideum*	(Benoit et al., 2000)
D-Ala-D-Ala peptide terminal‡	Vancomycin	83	98	*Lactococcus lactis*	(Gilbert et al., 2007)

The unbinding forces as determined by SMFS are given when known. *The most probable rupture force of the interaction at a loading rate of 1 nN s^{-1} is given when known. Numbers in parentheses are for activated receptors. †For these homotypic binding interactions, the adhesion between two cells that express the same adhesion receptor was probed. ‡The cell-surface receptor(s) assayed is not known or is not a specific protein. Where two or more rupture forces are given, different values have been published. PMN, polymorphonuclear cell.

Journal of Cell Science

receptors) that are immobilized to a surface. Dynamic SMFS probes these rupture forces at different loading rates (that is, applied force versus time) to determine the properties of the receptor-ligand energy landscape (Evans, 1998; Evans and Calderwood, 2007). These properties typically include the free energy (ΔG) that separates the bound state from the transition state, the distance that separates the bound state from the transition state (x_u) and the lifetime of the bound state at equilibrium (k_{off}). Although these in vitro measurements provide insights into the behavior of receptor-ligand interactions, they have limitations.

For example, receptors must be purified, which means removing them from their biological context, so one cannot be certain of their functional state. This is of particular concern with integrins that are known to have several substrate-binding affinity states. Furthermore, transmembrane receptors are purified in truncated forms, and therefore consist of only extracellular domains. This is problematic because it is known that some receptors are regulated through interactions with cytoplasmic factors, such as the regulation of integrins by paxillin (Rose et al., 2007).

In contrast to SMFS, SCFS enables single receptor-ligand interactions to be examined in their cellular environment. Using a living cell as a probe ensures that the receptors are native. In Table 1, we list the unbinding forces of receptor-ligand interactions that have been measured using SCFS. Several of these receptor-ligand pairs have also been studied using SMFS on solely purified proteins. The rupture forces that are measured by SCFS and SMFS are generally in agreement, but some show considerable deviation from each other. This might indicate that the strength of receptor-ligand interactions depends on the experimental conditions. Unfortunately, experimental conditions have not been standardized and are often not sufficiently documented to allow for rigorous comparisons, i.e. experiments are performed at different temperatures or the manner in which the bond is stressed is not clear. Moreover, it is possible that, although the binding strength that is measured by the two techniques is similar, the energy barrier that is crossed to break the bond differs. The external force that is applied to a bond defines the coordinate along which the bond is forced to break. In SMFS, receptor-ligand pairs are isolated, truncated and attached to solid surfaces, whereas in SCFS the receptors are in their cellular environment. Thus, bonds might break across different paths within the energy landscape of the binding interaction. Future dynamic SCFS experiments might show to what extent the biological context of receptors influences the energy barriers that separate bound and unbound states.

SCFS is not without limitations – the fact that SCFS uses a cell as a probe can also complicate certain aspects of single-molecule measurements. In contrast to the rigid stylus of the atomic force microscope that is used in SMFS, the applied forces in SCFS cause the cell to stretch and deform. In addition, because the mechanical response of cells to deformation is not necessarily linear, dynamic SCFS measurements are not as straightforward as SMFS measurements (Evans and Calderwood, 2007). Perhaps more crucial

Journal of Cell Science

is the multitude of possible specific and unspecific cell-surface interactions. Therefore, special care must be taken to ensure that the interactions that are recorded occur predominately, if not exclusively, between the receptor and ligand of interest. To this end, purified substrates and blocked surfaces are used. Cells can also be genetically modified to limit the number of possible receptors that are expressed, and rigorous control experiments that demonstrate the specificity of the interactions observed must be performed.

The use of a living cell to study single-molecule interactions at the cell surface has proved fruitful; however, it was shown early on that SCFS could be used to measure the dynamic-force spectrum of binding interactions (Chen and Moy, 2000). Making use of the advantages of living cells, Moy and co-workers demonstrated that the activation of leukocytes induced changes in the unbinding-energy landscape of the integrin LFA-1 (ITGAL) from its ligand ICAM1 (Zhang et al., 2002). Others have since studied the changes in binding dynamics of various integrins upon activation by antibodies (Li et al., 2003; Zhang et al., 2004) and magnesium (Wojcikiewicz et al., 2006). The properties of genetically modified receptors have also been studied (Alon et al., 2005).

Studying overall cell adhesion by SCFS

Cellular process, as opposed to single molecules, can be studied by increasing the cell-substrate contact time[4]. Not surprisingly, in most SCFS studies the strength of the adhesion between two cells or a cell and a substrate increases with contact time and contact area. Generally, retraction curves show that higher detachment forces are the result of increased numbers of adhesive interactions. However, significant cell-induced deviations are seen. For example, the high early adhesion forces that occur between cells that express the surface receptor Notch and its ligand Delta diminish as the receptors are cleaved and internalized as part of the signaling pathway (Ahimou et al., 2004). By contrast, Chinese hamster ovary (CHO) cells that express integrin α2β1 and are in contact with aligned type I collagen switch to an activated adhesion state (Taubenberger et al., 2007). This probably occurs as a result of the clustering of receptors into load-sharing entities and not because of the activation of individual integrin molecules. This phenomenon was observed for integrin-α2β1-mediated binding of Madin-Darby canine kidney (MDCK) cells to both type I and type IV collagen (J. Friedrichs, A. Manninen, D.J.M. and J.H., unpublished). These papers demonstrate that SCFS can be used to study dynamic and regulated adhesion processes that occur at the cellular level.

For the assessment of adhesion processes at a level that mimics the in vivo state, isolated primary cells have been used. For example, cells that were isolated from zebrafish embryos were used to examine the importance of non-canonical Wnt signaling for cell adhesion in early development, and the authors used SCFS to determine the specific adhesion of different types of primary cells to functionalized substrates as well as to other cells (Puech et al., 2005; Ulrich et al., 2005). Moreover, using the same system, the specific contributions of cell adhesion versus cell-cortex tension to cell sorting during zebrafish gastrulation have been clarified (Krieg et al., 2008).

Force-distance curves that trace cell detachment reveal the unbinding of individual receptor-ligand interactions and, thus, binding frequency (Gilbert et al., 2007). By simultaneously measuring the contact area using light microscopy, the number of active receptors per cell-surface area can be estimated and receptor-ligand attachment rates found (Gilbert et al., 2007). If combined with other techniques, such as FACS to determine the number of particular cell-surface receptors, SCFS allows the fraction of activated and inactivated receptors to be estimated. A search for functional states or environmental conditions that tune receptors might be possible with this combination. Recent SCFS studies on the adhesion of leukemic cells to bone-marrow stromal cells showed that the myeloid leukemia fusion protein BCR-ABL increased integrin β1 expression, which, in turn, increased the strength of the adhesion between the two cell types (Fierro et al., 2008). The addition of the anti-cancer drug imatinib mesylate suppressed the integrin-β1-dependent adhesion to the level of control cells.

Current limitations and data interpretation

Current SCFS set-ups do have some limitations. Adhesion measurements that use single cells are time consuming because only one cell can be characterized at a time. For statistical reasons, many detachment-force–distance curves must be recorded, which limits the length of the contact times that can reasonably be assayed. Furthermore, the almost unavoidable thermal drift in AFM complicates long-contact-time experiments (>20 minutes) and the tight adhesion of cells after longer contact times (>1 hour) exceeds the capability of the system[5]. Thus, SCFS is currently restricted to short contact times that range from milliseconds to ~20 minutes. There is also a high cost associated with SCFS; fortunately, inexpensive atomic force microscopes that have been specifically developed for SCFS should soon become available.

As with most new techniques, SCFS needs to mature. Presently, enthusiasm to publish comes, at times, second to rigorous examination of the data. Of particular concern are: (1) the need to establish controls that demonstrate the specificity of the molecular interaction being studied; (2) the temptation to over-interpret numerical data that are gleaned from unverified mathematical models; and (3) the difficulty in appreciating the complexity of both the physics and biology of the systems studied. This situation will improve as familiarity with the technique increases and standard experimental procedures and data-analysis norms are adopted.

Perspectives

The use of SCFS is still in its infancy and has much potential for development. This potential is based on the versatility of SCFS and the enormous variety of cell biological and medical applications to which it can be applied. Here, we have demonstrated that SCFS provides a 'force signature' of the cell-adhesion process and have shown how this tool has been used to study CAMs and the dynamics of regulated adhesion processes in living cells. In practice, all possible forms of cell adhesion can be studied, with limitations that include the restriction of experiments to short contact times and the high associated cost, as discussed above. The combination of AFM with advanced light-microscopy imaging has yet to be applied to its full advantage, and studies in which force

[4]To study single-molecule unbinding, contact times from 0 to 0.5 seconds are generally used. The rate at which bonds form depends on the system that is assayed.

[5]At high force, the adhesion between the cantilever and the cell is weaker than that between the substrate and the cell. Using concanavalin A to immobilize the cell to the cantilever, forces of up to 50 nN can be measured before cells detach from the cantilever. This force is, however, dependent on cell type.

measurements are correlated with changes in cell shape and structure will emerge in the foreseeable future. The development of single-cell force spectroscopes that have standardized cell-handling and analysis routines will also provide a possibility to expand the experimental parameters that can be addressed with this innovative technique.

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References

Ahimou, F., Mok, L. P., Bardot, B. and Wesley, C. (2004). The adhesion force of Notch with Delta and the rate of Notch signaling. *J. Cell Biol.* **167**, 1217-1229.

Alon, R., Feigelson, S. W., Manevich, E., Rose, D. M., Schmitz, J., Overby, D. R., Winter, E., Grabovsky, V., Shinder, V., Matthews, B. D. et al. (2005). Alpha4beta1-dependent adhesion strengthening under mechanical strain is regulated by paxillin association with the alpha4-cytoplasmic domain. *J. Cell Biol.* **171**, 1073-1084.

Baumgart, T. and Offenhausser, A. (2003). Polysaccharide-supported planar bilayer lipid model membranes. *Langmuir* **19**, 1730-1737.

Baumgartner, W., Hinterdorfer, P., Ness, W., Raab, A., Vestweber, D., Schindler, H. and Drenckhahn, D. (2000). Cadherin interaction probed by atomic force microscopy. *Proc. Natl. Acad. Sci. USA* **97**, 4005-4010.

Benoit, M. and Gaub, H. E. (2002). Measuring cell adhesion forces with the atomic force microscope at the molecular level. *Cells Tissues Organs* **172**, 174-189.

Benoit, M., Gabriel, D., Gerisch, G. and Gaub, H. E. (2000). Discrete interactions in cell adhesion measured by single-molecule force spectroscopy. *Nat. Cell Biol.* **2**, 313-317.

Bershadsky, A., Kozlov, M. and Geiger, B. (2006). Adhesion-mediated mechanosensitivity: a time to experiment, and a time to theorize. *Curr. Opin. Cell Biol.* **18**, 472-481.

Binnig, G., Quate, C. F. and Gerber, C. (1986). Atomic force microscope. *Phys. Rev. Lett.* **56**, 930-933.

Butt, H.-J. (1991). Measuring electrostatic, van der Waals, and hydration forces in electrolyte solutions with an atomic force microscope. *Biophys. J.* **60**, 1438-1444.

Chen, A. and Moy, V. T. (2000). Cross-linking of cell surface receptors enhances cooperativity of molecular adhesion. *Biophys. J.* **78**, 2814-2820.

Chiantia, S., Kahya, N. and Schwille, P. (2007). Raft domain reorganization driven by short- and long-chain ceramide: a combined AFM and FCS study. *Langmuir* **23**, 7659-7665.

Cisneros, D. A., Hung, C., Franz, C. M. and Muller, D. J. (2006). Observing growth steps of collagen self-assembly by time-lapse high-resolution atomic force microscopy. *J. Struct. Biol.* **154**, 232-245.

Ducker, W. A., Senden, T. J. and Pashley, R. M. (1991). Direct measurements of colloidal forces using an atomic force microscope. *Nature* **353**, 239-241.

Dufrene, Y. F. (2004). Using nanotechniques to explore microbial surfaces. *Nat. Rev. Microbiol.* **6**, 451-460.

Dupres, V., Menozzi, F. D., Locht, C., Clare, B. H., Abbott, N. L., Cuenot, S., Bompard, C., Raze, D. and Dufrene, Y. F. (2005). Nanoscale mapping and functional analysis of individual adhesins on living bacteria. *Nat. Methods* **2**, 515-520.

du Roure, O., Buguin, A., Feracci, H. and Silberzan, P. (2006). Homophilic interactions between cadherin fragments at the single molecule level: an AFM study. *Langmuir* **22**, 4680-4684.

Evans, E. (1998). Energy landscapes of biomolecular adhesion and receptor anchoring at interfaces explored with dynamic force spectroscopy. *Faraday Discuss.* **111**, 1-16.

Evans, E. A. and Calderwood, D. A. (2007). Forces and bond dynamics in cell adhesion. *Science* **316**, 1148-1153.

Evans, E., Ritchie, K. and Merkel, R. (1995). Sensitive force technique to probe molecular adhesion and structural linkages at biological interfaces. *Biophys. J.* **68**, 2580-2587.

Fierro, F. A., Taubenberger, A., Puech, P.-H., Ehninger, G., Bornhauser, M., Muller, D. J. and Illmer, T. (2008). BCR/ABL expression of myeloid progenitors increases alpha1-integrin mediated adhesion to stromal cells. *J. Mol. Biol.* **377**, 1082-1093.

Franz, C. M. and Muller, D. J. (2005). Analysing focal adhesion structure by AFM. *J. Cell Sci.* **118**, 5315-5323.

Friedrichs, J., Taubenberger, A., Franz, C. M. and Muller, D. J. (2007a). Cellular remodelling of individual collagen fibrils visualized by time-lapse AFM. *J. Mol. Biol.* **372**, 594-607.

Friedrichs, J., Torkko, J. M., Helenius, J., Teravainen, T. P., Fullekrug, J., Muller, D. J., Simons, K. and Manninen, A. (2007b). Contributions of galectin-3 and -9 to epithelial cell adhesion analyzed by single cell force spectroscopy. *J. Biol. Chem.* **282**, 29375-29383.

Fritz, J., Katopodis, A. G., Kolbinger, F. and Anselmetti, D. (1998). Force-mediated kinetics of single P-selectin/ligand complexes observed by atomic force microscopy. *Proc. Natl. Acad. Sci. USA* **95**, 12283-12288.

Garcia, A. J., Ducheyne, P. and Boettiger, D. (1997). Quantification of cell adhesion using a spinning disc device and application to surface-reactive materials. *Biomaterials* **18**, 1091-1098.

Gilbert, Y., Deghorain, M., Wang, L., Xu, B., Pollheimer, P. D., Gruber, H. J., Errington, J., Hallet, B., Haulot, X., Verbelen, C. et al. (2007). Single-molecule force spectroscopy and imaging of the vancomycin/D-Ala-D-Ala interaction. *Nano Lett.* **7**, 796-801.

Grandbois, M., Dettmann, W., Benoit, M. and Gaub, H. E. (2000). Affinity imaging of red blood cells using an atomic force microscope. *J. Histochem. Cytochem.* **48**, 719-724.

Hanley, W., McCarty, O., Jadhav, S., Tseng, Y., Wirtz, D. and Konstantopoulos, K. (2003). Single molecule characterization of P-selectin/ligand binding. *J. Biol. Chem.* **278**, 10556-10561.

Hanley, W. D., Wirtz, D. and Konstantopoulos, K. (2004). Distinct kinetic and mechanical properties govern selectin-leukocyte interactions. *J. Cell Sci.* **117**, 2503-2511.

Hansma, H. G., Kasuya, K. and Oroudjev, E. (2004). Atomic force microscopy imaging and pulling of nucleic acids. *Curr. Opin. Struct. Biol.* **14**, 380-385.

Hochmuth, F. M., Shao, J. Y., Dai, J. and Sheetz, M. P. (1996). Deformation and flow of membrane into tethers extracted from neuronal growth cones. *Biophys. J.* **70**, 358-369.

Kaplanski, G., Farnarier, C., Tissot, O., Pierres, A., Benoliel, A. M., Alessi, M. C., Kaplanski, S. and Bongrand, P. (1993). Granulocyte-endothelium initial adhesion. Analysis of transient binding events mediated by E-selectin in a laminar shear flow. *Biophys. J.* **64**, 1922-1933.

Kedrov, A., Janovjak, H., Sapra, K. T. and Muller, D. J. (2007). Deciphering molecular interactions of native membrane proteins by single-molecule force spectroscopy. *Annu. Rev. Biophys. Biomol. Struct.* **36**, 233-260.

Kemler, R. (1992). Classical cadherins. *Semin. Cell Biol.* **3**, 149-155.

Klebe, R. J. (1974). Isolation of a collagen-dependent cell attachment factor. *Nature* **250**, 248-251.

Krieg, M., Arboleda, Y., Puech, P.-H., Käfer, J., Graner, F., Muller, D. J. and Heisenberg, C. P. (2008). Tensile forces govern germ layer organization during gastrulation. *Nat. Cell Biol.* **10**, 429-436.

Lee, G. U., Kidwell, D. A. and Colton, R. J. (1994). Sensing discrete streptavidin-biotin interactions with atomic force microscopy. *Langmuir* **10**, 354-357.

Lehenkari, P. P. and Horton, M. A. (1999). Single integrin molecule adhesion forces in intact cells measured by atomic force microscopy. *Biochem. Biophys. Res. Commun.* **259**, 645-650.

Li, F., Redick, S. D., Erickson, H. P. and Moy, V. T. (2003). Force measurements of the alpha5beta1 integrin-fibronectin interaction. *Biophys. J.* **84**, 1252-1262.

Litvinov, R. I., Shuman, H., Bennett, J. S. and Weisel, J. W. (2002). Binding strength and activation state of single fibrinogen-integrin pairs on living cells. *Proc. Natl. Acad. Sci. USA* **99**, 7426-7431.

Lo, S. H. (2006). Focal adhesions: what's new inside. *Dev. Biol.* **294**, 280-291.

Marcus, W. D., McEver, R. P. and Zhu, C. (2004). Forces required to initiate membrane tether extrusion from cell surface depend on cell type but not on the surface molecule. *Mech. Chem. Biosyst.* **1**, 245-251.

Morgan, M. R., Humphries, M. J. and Bass, M. D. (2007). Synergistic control of cell adhesion by integrins and syndecans. *Nat. Rev. Mol. Cell Biol.* **8**, 957-969.

Moy, V. T., Florin, E.-L. and Gaub, H. E. (1994). Intermolecular forces and energies between ligands and receptors. *Science* **266**, 257-259.

Panorchan, P., George, J. P. and Wirtz, D. (2006a). Probing intercellular interactions between vascular endothelial cadherin pairs at single-molecule resolution and in living cells. *J. Mol. Biol.* **358**, 665-674.

Panorchan, P., Thompson, M. S., Davis, K. J., Tseng, Y., Konstantopoulos, K. and Wirtz, D. (2006b). Single-molecule analysis of cadherin-mediated cell-cell adhesion. *J. Cell Sci.* **119**, 66-74.

Puech, P. H., Taubenberger, A., Ulrich, F., Krieg, M., Muller, D. J. and Heisenberg, C. P. (2005). Measuring cell adhesion forces of primary gastrulating cells from zebrafish using atomic force microscopy. *J. Cell Sci.* **118**, 4199-4206.

Puech, P. H., Poole, K., Knebel, D. and Muller, D. J. (2006). A new technical approach to quantify cell-cell adhesion forces by AFM. *Ultramicroscopy* **106**, 637-644.

Puntheeranurak, T., Wildling, L., Gruber, H. J., Kinne, R. K. and Hinterdorfer, P. (2006). Ligands on the string: single-molecule AFM studies on the interaction of antibodies and substrates with the Na^+-glucose co-transporter SGLT1 in living cells. *J. Cell Sci.* **119**, 2960-2967.

Puntheeranurak, T., Wimmer, B., Castaneda, F., Gruber, H. J., Hinterdorfer, P. and Kinne, R. K. (2007). Substrate specificity of sugar transport by rabbit SGLT1: single-molecule atomic force microscopy versus transport studies. *Biochemistry* **46**, 2797-2804.

Radmacher, M. (2002). Measuring the elastic properties of living cells by the atomic force microscope. *Methods Cell Biol.* **68**, 67-90.

Razatos, A., Ong, Y. L., Sharma, M. M. and Georgiou, G. (1998). Molecular determinants of bacterial adhesion monitored by atomic force microscopy. *Proc. Natl. Acad. Sci. USA* **95**, 11059-11064.

Rose, D. M., Alon, R. and Ginsberg, M. H. (2007). Integrin modulation and signaling in leukocyte adhesion and migration. *Immunol. Rev.* **218**, 126-134.

Schmitz, J., Benoit, M. and Gottschalk, K. (2008). The importance of the nano-environment of adhesive receptors for cell adhesion. *Biophys. J.* (in press).

Sun, M., Graham, J. S., Hegedus, B., Marga, F., Zhang, Y., Forgacs, G. and Grandbois, M. (2005a). Multiple membrane tethers probed by atomic force microscopy. *Biophys. J.* **89**, 4320-4329.

Journal of Cell Science

Sun, Z., Martinez-Lemus, L. A., Trache, A., Trzeciakowski, J. P., Davis, G. E., Pohl, U. and Meininger, G. A. (2005b). Mechanical properties of the interaction between fibronectin and alpha5beta1-integrin on vascular smooth muscle cells studied using atomic force microscopy. *Am. J. Physiol.* **289**, H2526-H2535.

Taubenberger, A., Cisneros, D. A., Friedrichs, J., Puech, P. H., Muller, D. J. and Franz, C. M. (2007). Revealing early steps of alpha2beta1 integrin-mediated adhesion to collagen type I by using single-cell force spectroscopy. *Mol. Biol. Cell* **18**, 1634-1644.

Thie, M., Rospel, R., Dettmann, W., Benoit, M., Ludwig, M., Gaub, H. E. and Denker, H. W. (1998). Interactions between trophoblast and uterine epithelium: monitoring of adhesive forces. *Hum. Reprod.* **13**, 3211-3219.

Trache, A. and Meininger, G. A. (2005). Atomic force-multi-optical imaging integrated microscope for monitoring molecular dynamics in live cells. *J. Biomed. Opt.* **10**, 064023.

Trache, A., Trzeciakowski, J. P., Gardiner, L., Sun, Z., Muthuchamy, M., Guo, M., Yuan, S. Y. and Meininger, G. A. (2005). Histamine effects on endothelial cell fibronectin interaction studied by atomic force microscopy. *Biophys. J.* **89**, 2888-2898.

Ulrich, F., Krieg, M., Schotz, E. M., Link, V., Castanon, I., Schnabel, V., Taubenberger, A., Muller, D. J., Puech, P. H. and Heisenberg, C. P. (2005). Wnt11 functions in gastrulation by controlling cell cohesion through Rab5c and E-cadherin. *Dev. Cell* **9**, 555-564.

Wojcikiewicz, E. P., Abdulreda, M. H., Zhang, X. and Moy, V. T. (2006). Force spectroscopy of LFA-1 and its ligands, ICAM-1 and ICAM-2. *Biomacromolecules.* **7**, 3188-3195.

Zhang, X., Wojcikiewicz, E. and Moy, V. T. (2002). Force spectroscopy of the leukocyte function-associated antigen-1/intercellular adhesion molecule-1 interaction. *Biophys. J.* **83**, 2270-2279.

Zhang, X., Craig, S. E., Kirby, H., Humphries, M. J. and Moy, V. T. (2004). Molecular basis for the dynamic strength of the integrin alpha4beta1/VCAM-1 interaction. *Biophys. J.* **87**, 3470-3478.

Zhang, X., Wojcikiewicz, E. P. and Moy, V. T. (2006). Dynamic adhesion of T lymphocytes to endothelial cells revealed by atomic force microscopy. *Exp. Biol. Med. Maywood* **231**, 1306-1312.

Zhuang, X. and Rief, M. (2003). Single-molecule folding. *Curr. Opin. Struct. Biol.* **13**, 88-97.