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Investigating the Dynamics of Interactions on Living Cells via Atomic Force Microscopy

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Introduction

In biology, the ability to visualize the structure and function of macromolecules has long been a central goal and microscopy has been instrumental in this pursuit, with conventional optical microscopes providing insights into protein localization. However, their spatial resolution, limited to hundreds of nanometers, falls short of resolving single-molecule localization and interaction. As a result, our understanding of protein interaction dynamics often relies on interpreting data that cannot directly capture these fine molecular events. However, in the 1980s, a new class of microscopy techniques emerged—scanning probe microscopies (SPM)—which fundamentally changed how surfaces could be examined. SPM techniques, such as the scanning tunnelling microscope (STM) (Binnig, Rohrer et al. 1982) and atomic force microscope (AFM) (Binnig, Quate et al. 1986), involve scanning a physical probe across a sample's surface to map its topography at nanometer-scale resolution. STM, introduced in 1981, utilizes tunnelling current between the probe and sample at extremely short distances to image surfaces. Five years later, AFM was developed, which measures local interaction forces between a sharp tip and a surface to reconstruct a detailed topographical image.

AFM's ability to capture high-resolution images (1-50 nm) far surpasses the diffraction limit of conventional optical microscopy, making it a powerful tool for biological research. Furthermore, AFM's versatility allows it to operate in various environments—air, vacuum, or liquid—making it particularly useful for studying biological systems under near-physiological conditions (Hansma, Elings et al. 1988, Meyer and Amer 1988, Weisenhorn, Hansma et al. 1989). This capability, combined with refinements aimed at minimizing sample damage, has made AFM a preferred technique for studying biological materials in their native state. Early AFM applications included high-resolution imaging of proteins (Arakawa, Umemura et al. 1992), lipid bilayers (Egger, Ohnesorge et al. 1990) , DNA (Weisenhorn, Egger et al. 1991), microbes (Kuznetsov, Malkin et al. 2001) and cells (Butt, Wolff et al. 1990). Additionally, by functionalizing AFM tips and surfaces with biomolecules, researchers have been able to probe the biophysical properties of molecular interactions, as demonstrated in early studies on chemical interactions and receptor-ligand interactions (Florin, Moy et al. 1994, Frisbie, Rozsnyai et al. 1994, Lee, Kidwell et al. 1994).

The main components of an AFM system include the probe, sample stage, piezoelectric scanner, optical detection system (laser beam and photodetector), and feedback system (**Figure 1**). The probe consists of a soft cantilever with an integrated sharp tip, typically made from silicon or silicon nitride, with a tip radius ranging from 1 to 100 nm for high-resolution imaging. The movement of the tip or sample is controlled by piezoelectric materials that expand or contract in response to voltage changes, allowing for precise scanning of the sample in three dimensions. As the tip interacts with the sample, the cantilever deflects, and these deflections are detected by reflecting a laser beam off the cantilever onto a position-sensitive photodetector. The photodiode voltage signal [V], which represents the cantilever deflection, is converted into actual tip deflection [m] using the system's sensitivity [nm/V], a calibration factor that correlates the voltage output from the photodiode with the cantilever movement. The deflection (d) is then used to calculate the interaction force (F [N]) through Hooke's law: F = -k.d, where k is the cantilever spring constant [N/m].

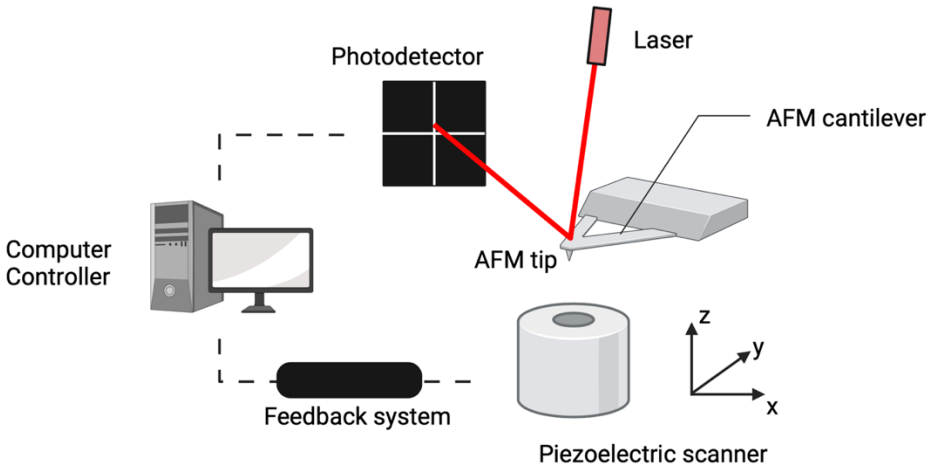

Figure 1. Main components of an AFM. The piezoelectric scanner enables precise sample movements in all three dimensions (x, y, z). The AFM cantilever with a sharp tip interacts with the sample surface. At the same time, a laser beam reflects off the back of the cantilever onto a photodiode, which detects deflections caused by surface interactions. A computer controller and feedback system regulate the scanning process and adjust the sample position depending on the setpoint.

In contact mode AFM, the tip of the cantilever makes direct contact with the sample surface as it scans. Variations in surface topography cause the cantilever to deflect, and a feedback loop maintains constant deflection by adjusting the sample's height, ensuring a consistent force between the tip and the surface. This process enables the AFM to generate a high-resolution surface map. Although contact mode is well-suited for imaging hard surfaces and achieving atomic-level resolution, it can damage softer samples due to the continuous

force applied. To mitigate this, amplitude modulation (AM) or tapping mode was developed, where the tip intermittently contacts the surface, reducing the risk of sample damage. In this mode, the cantilever oscillates at or near its resonant frequency, and the tip briefly touches the sample with each oscillation cycle. AM-AFM has become the most widely used mode for imaging biological samples in solution due to its simplicity, stability, and minimal disturbance to delicate structures. It provides high-resolution images without causing significant deformation, making it ideal for investigating soft biomolecules and living cells. Additionally, other non-contact modes, such as frequency modulation (FM-AFM), have been introduced, further enhancing AFM's sensitivity in detecting nanoscale surface interactions (for further details please refer to (Muller and Dufrêne 2008)).

With ongoing advancements, AFM has become an essential tool in biophysics and molecular biology, offering unparalleled insights into the nanomechanical properties of living cells and the molecular interactions that govern cellular functions. This technique has been particularly valuable in studying how membrane receptors mediate various biological responses. This review shows the key developments that have enabled the use of AFM to probe mammalian cells under near-physiological conditions. Through this lens, I will showcase the studies where AFM has been applied to investigate the biophysical properties of cell surfaces, focusing on how single-molecules and viruses interact with cell membrane receptors. These studies not only demonstrate the versatility of AFM in characterizing complex biological systems but also reveal how the technique can provide critical insights into the molecular mechanisms driving cell surface dynamics and interactions. By capturing these interactions in real-time and under conditions closely mimicking those in living organisms, our research has contributed to a deeper understanding of cellular responses at the nanoscale.

AFM as a sensor for probing and mapping single-molecule interactions

Originally developed as an imaging tool, AFM soon showed potential for broader applications. This led to the development of the force spectroscopy mode. This mode allows force measurements at the single-molecule level with piconewton (pN) sensitivity by successively advancing and retracting the tip (**Figure 2A**). In AFM force spectroscopy, the force exerted by or experienced by the AFM tip is recorded as a function of time (force-time, FT) (**Figure 2B**) or tip-sample distance (force-distance, FD) (**Figure 2C**), enabling the study of various (bio)physical properties. The recording of force curves begins with the AFM cantilever

positioned far from the sample (**Figure 2**, label 1), where no interaction occurs, and the measured force is zero (baseline). As the cantilever approaches the sample, the tip moves closer until it contacts the surface (**Figure 2**, label 2), causing an upward deflection of the cantilever. The tip is then pushed further into the sample, reaching a predefined force setpoint (**Figure 2**, label 3), allowing the analysis of the mechanical properties of the sample, such as elasticity, stiffness, and deformation. At this point, the approach stops, and the tip begins to retract. During retraction, the force gradually decreases as the tip moves away from the sample. If a molecular interaction is present, this can cause the cantilever to bend downward (**Figure 2**, label 4), leading to a characteristic signal in the force curve. After the interaction breaks, the cantilever jumps to zero-force (**Figure 2**, label 5). If no interaction occurs, the signal follows the baseline, with the tip returning directly to its original position. In addition to single-point measurements, AFM can perform force mapping, where an array of force curves is collected across a defined area of the sample. For each pixel of the map, the AFM tip undergoes an approach-retraction cycle, either using a linear (**Figure 3A**) or oscillating (**Figure 3B**) movement. This extension of force spectroscopy allows for detailed spatial mapping of mechanical, biological and biophysical properties across the sample surface.

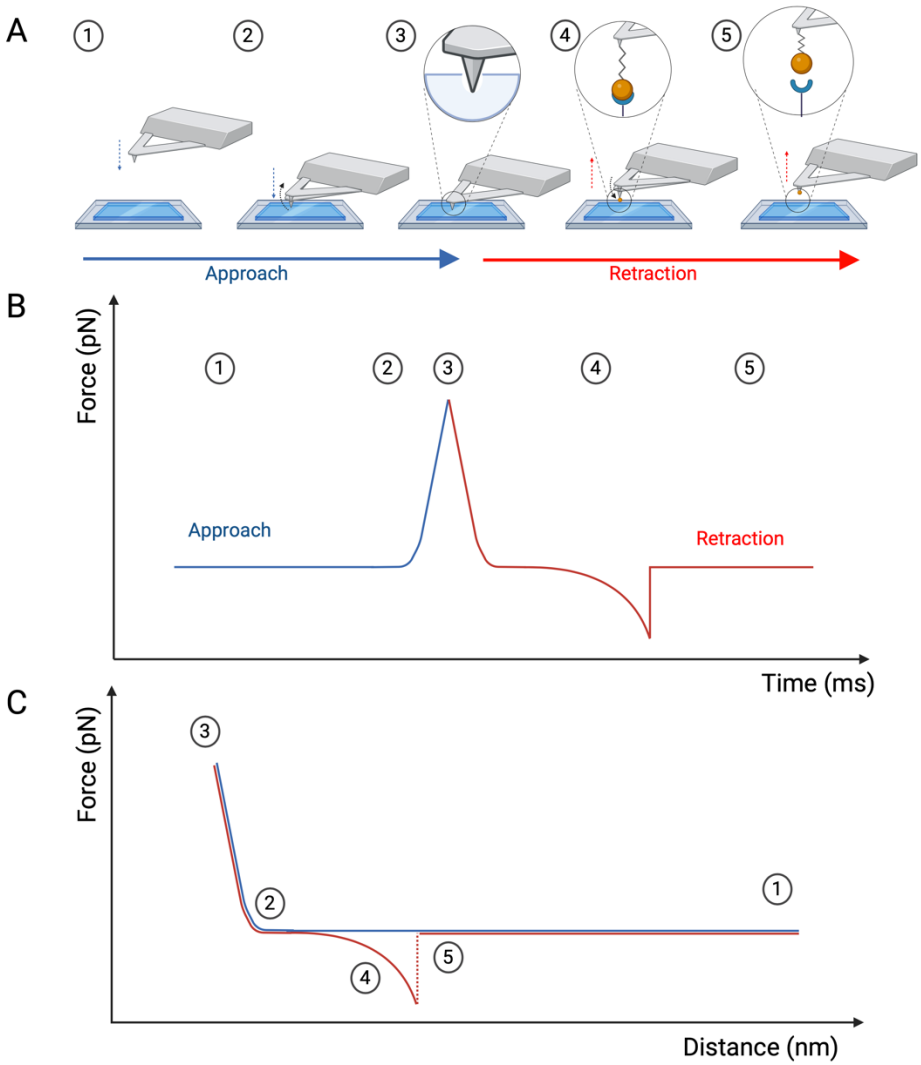

Figure 2. AFM force spectroscopy overview. (A) Schematic representation of the AFM tip's approach and retraction from the sample surface, highlighting the key interaction stages. (B) Force-time (FT) curve illustrating the force exerted by the AFM tip over time during the approach and retraction phases, with numbered labels corresponding to the stages in panel A. (C) Force-distance (FD) curve displaying the same interaction, where the force is plotted against the tip-sample distance. As the cantilever approaches the sample (label 1), the force remains at baseline. Upon contact (label 2), upward deflection occurs, followed by a predefined force setpoint (label 3) to assess the sample's mechanical properties. During retraction (label 4), if an interaction is present, downward bending of the cantilever is observed, leading to a characteristic signal until the interaction breaks (label 5). If no interaction occurs, the force returns directly to baseline.

A wide range of AFM tip functionalization strategies have been developed over the years to enhance measurement specificity (**Figure 3C**) (Riener, Kienberger et al. 2003, Hinterdorfer and Dufrêne 2006, Ebner, Wildling et al. 2007, Wildling, Unterauer et al. 2011, Koehler, Lo Giudice et al. 2020). Chemical groups or biomolecules are often chemically attached to the tip via short linkers, while the rest of the tip apex is blocked using spacer molecules to reduce non-specific interactions. However, with high ligand coverage, there is a risk of probing multiple binding events, which can also lead to increased steric hindrance that interferes with

the binding process. To manage ligand density, the typical molecule-to-spacer ratio is maintained very low (1:100 to 1:1000) (Müller, Dumitru et al. 2020). Chemical force microscopy (CFM) is widely used to probe the chemically heterogeneous surface of samples by mapping surface properties like hydrophobicity. In this case, gold tips are modified with self-assembled monolayers of alkanethiols terminated with specific functional groups (e.g., hydroxyl or methyl terminals) (Figure 3C, *left*) (Frisbie, Rozsnyai et al. 1994, Noy, Frisbie et al. 1995, Alsteens, Dague et al. 2007). While rigid and short linkers may limit biomolecular conformational mobility, potentially reducing the effectiveness of interactions between binding partners, long and flexible linkers like poly(ethylene glycol) (PEG) offer greater mobility and orientational freedom, improving molecular recognition. Linkers with lengths of around 6-10 nm (18-27 ethylene glycol units) provide an optimal balance between flexibility and lateral resolution (Figure 3C, *right*) (Riener, Kienberger et al. 2003). Additionally, during tip retraction, PEG linkers exhibit characteristic stretching behavior, which serves as a molecular fingerprint for identifying specific unbinding or unfolding events. This approach is preferable to probe the interaction between biomolecules.

Controlling the orientation of grafted ligands on the AFM tip is crucial for many studies involving single-molecule force spectroscopy (SMFS). Methods that use covalent bonding via randomly distributed amino acid residues often result in an uncontrolled orientation of the molecules on the AFM tip (Ott, Jobst et al. 2017). To address this, site-specific attachment strategies have been developed, such as using the affinity between tetravalent nickel and histidine to immobilize His_6-tagged proteins on AFM tips (Kienberger, Kada et al. 2000, Verbelen, Gruber et al. 2007) (Figure 3C). Additionally, proteins with a free cysteine or thiol-modified oligonucleotides can be immobilized at low concentrations using thiol-maleimide chemistry (Figure 3C), with tris(2-carboxyethyl)phosphine (TCEP) used to reduce disulfides to reactive sulfhydryl groups. As free cysteines are rare, they readily react with maleimide and bare-gold surfaces. Alternatively, free amines present on proteins or viruses (leading to the single-virus force spectroscopy (SVFS) mode) can be used to covalently attached the ligand in an unoriented manner. To this end heterobifunctional linker bearing a N-hydroxysuccinimide on one side and an aldehyde group on the other side a frequently used. Aldehyde functions are particularly useful for covalently coupling of proteins, as they react with amino groups (-NH2), which are present in most proteins, including virus surface proteins. This aldehyde function, in

combination with the NHS function present in this linker, may result in loop formation between adjacent NH2 groups. However, coupling of amino groups to aldehydes is much slower than to NHS esters; NH2 functions on the tip are rapidly consumed by reaction with NHS esters, allowing most aldehyde functions to be free for coupling of proteins (Wildling, Unterauer et al. 2011). Other advanced strategies include enzyme-assisted methods using sortase A transpeptidase from S. aureus, which recognizes the LPXTG motif at the C-terminus of the target protein and ligates it to a second protein with an N-terminal oligo G motif (Theile, Witte et al. 2013, Dumitru, Conrard et al. 2018), and copper(I)-catalyzed azide-alkyne cycloaddition (Chen, Ning et al. 2009). Both approaches enable site-specific tethering of biomolecules with controlled spatial orientation.

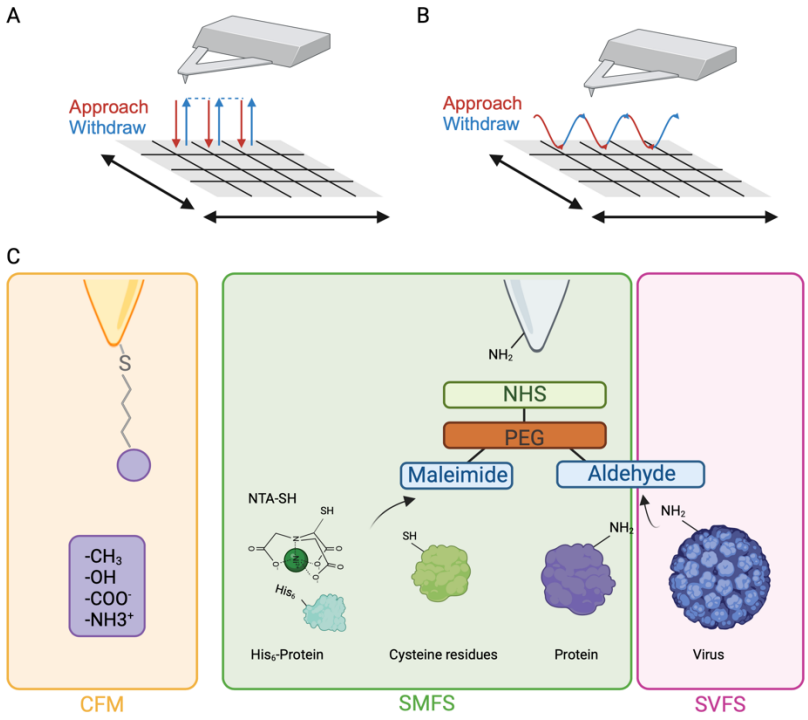

Figure 3. Principle of AFM mapping and tip functionalization strategies. (A-B) Schematic representation of AFM force mapping, where the AFM tip undergoes approach-retraction cycles for each pixel across the sample surface. The tip movement can follow either a linear (rectangular) path (A) or an oscillating (sinusoidal) waveform (B). (C) Various AFM tip functionalization strategies are shown. Left: Chemical functionalization of tips for chemical force microscopy, such as gold tips modified with self-assembled monolayers of alkanethiols. Right: Biomolecule attachment using flexible linkers like poly(ethylene glycol) (PEG), offering improved mobility for molecular interactions.

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Model surfaces, such as gold-coated or mica substrates, provide a simplified platform for studying ligand-receptor interactions, improving the throughput of SMFS measurements (Li

2014). For gold surfaces, His_6-tagged proteins can be immobilized using NTA-terminated and PEG-terminated alkanethiols, followed by Ni^{2+} ions to bind the His_6 tag. This ensures proper receptor orientation, keeping binding sites accessible. However, because non-covalent bonds are formed, the complexes can break under low forces, limiting suitability for high-force experiments (Kienberger, Kada et al. 2000, Wasserberg, Cabanas-Danés et al. 2017). Covalent attachment using NHS/EDC chemistry offers an alternative. Here, thiols interact with the gold surface, and activated COOH groups bind proteins via amino groups. However, multiple lysine residues may interact with the surface, reducing control over molecular orientation (Fischer 2010). While model surfaces simplify interactions, they may not fully reflect receptor behavior on living cells. For instance, for membrane receptors, the surrounding lipid environment plays a crucial role in their structure and function. Thus, strategies that maintain a more physiological context can be preferable for studying receptor-ligand interactions. Approaches such as reconstituting the protein in lipid membranes (Alsteens, Pfreundschuh et al. 2015) or nanodiscs (Zocher, Roos et al. 2012) help preserve native-like conditions by providing a lipid bilayer that mimics the cellular membrane. Cell surfaces are more complex, consisting of various lipids and proteins.

From chemical to biological characterization

Over the last decades, AFM force spectroscopy has become a powerful technique for investigating various samples' chemical and biological properties with nanometer-scale precision. While hydrophobic forces were historically studied using the surface force apparatus, AFM has made it possible to examine these interactions at much higher spatial resolution (~10 nm). This has enabled researchers to probe the hydrophobic characteristics of a wide range of surfaces, including colloids, oligopeptides, and living cells (Yoon, Flinn et al. 1997, Nguyen, Nalaskowski et al. 2003, Alsteens, Dague et al. 2007, Beaussart, El-Kirat-Chatel et al. 2014). Functionalizing AFM tips with methyl-terminated monolayers allows short-range hydrophobic forces to be selectively measured while minimizing the influence of longer-range forces like electrostatic interactions. Recent advancements, such as multifrequency AFM (MF-AFM), have enabled detailed mapping of the protein-water interface and sub-molecular structures on membrane proteins like aquaporin-0 (AQP0). This approach revealed a higher affinity of water for the region surrounding AQP0 tetramers, while also providing sub-nanometer resolution of both the interfacial water and the protein itself, offering insights into its lattice arrangement

and mechanical properties (Ricci, Quinlan et al. 2017). Beyond hydrophobicity, AFM has also been used to investigate electrostatic forces, employing bare or functionalized tips to detect charge variations on biological membranes with sub-nanometer accuracy. For example, FD curve-based AFM has provided insights into the electrostatic field and potential of pore-forming proteins such as OmpF, allowing researchers to quantify how these fields guide charged molecules and ions through transmembrane pores, significantly advancing our understanding of their function in their native state (Pfreundschuh, Hensen et al. 2013).

AFM force spectroscopy also emerged as a transformative tool in the investigation of molecular interactions, particularly receptor-ligand binding dynamics. Pioneering applications of AFM have included the direct measurement of forces between complementary strands of DNA (Lee, Chrisey et al. 1994), as well as mechanical investigations of protein structures through stretching and unfolding (Rief, Gautel et al. 1997). These foundational studies have paved the way for a broader exploration of molecular interactions at the nanoscale. In addition to its capabilities as a nanoscopic imaging tool and force spectroscopy instrument, AFM has evolved to enable simultaneous imaging and quantification of biomolecular interactions. Initial work on streptavidin-patterned surfaces using a biotinylated tip has been expanded to include living red blood cells (Ludwig, Dettmann et al. 1997, Grandbois, Dettmann et al. 2000), highlighting AFM's capacity to provide insights into complex biological systems and demonstrating the concept of "affinity imaging", which allows for the identification and localization of specific biological interactions. Following this, force mapping became a critical technique, applied extensively across various bacterial and mammalian cells. Concurrently, the development of dynamic recognition modes has led to improvements in both time and spatial resolution, enhancing our understanding of molecular interactions. Enhanced force-distance (FD) curve methodologies and the coupling of AFM with optical microscopy techniques—ranging from epifluorescence to super-resolution—have further amplified AFM's capabilities. The complementary information gleaned from these dual techniques provides unparalleled insights into the relationship between supramolecular bond formation and biological functionality.

Recent innovations in AFM tip manipulation have also contributed to its versatility. For instance, Koehler et al. introduced photocleavable linkers to control ligand-binding specificity in AFM force spectroscopy experiments, allowing for the precise delivery of single molecules

with high spatiotemporal resolution(Koehler, Lo Giudice et al. 2020). Fluidic Force Microscopy (Fluid-FM) technology has further expanded AFM's capabilities, enabling the trapping and release of beads, cells, and other biomolecules via pressure-controlled nanopipettes, thus allowing for the localization of specific adhesion events at resolutions as fine as ~10 nm on cellular surfaces(Meister, Gabi et al. 2009).

Given these advances, it is not surprising that AFM has gained importance in medical studies, providing valuable information that enhances our understanding of health, disease and treatment efficacy. For example, a study shown that degradation of the cellular glycocalyx in diabetes correlates with disease progression and endothelial cell stiffening has been observed in mouse models (Targosz-Korecka, Jaglarz et al. 2017). Similarly, AFM has been used in cancer therapies to assess how drugs like taxanes increase cancer cell rigidity by targeting microtubules(Rotsch and Radmacher 2000), while non-cytoskeletal drugs, such as chitosan, indirectly alter cancer cell mechanics by affecting glycolysis (Lekka, Laidler et al. 2001). This ability to measure drug-induced changes at the nanoscale highlights AFM's critical role in evaluating treatment effects on cellular behavior.

The following sections delve into the application of AFM force spectroscopy for probing interactions between individual ligands and receptors, both in reconstituted systems and directly on living cells. This exploration includes how AFM has been utilized to study virus-host interactions, providing valuable insights into viral infectivity and receptor recognition. Additionally, probing cell surface receptors using AFM can be linked to physiological responses in living cells, offering a deeper understanding of the functional consequences of these molecular interactions.

AFM as a sensor to probe receptor-ligand interactions

In terms of quantitative measurements, AFM-based single-molecule force spectroscopy (SMFS) has shown that the rupture forces of biomolecular complexes, such as antibody-antigen (Hinterdorfer, Baumgartner et al. 1996) interactions or DNA strand separations (Lee, Chrisey et al. 1994), typically range from 20-150 pN, depending on the sample and experimental conditions (Allen, Chen et al. 1997). The streptavidin-biotin bond, previously considered to be the strongest receptor-ligand interaction, exhibits break forces of approximately 200 pN (Merkel, Nassoy et al. 1999). However, recent research has shown that staphylococcal adhesins

can bind their ligands with forces approaching ~2000 pN, a force range comparable to that required to break covalent bonds (Herman, El-Kirat-Chatel et al. 2014). This finding highlights the evolutionary adaptations of bacterial pathogens that enable robust attachment to protein-coated biomaterials and host cells.

Moreover, the use of biochemical probes, combined with technological advancements in AFM force-mapping, now allows for the localization of specific binding sites with remarkable precision, achieving resolutions of 1 nm or better. For instance, AFM with Ni^{2+}-NTA functionalized tips has successfully imaged proteins and located interaction sites, such as histidine tags, on various biomolecules, including spindle assembly abnormal protein 6 homologue (SAS-6) (Pfreundschuh, Alsteens et al. 2014). Such strategies have facilitated the collection of multiparametric images that characterize the structure, adhesion, and elasticity of *E. coli* infected by his-tagged bacteriophages (Alsteens, Trabelsi et al. 2013), revealing viral assembly machinery within soft nanodomains. By applying a sinusoidal motion to the AFM tip, the ligand-receptor bond is ruptured at various loading rates, providing data to reconstruct the free-energy landscape. This approach was first used on the protease-activated receptor 1 (PAR1), a GPCR involved in coagulation and inflammation (Alsteens, Pfreundschuh et al. 2015). Thrombin cleaves PAR1's N-terminal, exposing the tethered thrombin receptor-activating peptide (TRAP), which activates the receptor. Tethering the SFLLRN sequence to the cantilever tip enabled to mimic TRAP activation, allowing to extract the specific interaction with PAR1. The bond rupture forces ranged from 40-150 pN, with a dissociation constant (Kd) of ~350 nM, in agreement with functional assays. Changes in ligand sequence, such as SFLLAN and SALLRN, significantly reduced binding affinity, highlighting key amino acids for high-affinity interactions.

Building on these advances, the multiplex capabilities of FD-based AFM were applied to investigate the dynamic binding of glucagon to the glucagon receptor (GCGR), a class B GPCR. While static structural methods like X-ray crystallography and cryo-EM have provided valuable snapshots of GCGR in its inactive and active states, they fall short in capturing the dynamic conformational changes that occur during receptor activation. FD-based AFM filled this gap, revealing new details about the stepwise "dock and lock" mechanism of GCGR activation. In this mechanism, glucagon initially binds to the extracellular domain (ECD) of the receptor, triggering a conformational transition that eventually stabilizes further interactions with the transmembrane domain (TMD), leading to full activation of the receptor. By analyzing receptor

height and stiffness during ligand binding, FD-based AFM provided direct evidence of these conformational transitions. The experiments showed two distinct receptor populations corresponding to inactive and active GCGR states, with glucagon binding driving the transition to the active form. Interestingly, the stepwise nature of this process was reflected in the time-resolved AFM mapping, which revealed progressive stiffening of the receptor-ligand complex over time. This stiffening is thought to result from mechanical coupling between the ECD and TMD, facilitating structural rearrangements necessary for G-protein recruitment and intracellular signaling. These findings highlight the importance of mechanical forces in GPCR signal transduction, providing novel insights into how biochemical ligand binding translates into mechanical changes that drive receptor activation.

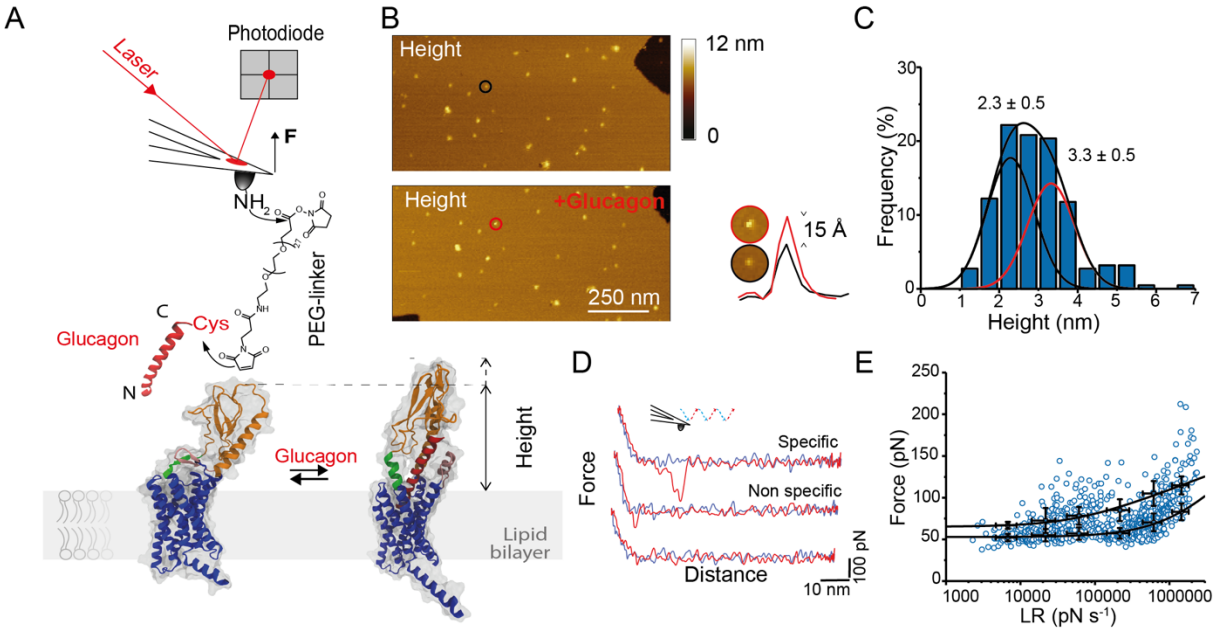

Figure 4. Dynamic binding of glucagon to the glucagon receptor (GCGR) investigated using force-distance (FD)-based AFM. (A) Schematic of the AFM tip functionalization with glucagon, covalently immobilized via a C-terminal cysteine using a heterobifunctional linker. (B) Topographical overview of apo-GCGRs before (top) and after (bottom) 30 minutes of incubation with glucagon. Zoomed topographs and cross-sections of GCGRs extracted from black (inactive) and red (active) circles show a clear increase in receptor height upon glucagon binding. (C) Height distribution of glucagon-GCGR complexes acquired in situ with glucagon-functionalized AFM tips, highlighting the transition between inactive and active receptor states. (D) Representative force-distance curves showing specific binding, non-specific interactions, and no adhesion events. (E) Force vs. loading rate plots for glucagon-GCGR interactions (n = 832), with Friddle-Noy-De Yoreo fits (dashed lines) reconstructing the ligand-receptor binding free energy landscape for the two distinct binding states. These data reveal stepwise receptor activation and the role of mechanical forces in GCGR signaling. Adapeted from (Lo Giudice, Zhang et al. 2020).

While FD-based AFM has been instrumental in revealing the mechanical underpinnings of GPCR activation, its utility extends beyond membrane receptors to intracellular processes, such as

DNA-protein interactions. SMFS has proven highly effective in probing the dynamic assembly of transposon complexes at the single-molecule level by measuring the binding strength (rupture force) between wild-type TnpA, as well as its hyperactive mutants TnpA3X and TnpAS911R, and DNA substrates (Fernandez, Shkumatov et al. 2023). This approach allowed for the detailed characterization of the kinetics and thermodynamics governing the transition from the single-end complex (SEC) to the paired-end complex (PEC), providing quantitative insights into conformational changes that were previously inaccessible via conventional biochemical methods. The ability of AFM to resolve such dynamic molecular events complements its application in receptor studies, illustrating its broader potential to illuminate complex intracellular mechanisms.

AFM as a sensor to probe interactions on mammalian cells

The exploration of mammalian cells under physiologically relevant conditions has also accelerated. Researchers have utilized AFM tips functionalized with various probes to study cellular nano-organization and adhesion phenomena. For example, the investigation of glycosylphosphatidylinositol (GPI) domains in neurons has provided insights into cellular stiffness (Roduit, van der Goot et al. 2008). In this study, AFM in force-volume imaging mode, combined with tips coated with a GPI-anchor protein-specific binding bacterial toxin aerolysin from *Aeromonas hydrophila*, was used to explore living cell surfaces. When the aerolysin molecules interacted with proteins in the membrane, the cantilever exhibited binding-unbinding events, indicative of the presence of GPI domains. By analyzing the force-distance curves and fitting the indentation data to the Hertz model, the local mechanical properties of these domains were measured. These measurements revealed that GPI domains are approximately 30% stiffer than surrounding regions of the membrane, and the domain size was estimated to be less than 70 nm. The stiffness of these domains was shown to depend on cholesterol, as treatment with methyl-β-cyclodextrin (MβCD), which depletes cholesterol, reduced the stiffness of GPI domains to match that of the surrounding membrane, further confirming the method's accuracy. Additionally, control experiments with mutant aerolysin and specific antibodies confirmed the specificity of these interactions. This work highlights the capability of AFM not only to probe the mechanical properties of cell membranes but also to study the biophysical behaviors of membrane microdomains in living cells, opening new avenues for understanding membrane-associated processes, including cell signaling and

adhesion mechanisms. Furthermore, this approach offers a valuable tool for studying membrane-related dynamics in various physiological and pathological contexts, such as cancer or neurodegenerative diseases.

Recent developments have enabled to address the study at high-resolution of the localization of specific lipids within membranes through the development of functionalized AFM probes. For instance, AFM tips functionalized with toxin fragments (θ -D4 and Lysenin derivatives) were developed to specifically target cholesterol (Chol) and sphingomyelin (SM) domains within DOPC lipid bilayers (Dumitru, Conrard et al. 2018). This force-distance curve-based AFM technique allowed for direct and label-free identification of lipid-enriched domains, demonstrating the preferential localization of specific lipids. Combination with mechanical mapping provided additional evidence of lateral heterogeneities in lipid membranes, highlighting variations in Young's modulus. This technique was then applied to breast cancer cells to investigate the role of cholesterol in membrane mechanics during cancer progression (Dumitru, Mohammed et al. 2020). θ -toxin derivatized AFM tips were used to study the plasma membrane cholesterol distribution in three MCF10 cell lines representing different stages of breast cancer progression: MCF10A (benign), MCF10AT (premalignant), and MCF10CA1a (malignant). Mechanical analysis revealed a significant softening of cells with increasing malignancy, accompanied by a stiffening of the plasma membrane, which was attributed to an increase in cholesterol content at the external leaflet of the membrane. This study provided the first *in situ*, label-free quantification of cholesterol's contribution to membrane stiffness on living cells, demonstrating how cholesterol enrichment enhances These findings open new avenues for therapeutic strategies targeting cholesterol metabolism in cancer treatment.

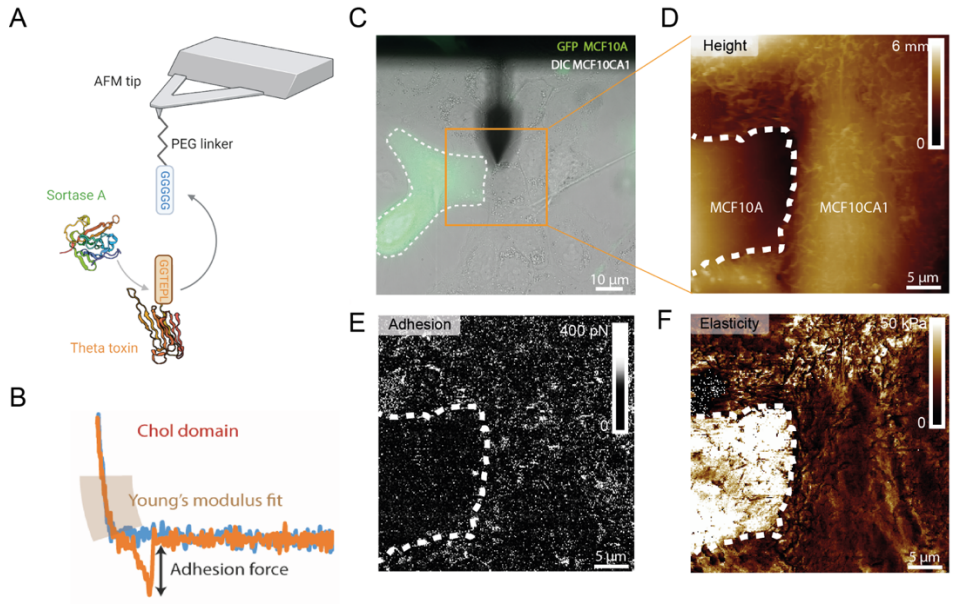

Figure 5. Probing cholesterol-enriched domains in breast cancer cells using FD-AFM with θ -toxin functionalized tips. (A) Schematic of AFM tip functionalization with the θ -toxin via a sortase A-mediated reaction. (B) θ -toxin functionalized AFM tip probing plasma membrane cholesterol. Specific unbinding events (F > 100 pN) indicate cholesterol-enriched domains, while areas lacking cholesterol show minimal adhesion (F < 30 pN). (C-F) Co-cultured MCF10A (benign, GFP-labeled) and MCF10CA1a (malignant, unlabeled) cells. (D) FD-AFM height image, (E) adhesion map showing higher cholesterol in MCF10CA1a, and (F) Young's modulus map showing higher stiffness in MCF10A. Adapted from (Dumitru, Mohammed et al. 2020)

AFM as a sensor to probe virus-host interactions

AFM has proven to be a powerful tool for studying virus-host interactions at the molecular level. Over twenty years ago, AFM force spectroscopy revolutionized the investigation of ligand-receptor bonds, opening new possibilities for probing virus binding to cell membranes. In this context, AFM has been employed to study the interaction of vesicular stomatitis virus (VSV) with membranes of varying phospholipid compositions, providing key insights into viral attachment mechanisms. In an early study, VSV was non-specifically adsorbed onto an AFM tip, and its interactions with membranes composed of phosphatidylcholine and phosphatidylserine (PC-PS, 3:1), phosphatidylcholine alone (PC), and phosphatidylcholine with cardiolipin (PC-CL, 3:1) were assessed (Carneiro, Bianconi et al. 2002). AFM approach-retraction cycles revealed strong adhesion forces between VSV and both PC-PS and PC-CL membranes, averaging 690 pN and 1,500 pN, respectively, suggesting that negatively charged phospholipids facilitate viral attachment. In contrast, little to no interaction was observed between VSV and PC-only membranes, underscoring the crucial role of membrane composition

in viral binding. A few years later, the same team of researchers revealed a high specificity of the G protein for membranes containing PS (Carneiro, Lapido-Loureiro et al. 2006). These findings highlight AFM's versatility in directly measuring virus-membrane interactions, paving the way for a deeper understanding of viral attachment specificity at the nanoscale.

Building on advancements of advanced functionalization protocols, covalent attachment of individual viruses to AFM tips through chemical linkers enabled more precise and reliable probing of virus-receptor interactions. This approach allows for the controlled study of viral binding dynamics, not only with purified receptors but also directly on the surface of living mammalian cells. For example, human rhinovirus (HRV) was immobilized on an AFM cantilever via a flexible polyethylene glycol linker, and its interaction with LDL receptors was analyzed (Rankl, Kienberger et al. 2008). By measuring the unbinding forces between the virus and receptor molecules, Rankl *et al.* could quantify receptor-virus binding events and estimate binding kinetic rates. The real-time nature of these single-molecule experiments allows for dynamic observation of molecular interactions, capturing rare events that are often missed by conventional ensemble techniques. These methods revealed that virus binding is dependent on multiple receptor interactions, and the distribution of rupture forces followed a multimodal pattern, indicating the involvement of several receptors in virus attachment. Additionally, binding kinetic on- and off-rates were determined, providing valuable insights into the virus-receptor binding kinetics that underpin viral entry mechanisms. This innovative use of AFM has expanded our understanding of the early stages of viral infection and the molecular forces driving virus attachment to host cells.

The next key development in studying virus-cell interactions involved combining AFM with confocal microscopy and an environmental chamber to maintain mammalian cells in physiological conditions for long-term experiments, by controlling temperature, relative humidity and gas composition (Alsteens, Newton et al. 2016) (Figure 6A). This system integrates the high-resolution capabilities of scanning probe microscopy with the broad imaging advantages of light microscopy, offering a powerful method for investigating dynamic virus-receptor interactions. Through a process called force mapping, AFM tip measurements are taken at multiple points on the cell surface, generating high-resolution spatial adhesion maps. These maps translate the thermodynamic properties of virus-receptor interactions into detailed images, enabling the observation of the mechanics of viral entry.

Rabies viruses pseudotyped with EnvA proteins, covalently attached to the AFM tip, were probed towards MDCK cells expressing the TVA receptor fused to fluorescent proteins (Figure 6B-E). The combined AFM and confocal microscopy approach allowed detection of precise EnvA–TVA contacts in real time, revealing that the virus rapidly forms multiple binding interactions after initial contact (Figure 6B), suggesting a cooperative binding mechanism. This technique provides not only a deeper understanding of virus entry mechanisms but also enables quantitative analysis of viral binding kinetics directly on living cells. It also opens new possibilities for studying how receptor clustering and lateral organization impact virus binding and how multivalency multiple simultaneous receptor-virus interactions—plays a role in viral attachment stability. In this context, the role of multivalent binding during the early attachment phase of herpesvirus was investigated (Delguste, Zeippen et al. 2018). While multiple parallel interactions would allow strong virus attachment, fewer bonds could be favored to enable lateral diffusion towards specific receptors and facilitate the efficient release of progeny virions. However, the molecular mechanisms governing the regulation of multivalency in virus attachment remain poorly understood. Using AFM, multivalent attachment of a single herpesvirus was probed at the single-virion level, providing the first direct evidence of how a herpesvirus surface glycoprotein acts as a negative regulator in the initial binding step. The experiments demonstrated that gp150, a herpesvirus glycoprotein, limits the number of interactions with cell surface glycosaminoglycans, regulating both virus attachment and release. This mechanism highlights the balance between attachment strength and mobility, where too many bonds could impede viral entry by spatially confining the virus, while fewer bonds enable dynamic movement across the cell surface.

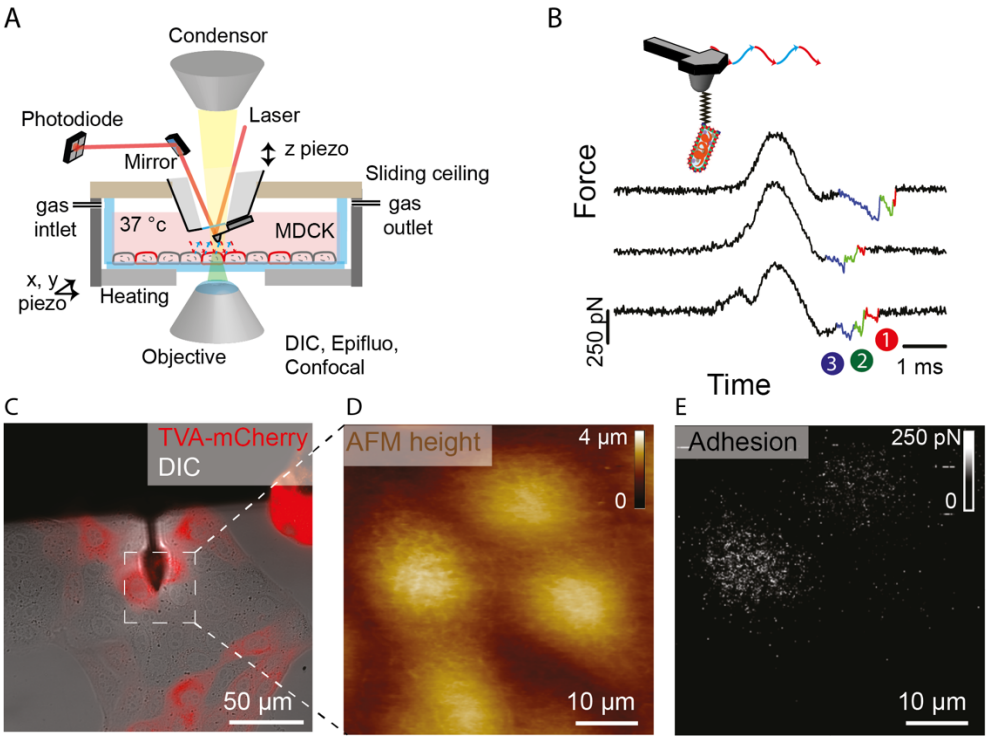

Figure 6. Integration of AFM with confocal microscopy for studying virus-cell interactions under physiological conditions. (**A**) Schematic of the experimental setup combining AFM with optical microscopy in a temperature- and gas-controlled environmental chamber. (**B**) Force-time curves recorded during the interaction between rabies viruses pseudotyped with EnvA proteins (attached to the AFM tip) and MDCK cells expressing TVA receptors. The graph highlights multiple rupture events (blue, green, and red), indicative of cooperative virus-receptor binding interactions. (**C**) Imaging of mixed cultures of wild-type MDCK cells and MDCK–TVA cells expressing fluorescent proteins (red). (**D**) FD-based AFM height image of cells in the dashed region shown in panel C, displaying the surface topography of the cells. (**E**) Corresponding adhesion map, generated through force mapping, showing the spatial distribution of virus-cell binding events. Adapted from (Alsteens, Newton et al. 2016)

Initial interactions of viruses with surface glycans can, on the opposite, activate binding to entry receptors. For example, the binding steps of reovirus were explored using AFM, revealing that the interaction of its σ 1 protein with sialic acids (α -SA) induces a conformational shift toward an extended form, increasing avidity for the junctional adhesion molecule A (JAM-A) receptor(Koehler, Aravamudhan et al. 2019). Through single-virus force spectroscopy, the binding strengths of reovirus to both α-SA and JAM-A were quantified, and the kinetic parameters characterizing these interactions were extracted. AFM experiments conducted on both model surfaces and living cells showed that reovirus binding favors multivalent interactions, specifically three parallel interactions with α-SA and two to three with JAM-A. This suggests that each receptor-binding domain within the trimeric structure of σ 1 independently engages with α-SA and JAM-A receptors, enhancing binding avidity through multivalency. While the affinity for single protein-glycan interactions, such as those between σ 1 and α-SA, is relatively low (in the millimolar range), the overall avidity increases

significantly to nanomolar levels due to the cumulative effect of multiple bonds. Evidence of cooperativity between the reovirus attachment factor α-SA and the specific entry receptor JAM-A was also found. It was observed that the binding of the σ 1 tail to α-SA enhances the subsequent binding of the σ 1 head to JAM-A, suggesting a conformational change triggered by the initial glycan interaction. This mechanism illustrates how surface glycans mediate viral targeting and offers potential strategies to manipulate reovirus infectivity for therapeutic purposes, such as vaccine design and oncolytic virus development (Mohammed, Koehler et al. 2021).

With these advancements, atomic force microscopy (AFM) has significantly enhanced our understanding of viral entry mechanisms and provided invaluable insights into the binding stages of various viruses. For instance, shortly after the emergence of the COVID-19 pandemic, AFM demonstrated how the spike glycoprotein of SARS-CoV-2 binds to the ACE2 receptor (Yang, Petitjean et al. 2020), facilitating the identification of potential inhibitor peptides and molecules that target the virus's early attachment stages (Yang, Petitjean et al. 2020, Petitjean, Chen et al. 2022, Petitjean, Lecocq et al. 2022). Additionally, investigations into SARS-CoV-2 variants have yielded critical insights into the molecular basis of increased infectivity linked to mutations in the receptor-binding domain, as well as the involvement of other co-receptors and attachment factors (Koehler, Ray et al. 2021, Ray, Minh Tran et al. 2024). Studies on reovirus have highlighted the cooperative interactions between viral attachment proteins and cell receptors, particularly the role of β1 integrins in mediating virus internalization(Koehler, Petitjean et al. 2021). Research on the Ebola virus has identified TIM-1 and DC-SIGNR as high-affinity receptors for viral binding, underscoring the complexity of the binding process (Zhang, Yang et al. 2022). Collectively, these studies illustrate the broad applicability of AFM in elucidating the intricate dynamics of virus-cell interactions, paving the way for innovative antiviral strategies.

AFM as a sensor to study intracellular signalling

Although AFM is primarily recognized as a surface technique due to its reliance on a physical probe, it is also effective for investigating biological processes occurring within cells. Cells continuously sense and respond to alterations in their surrounding environment, adapting to a variety of biochemical and mechanical stimuli that can modify their state. In a groundbreaking

study, the application of mechanical stimuli via an AFM cantilever on osteoblasts was shown to induce an intracellular calcium response, which is transmissible to neighboring cells. These neighboring cells possess connexins and functional gap junctions, enabling efficient intercellular communication (Charras and Horton 2002). In the field of immunology, AFM has been used to unravel the mechanisms by which human peroxiredoxin 5 (PRDX5) acts as a damage-associated molecular pattern (DAMP) (Knoops, Becker et al. 2018). AFM experiments revealed specific binding interactions between PRDX5 and TLR4 and evidence on living macrophage-differentiated THP-1 cells that PRDX5 binding induces a rapid mechano-response, leading to increased cellular stiffness (Figure 7A-D). This finding is particularly relevant for understanding the mechanisms of inflammation-related stiffness in vascular diseases (Knoops, Becker et al. 2018). Additionally, AFM has proven invaluable in elucidating the dynamics of nanoscale plasma membrane deformation, which recruits BAR domain proteins and actin structures. it was demonstrated that a distinct subset of BAR domain proteins is recruited to each plasma membrane deformation size, with their affinity for specific curvature radii. To capture the dynamics of CDC42 and local actin polymerization upon acute membrane deformation—occurring within 10 minutes—AFM experiments were conducted on live cells using a combination of FluidFM and confocal microscopy. Fluorescent 500-nm particles were captured at the tip of the FluidFM probe and pulled toward the apical plasma membrane of cells coexpressing LifeAct-mCherry and various GFP-tagged CDC42 constructs. Upon the transient expression of wild-type CDC42, a peak of actin polymerization was observed within 2 minutes following the induction of deformation, followed by a gradual decrease in the signal (Ledoux, Zanin et al. 2023). Similarly, AFM coupled to FluidFM and confocal microscopy has been used to investigate virus endocytosis, particularly in elucidating the role of β1 integrin in clathrin recruitment (Koehler, Petitjean et al. 2021). The study demonstrated a direct interaction between reovirus and β1 integrin, providing insights into how this interaction triggers clathrin-mediated endocytosis (Figure 7E-G). Using FluidFM, nanoparticles (NPs) functionalized with fluorescently labeled reovirus particles (T3SA+) were positioned onto the surface of living CHO cells expressing JAM-A and clathrin tagged with blue-fluorescent protein (BFP). By precisely controlling the contact force between the virus-bound NPs and the cell membrane, the researchers observed a rapid increase in BFP signal, indicating clathrin recruitment to the plasma membrane within 50 seconds after contact. This process was dependent on the binding of β1 integrin, as blocking integrin interaction with cRGD or KGE

peptides, or manipulating viral proteins via Neu5Ac-induced changes, abolished clathrin recruitment (**Figure 7G**). This real-time observation of clathrin dynamics using FluidFM allowed to pinpoint that β1 integrin binding is crucial for the activation of the clathrin-mediated uptake pathway. Notably, reovirus particles that poorly interacted with β1 integrin did not show clathrin recruitment, underscoring the specificity of this interaction in promoting endocytosis. Thus, the combination of AFM with FluidFM, optical microscopy and mechanics approaches introduces new capabilities to AFM, enabling the tracking of intracellular processes in real-time with high spatial and temporal resolution, thereby expanding its potential to study dynamic interactions during virus entry and other complex cellular mechanisms.

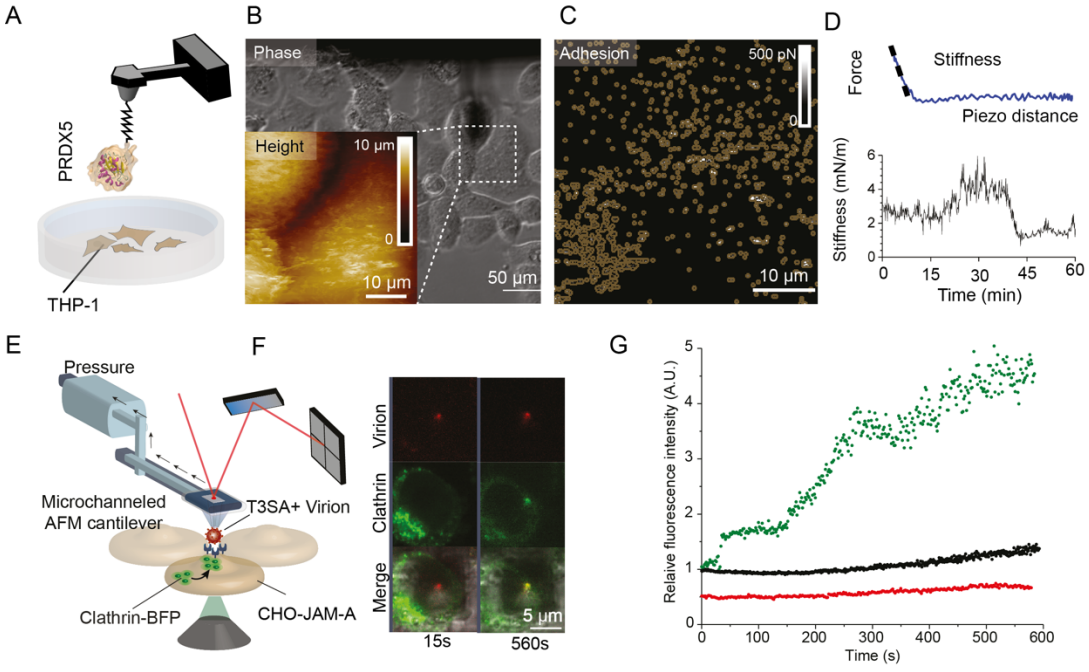

Figure 7. AFM as a sensor to study intracellular signaling and virus endocytosis. (A-D) PRDX5-TLR4 interaction studied using FD-based AFM on living THP-1 cells. (A) Schematic of AFM setup with PRDX5-functionalized tip probing cells under physiological conditions. (B) Phase image of THP-1 cells with AFM tip above (shadow visible). Inset: Height image in dashed square. (C) Adhesion channel image showing PRDX5-TLR4 binding. (D) Force vs piezo-distance curve with stiffness extracted from the contact region, showing time-dependent stiffness increase due to PRDX5 binding. (E-G) Reovirus early-endocytosis visualized with FluidFM and confocal microscopy. (E) Fluorescent T3SA+ reovirus nanoparticles trapped by FluidFM probe and brought into contact with clathrin-BFP CHO-JAM-A cells. (F) Time-lapse images showing clathrin (green) recruitment to reovirus (red) contact site. (G) Clathrin recruitment over time, inhibited by integrin blockers (black, red) or Neu5Ac, but present without blockers (green).Adapted from (Knoops, Becker et al. 2018) (Koehler, Petitjean et al. 2021).

The combined fluorescence-nanoscopic approach utilized in this study promises to further elucidate the roles of various ligands in immune receptor activation and may aid in developing

novel anti-inflammatory drugs by quantifying the effects of molecules that modulate receptor activation.

Concluding remarks and perspective

AFM has made significant strides in the study of receptor-ligand interactions and intracellular processes at the single-molecule level. Its ability to provide high-resolution imaging, force measurements, and real-time tracking under near-physiological conditions has proven invaluable in advancing our understanding of complex biological systems. From studying virus-host interactions to probing intracellular signaling pathways, AFM has expanded the frontiers of nanobiophysics. The combination of AFM with advanced techniques like FluidFM and confocal microscopy has introduced new capabilities, allowing researchers to visualize dynamic processes such as viral endocytosis, protein recruitment, and actin polymerization with unprecedented spatial and temporal precision. These innovations have positioned AFM as a versatile tool capable of addressing questions that extend beyond surface interactions, offering insights into cellular and molecular mechanisms critical for health and disease. Looking ahead, continued improvements in AFM technology, such as integrating additional optical and mechanical methodologies, will further enhance its application scope. This will enable even deeper exploration of cellular mechanics, membrane receptor functions, and virus-cell interactions. The future of AFM promises not only to refine our understanding of fundamental biological processes but also to contribute to the development of therapeutic strategies targeting molecular mechanisms involved in disease progression and immune responses.

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Biography: David Alsteens

References

Allen, S., X. Y. Chen, J. Davies, M. C. Davies, A. C. Dawkes, J. C. Edwards, C. J. Roberts, J. Sefton, S. J. B. Tendler and P. M. Williams (1997). "Detection of antigen-antibody binding events with the atomic force microscope." Biochemistry **36**(24): 7457-7463.

Alsteens, D., E. Dague, P. G. Rouxhet, A. R. Baulard and Y. F. Dufrene (2007). "Direct measurement of hydrophobic forces on cell surfaces using AFM." Langmuir **23**(24): 11977-11979.

Alsteens, D., R. Newton, R. Schubert, D. Martinez-Martin, M. Delguste, B. Roska and D. J. Muller (2016). "Nanomechanical mapping of first binding steps of a virus to animal cells." Nat Nanotechnol.

Alsteens, D., M. Pfreundschuh, C. Zhang, P. M. Spoerri, S. R. Coughlin, B. K. Kobilka and D. J. Muller (2015). "Imaging G protein-coupled receptors while quantifying their ligand-binding free-energy landscape." Nat Methods **12**(9): 845-851.

Alsteens, D., H. Trabelsi, P. Soumillion and Y. F. Dufrene (2013). "Multiparametric atomic force microscopy imaging of single bacteriophages extruding from living bacteria." Nature Communications **4**: 2926.

Arakawa, H., K. Umemura and A. Ikai (1992). "Protein images obtained by STM, AFM and TEM." Nature **358**(6382): 171-173.

Beaussart, A., S. El-Kirat-Chatel, R. M. Sullan, D. Alsteens, P. Herman, S. Derclaye and Y. F. Dufrene (2014). "Quantifying the forces guiding microbial cell adhesion using single-cell force spectroscopy." Nat Protoc **9**(5): 1049-1055.

Binnig, G., C. F. Quate and C. Gerber (1986). "Atomic Force Microscope." Physical Review Letters **56**(9): 930-933.

Binnig, G., H. Rohrer, C. Gerber and E. Weibel (1982). "Tunneling through a controllable vacuum gap." Appl. Phys. Lett. **40**(2): 178-180.

Butt, H. J., E. K. Wolff, S. A. Gould, B. Dixon Northern, C. M. Peterson and P. K. Hansma (1990). "Imaging cells with the atomic force microscope." J Struct Biol **105**(1-3): 54-61.

Carneiro, F. A., M. L. Bianconi, G. Weissmüller, F. Stauffer and A. T. D. Poian (2002). "Membrane Recognition by Vesicular Stomatitis Virus Involves Enthalpy-Driven Protein-Lipid Interactions." Journal of Virology **76**(8): 3756-3764.

Carneiro, F. A., P. A. Lapido-Loureiro, S. M. Cordo, F. Stauffer, G. Weissmüller, M. L. Bianconi, M. A. Juliano, L. Juliano, P. M. Bisch and A. T. D. Poian (2006). "Probing the interaction between vesicular stomatitis virus and phosphatidylserine." European Biophysics Journal **35**(2): 145-154.
Charras, G. T. and M. A. Horton (2002). "Single Cell Mechanotransduction and Its Modulation Analyzed by Atomic Force Microscope Indentation." Biophysical Journal **82**(6): 2970-2981.
Chen, G., X. Ning, B. Park, G. J. Boons and B. Xu (2009). "Simple, clickable protocol for atomic force microscopy tip modification and its application for trace ricin detection by recognition imaging." Langmuir **25**(5): 2860-2864.
Delguste, M., C. Zeippen, B. Machiels, J. Mast, L. Gillet and D. Alsteens (2018). "Multivalent binding of herpesvirus to living cells is tightly regulated during infection." Science Advances **4**(8).
Dumitru, A. C., L. Conrard, C. Lo Giudice, P. Henriet, M. Veiga-da-Cunha, S. Derclaye, D. Tyteca and D. Alsteens (2018). "High-resolution mapping and recognition of lipid domains using AFM with toxin-derivatized probes." Chemical Communications **54**(50): 6903-6906.
Dumitru, A. C., D. Mohammed, M. Maja, J. Yang, S. Verstraeten, A. del Campo, M.-P. Mingeot-Leclercq, D. Tyteca and D. Alsteens (2020). "Label-Free Imaging of Cholesterol Assemblies Reveals Hidden Nanomechanics of Breast Cancer Cells." Adv. Sci. **7**(22): 2002643.
Ebner, A., L. Wildling, A. S. M. Kamruzzahan, C. Rankl, J. Wruss, C. D. Hahn, M. Holzl, R. Zhu, F. Kienberger, D. Blaas, P. Hinterdorfer and H. J. Gruber (2007). "A new, simple method for linking of antibodies to atomic force microscopy tips." Bioconjugate Chemistry **18**(4): 1176-1184.
Egger, M., F. Ohnesorge, A. L. Weisenhorn, S. P. Heyn, B. Drake, C. B. Prater, S. A. C. Gould, P. K. Hansma and H. E. Gaub (1990). "Wet lipid-protein membranes imaged at submolecular resolution by atomic force microscopy." Journal of Structural Biology **103**(1): 89-94.
Fernandez, M., A. V. Shkumatov, Y. Liu, C. Stulemeijer, S. Derclaye, Rouslan G. Efremov, B. Hallet and D. Alsteens (2023). "AFM-based force spectroscopy unravels stepwise formation of the DNA transposition complex in the widespread Tn3 family mobile genetic elements." Nucleic Acids Research **51**(10): 4929-4941.
Fischer, M. J. (2010). "Amine coupling through EDC/NHS: a practical approach." Methods Mol Biol **627**: 55-73.
Florin, E. L., V. T. Moy and H. E. Gaub (1994). "Adhesion forces between individual ligand receptor pairs." Science **264**(5157): 415-417.
Frisbie, C. D., L. F. Rozsnyai, A. Noy, M. S. Wrighton and C. M. Lieber (1994). "Functional-group imaging by chemical force microscopy." Science **265**(5181): 2071-2074.
Grandbois, M., W. Dettmann, M. Benoit and H. E. Gaub (2000). "Affinity imaging of red blood cells using an atomic force microscope." Journal of Histochemistry and Cytochemistry **48**(5): 719-724.
Hansma, P. K., V. B. Elings, O. Marti and C. E. Bracker (1988). "Scanning tunneling microscopoy and atomic force microscopy : application to biology and technology." Science **247**: 209-216.
Herman, P., S. El-Kirat-Chatel, A. Beaussart, J. A. Geoghegan, T. J. Foster and Y. F. Dufrene (2014). "The binding force of the staphylococcal adhesin SdrG is remarkably strong." Mol Microbiol **93**(2): 356-368.
Hinterdorfer, P., W. Baumgartner, H. J. Gruber, K. Schilcher and H. Schindler (1996). "Detection and localization of individual antibody-antigen recognition events by atomic force microscopy." Proceedings of the National Academy of Sciences of the United States of America **93**(8): 3477-3481.
Hinterdorfer, P. and Y. F. Dufrêne (2006). "Detection and localization of single molecular recognition events using atomic force microscopy." Nature Methods **3**(5): 347-355.
Kienberger, F., G. Kada, H. J. Gruber, V. Pastushenko, C. Riener, M. Trieb, H. G. Knaus, H. Schindler and P. Hinterdorfer (2000). "Recognition Force Spectroscopy Studies of the NTA-His_6 Bond." Single Molecules **1**: 59-65.
Knoops, B., S. Becker, M. A. Poncin, J. Glibert, S. Derclaye, A. Clippe and D. Alsteens (2018). "Specific Interactions Measured by AFM on Living Cells between Peroxiredoxin-5 and TLR4: Relevance for Mechanisms of Innate Immunity." Cell Chem. Biol. **25**(5): 550-559.e553.
Knoops, B., S. Becker, M. A. Poncin, J. Glibert, S. Derclaye, A. Clippe and D. Alsteens (2018). "Specific Interactions Measured by AFM on Living Cells between Peroxiredoxin-5 and TLR4: Relevance for Mechanisms of Innate Immunity." Cell Chemical Biology **25**(5): 550-559.e553.
Koehler, M., P. Aravamudhan, C. Guzman-Cardozo, A. C. Dumitru, J. Yang, S. Gargiulo, P. Soumillion, T. S. Dermody and D. Alsteens (2019). "Glycan-mediated enhancement of reovirus receptor binding." Nature Communications **10**(1): 4460.
Koehler, M., C. Lo Giudice, P. Vogl, A. Ebner, P. Hinterdorfer, H. J. Gruber and D. Alsteens (2020). "Control of Ligand-Binding Specificity Using Photocleavable Linkers in AFM Force Spectroscopy." Nano Lett. **20**(5): 4038-4042.

Koehler, M., S. J. L. Petitjean, J. Yang, P. Aravamudhan, X. Somoulay, C. Lo Giudice, M. Poncin, A. Dumitru, T. S. Dermody and D. Alsteens (2021). "Reovirus directly engages integrin to recruit clathrin for entry into host cells." Nature Communications **12**: 2149.
Koehler, M., A. Ray, R. A. Moreira, B. Juniku, A. B. Poma and D. Alsteens (2021). "Molecular insights into receptor binding energetics and neutralization of SARS-CoV-2 variants." Nature Communications **12**(1): 6977.
Kuznetsov, Y. G., A. J. Malkin, R. W. Lucas, M. Plomp and A. McPherson (2001). "Imaging of viruses by atomic force microscopy." Journal of General Virology **82**: 2025-2034.
Ledoux, B., N. Zanin, J. Yang, V. Mercier, C. Coster, C. Dupont-Gillain, D. Alsteens, P. Morsomme and H.-F. Renard (2023). "Plasma membrane nanodeformations promote actin polymerization through CIP4/CDC42 recruitment and regulate type II IFN signaling." Science Advances **9**(50): eade1660.
Lee, G. U., L. A. Chrisey and R. J. Colton (1994). "Direct measurement of the forces between complementary strands of DNA." Science **266**(5186): 771-773.
Lee, G. U., D. A. Kidwell and R. J. Colton (1994). "Sensing discrete streptavidin biotin interactions with atomic force microscopy." Langmuir **10**(2): 354-357.
Lekka, M., P. Laidler, J. Ignacak, M. Łabedz, J. Lekki, H. Struszczyk, Z. Stachura and A. Z. Hrynkiewicz (2001). "The effect of chitosan on stiffness and glycolytic activity of human bladder cells." Biochim Biophys Acta **1540**(2): 127-136.
Li, L. i. (2014). "Progress in measuring biophysical properties of membrane proteins with AFM single-molecule force spectroscopy." Chinese science bulletin = 科学通报（英文版） **59**(22): 2717-2725.
Lo Giudice, C., H. Zhang, B. Wu and D. Alsteens (2020). "Mechanochemical Activation of Class-B G-Protein-Coupled Receptor upon Peptide-Ligand Binding." Nano Letters **20**(7): 5575-5582.
Ludwig, M., W. Dettmann and H. E. Gaub (1997). "Atomic force microscope imaging contrast based on molecular recognition." Biophysical Journal **72**(1): 445-448.
Meister, A., M. Gabi, P. Behr, P. Studer, J. Vörös, P. Niedermann, J. Bitterli, J. Polesel-Maris, M. Liley, H. Heinzelmann and T. Zambelli (2009). "FluidFM: Combining atomic force microscopy and nanofluidics in a universal liquid delivery system for single cell applications and beyond." Nano Letters **9**(6): 2501-2507.
Merkel, R., P. Nassoy, A. Leung, K. Ritchie and E. Evans (1999). "Energy landscapes of receptor-ligand bonds explored with dynamic force spectroscopy." Nature **397**(6714): 50-53.
Meyer, G. and N. M. Amer (1988). "Novel Optical Approach to Atomic Force Microscopy." Applied Physics Letters **53**(12): 1045-1047.
Mohammed, D., M. Koehler, A. C. Dumitru, P. Aravamudhan, D. M. Sutherland, T. S. Dermody and D. Alsteens (2021). "Altered Glycan Expression on Breast Cancer Cells Facilitates Infection by T3 Seroptype Oncolytic Reovirus." Nano Letters **21**(22): 9720-9728.
Muller, D. J. and Y. F. Dufrêne (2008). "Atomic force microscopy as a multifunctional molecular toolbox in nanobiotechnology." Nature Nanotechnology **3**(5): 261-269.
Müller, D. J., A. C. Dumitru, C. Lo Giudice, H. E. Gaub, P. Hinterdorfer, G. Hummer, J. J. De Yoreo, Y. F. Dufrêne and D. Alsteens (2020). "Atomic Force Microscopy-Based Force Spectroscopy and Multiparametric Imaging of Biomolecular and Cellular Systems." Chemical Reviews.
Nguyen, A. V., J. Nalaskowski, J. D. Miller and H.-J. Butt (2003). "Attraction between hydrophobic surfaces studied by atomic force microscopy." International Journal of Mineral Processing **72**(1-4): 215-225.
Noy, A., C. D. Frisbie, L. F. Rozsnyai, M. S. Wrighton and C. M. Lieber (1995). "Chemical force microscopy exploiting chemically-modified tips to quantify adhesion, friction, and functional-group distributions in molecular assemblies." Journal of the American Chemical Society **117**(30): 7943-7951.
Ott, W., M. A. Jobst, C. Schoeler, H. E. Gaub and M. A. Nash (2017). "Single-molecule force spectroscopy on polyproteins and receptor-ligand complexes: The current toolbox." J Struct Biol **197**(1): 3-12.
Petitjean, S. J. L., W. Chen, M. Koehler, R. Jimmidi, J. Yang, D. Mohammed, B. Juniku, M. L. Stanifer, S. Boulant, S. P. Vincent and D. Alsteens (2022). "Multivalent 9-O-Acetylated-sialic acid glycoclusters as potent inhibitors for SARS-CoV-2 infection." Nature Communications **13**(1): 2564.
Petitjean, S. J. L., M. Lecocq, C. Lelong, R. Denis, S. Defrère, P.-A. Mariage, D. Alsteens and C. Pilette (2022). "Salvia miltiorrhiza Bunge as a Potential Natural Compound against COVID-19." Cells **11**(8): 1311.
Pfreundschuh, M., D. Alsteens, M. Hilbert, M. O. Steinmetz and D. J. Muller (2014). "Localizing chemical groups while imaging single native proteins by high-resolution atomic force microscopy." Nano Lett **14**(5): 2957-2964.
Pfreundschuh, M., U. Hensen and D. J. Muller (2013). "Quantitative imaging of the electrostatic field and potential generated by a transmembrane protein pore at subnanometer resolution." Nano Lett **13**(11): 5585-5593.

Rankl, C., F. Kienberger, L. Wildling, J. Wruss, H. J. Gruber, D. Blaas and P. Hinterdorfer (2008). "Multiple receptors involved in human rhinovirus attachment to live cells." Proceedings of the National Academy of Sciences of the United States of America **105**(46): 17778-17783.
Ray, A., T. T. Minh Tran, R. d. Santos Natividade, R. A. Moreira, J. D. Simpson, D. Mohammed, M. Koehler, S. J. L Petitjean, Q. Zhang, F. Bureau, L. Gillet, A. B. Poma and D. Alsteens (2024). "Single-Molecule Investigation of the Binding Interface Stability of SARS-CoV-2 Variants with ACE2." ACS Nanoscience Au **4**(2): 136-145.
Ricci, M., R. A. Quinlan and K. Voïtchovsky (2017). "Sub-nanometre mapping of the aquaporin–water interface using multifrequency atomic force microscopy." Soft Matter **13**(1): 187-195.
Rief, M., M. Gautel, F. Oesterhelt, J. M. Fernandez and H. E. Gaub (1997). "Reversible unfolding of individual titin immunoglobulin domains by AFM." Science **276**(5315): 1109-1112.
Riener, C. K., F. Kienberger, C. D. Hahn, G. M. Buchinger, I. O. C. Egwim, T. Haselgrubler, A. Ebner, C. Romanin, C. Klampfl, B. Lackner, H. Prinz, D. Blaas, P. Hinterdorfer and H. J. Gruber (2003). "Heterobifunctional crosslinkers for tethering single ligand molecules to scanning probes." Analytica Chimica Acta **497**(1-2): 101-114.
Roduit, C., F. G. van der Goot, P. Los Rios, A. Yersin, P. Steiner, G. Dietler, S. Catsicas, F. Lafont and S. Kasas (2008). "Elastic membrane heterogeneity of living cells revealed by stiff nanoscale membrane domains." Biophysical Journal **94**(4): 1521-1532.
Rotsch, C. and M. Radmacher (2000). "Drug-induced changes of cytoskeletal structure and mechanics in fibroblasts: An atomic force microscopy study." Biophysical Journal **78**(1): 520-535.
Targosz-Korecka, M., M. Jaglarz, K. E. Malek-Zietek, A. Gregorius, A. Zakrzewska, B. Sitek, Z. Rajfur, S. Chlopicki and M. Szymonski (2017). "AFM-based detection of glycocalyx degradation and endothelial stiffening in the db/db mouse model of diabetes." Sci Rep **7**(1): 15951.
Theile, C. S., M. D. Witte, A. E. Blom, L. Kundrat, H. L. Ploegh and C. P. Guimaraes (2013). "Site-specific N-terminal labeling of proteins using sortase-mediated reactions." Nat Protoc **8**(9): 1800-1807.
Verbelen, C., H. J. Gruber and Y. F. Dufrêne (2007). "The NTA-His_6 bond is strong enough for AFM single-molecular recognition studies." Journal of Molecular Recognition **20**(6): 490-494.
Wasserberg, D., J. Cabanas-Danés, J. Prangsma, S. O'Mahony, P.-A. Cazade, E. Tromp, C. Blum, D. Thompson, J. Huskens, V. Subramaniam and P. Jonkheijm (2017). "Controlling Protein Surface Orientation by Strategic Placement of Oligo-Histidine Tags." ACS Nano **11**(9): 9068-9083.
Weisenhorn, A. L., M. Egger, F. Ohnesorge, S. A. C. Gould, S. P. Heyn, H. G. Hansma, R. L. Sinsheimer, H. E. Gaub and P. K. Hansma (1991). "Molecular-resolution images of Langmuir-Blodgett films and DNA by atomic force microscopy." Langmuir **7**(1): 8-12.
Weisenhorn, A. L., P. K. Hansma, T. R. Albrech and C. F. Quate (1989). "Forces in atomic force microscope in air and water." Appl. Phys. Lett. **54**(26): 2651-2653.
Wildling, L., B. Unterauer, R. Zhu, A. Rupprecht, T. Haselgrübler, C. Rankl, A. Ebner, D. Vater, P. Pollheimer, E. E. Pohl, P. Hinterdorfer and H. J. Gruber (2011). "Linking of sensor molecules with amino groups to amino-functionalized AFM tips." Bioconjugate Chemistry **22**(6): 1239-1248.
Yang, J., S. J. L. Petitjean, M. Koehler, Q. Zhang, A. C. Dumitru, W. Chen, S. Derclaye, S. P. Vincent, P. Soumillion and D. Alsteens (2020). "Molecular interaction and inhibition of SARS-CoV-2 binding to the ACE2 receptor." Nature Communications **11**(1): 4541.
Yoon, R.-H., D. H. Flinn and Y. I. Rabinovich (1997). "Hydrophobic Interactions between Dissimilar Surfaces." Journal of Colloid and Interface Science **185**(2): 363-370.
Zhang, Q., J. Yang, S. Tillieux, Z. Guo, R. d. S. Natividade, M. Koehler, S. Petitjean, Z. Cui and D. Alsteens (2022). "Stepwise Enzymatic-Dependent Mechanism of Ebola Virus Binding to Cell Surface Receptors Monitored by AFM." Nano Letters **22**(4): 1641-1648.
Zocher, M., C. Roos, S. Wegmann, P. D. Bosshart, V. Dötsch, F. Bernhard and D. J. Müller (2012). "Single-Molecule Force Spectroscopy from Nanodiscs: An Assay to Quantify Folding, Stability, and Interactions of Native Membrane Proteins." ACS Nano **6**(1): 961-971.