High-Resolution Functional Imaging of Native Proteins using Force Distance Curve Based Atomic Force Microscopy

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Physicists are made of atoms. A physicist is an attempt by an atom to understand itself.

– Michio Kaku
1 Introduction

Preamble

Life would not exist without molecular interactions holding biomacromolecules together and regulating their functional states. In fact, all cellular processes are governed by the outcome of a sensitive balance of these molecular driving forces. Hereby proteins, lipids, nucleic acids, and other biomolecules form the highly sophisticated molecular machinery of the living cell that works and responds to its environment in a complex manner. Among these biological key players proteins are the ones that perform most of the work in living cells. They are involved in DNA replication, protein production, -transformation, -secretion, metabolism, cell division and the flow of materials or information across the cell boundaries. Understanding how cells work requires understanding how proteins function. Furthermore, understanding how proteins function requires understanding how they interact with their environment.

For the molecular recognitions of a cell wall's environment and for the transduction of signals transmembrane proteins are vital. The focus of this PhD thesis lies on the investigation of these lipid embedded proteins.

In this introductory chapter the state of the art in membrane protein research is presented with a focus on its structure-function relationship. Furthermore, we will turn our attention on the application of atomic force microscopy (AFM), which in this thesis served as a nanoscopic tool, able to decipher the nature of membrane proteins and soluble proteins in its structural details as well as in its functionality when interacting with other molecules. We will describe what kind of intermolecular forces can be detected using the AFM. In detail we will deal with electrostatic forces such as detecting an electrostatic field and potential by the AFM. Later we turn our focus to AFM tip functionalization and how to detect, localize and quantify ligand-receptor bonds.
The main question raised were: How can we investigate a membrane proteins structure and function in a native environment? How can we localize and quantitate molecular recognition and binding properties of the interaction partners of these proteins?

The cellular membrane

Cell membranes are crucial to the survival of the cell. In eukaryotic cells the plasma membrane separates the cytosolic from the extracellular space. Membranes are also key component inside the eukaryotic cell. They delimit for example the endoplasmic reticulum, the Golgi apparatus and mitochondria\(^2\). Ion gradients across the membrane provide the electrochemical energy needed for ATP productions, drive the transmembrane movement of solutes or, as in neurones, provide a mechanism for the transmission of electrical signals\(^3\). Being a relative impermeable barrier for soluble proteins the membrane is equipped with membrane proteins that selectively allow or inhibit the solutes to cross this barrier\(^4\).

Furthermore, all plasma membranes contain proteins that are responsible for sensing external signals, thereby allowing the cell to adapt to its environmental cues. Internal signal cascades are frequently triggered by conformational changes of receptor proteins and finally transmit these signals downstream. These receptor proteins, opposed to transporter proteins, transfer information rather than molecules across the membrane\(^5\).

Membrane Proteins

Membrane proteins make up about one third of the genes of every animals genome\(^6\) and constitute about 50% of nowadays drug targets\(^7\). They play an important role in signal processing, energy conversion, molecular transport and cell adhesion. Membrane proteins continue to be among the most challenging targets in structural biology. Unfortunately, they are more diffi-
cult to characterize than soluble proteins, since their integrity is hampered when removed from their native membrane. As amphiphilic molecules they are exposing a hydrophobic core, are insoluble and do not easily tend to form crystals, compared to soluble proteins. Thus, of the predicted structure determination through X-ray diffraction experiments is more difficult. Even when membrane proteins are solubilized in detergent or organic solvent, it has been shown from NMR experiments of membrane proteins with detergents and with phospholipids that the structure and dynamics under near-native conditions (phospholipids) vary from those of non-native conditions (detergents)⁸. Several techniques, such as UV-Vis spectroscopy, NMR and crystallization techniques are impaired when one tries to investigate membrane proteins that are still residing in lipid bilayers. Therefore, a method that enables to investigate structure and function of membrane proteins in near-physiological conditions is needed.

In the field of biophysics, molecular- and structural biology one nanoscopic tool has become apparent to be indispensable for the investigation of structure and function of membrane proteins - the AFM. This scanning probe microscope technique was first introduced in 1986 by Binning, Gerber and Quate⁹ and is based on the measurement of interactions between a sharp tip and the atoms of the sample surface. Being capable to work in liquids, AFM measurements can be performed in near-physiological conditions. In the last two decades it found its way into the investigation of biological systems¹⁰.

**Aim of this thesis**

In order to understand the function of a membrane protein it is important to determine its structure at a high resolution and to simultaneously quantify and structurally map its biophysical and -chemical properties in the living cell or at least close to physiologically relevant conditions. As intracellular conditions are very heterogeneous, the properties of the molecular machines such as assembled membrane proteins depend on the machines' location
within the cell. Additionally, the structural and functional properties vary between the individuals. Conventional bulk methods to determine structural (X-ray, NMR, circular dichroism spectroscopy) or functional details (UV-Vis spectroscopy, enzymatic assays, calorimetry) extract information only from an assembly of molecules. Nevertheless, it is a major challenge to quantify and structurally map physical and chemical properties on single molecular machines to assess their functional states individually and at a high resolution.

One goal of this thesis is to record high resolution topographies of native membrane proteins using AFM and to quantify and visualize features such as electrostatic properties by analyzing interaction forces.

Communication between a cell and its environment happens mostly through signal processing mediated by transmembrane proteins. Most of the times, a signal-triggering molecule is acting as ligand and forming a complex with its receptor, a transmembrane protein. These ligand-receptor interactions make up one of the greatest regulation mechanisms of the cell. It is a current need to decipher these on a molecular basis and quantify their thermodynamic and kinetic properties while resolving their structure and molecular assembly in a cell or at least at physiologically relevant conditions. As a second goal of this thesis, we want to combine AFM high-resolution imaging with the quantification of ligand receptor interactions to gain insights into the structural and energetic properties of this important mechanism in biology.

Atomic force microscopy

The concept

The AFM originally conceived as a high-resolution imaging tool of surfaces operated in air and vacuum. Fortunately, a few years later and most important for biological applications it was applicable also in buffered solutions. The instrument mainly consists of four building blocks (see Fig. 1).
sharp AFM tip that is attached at the end of a flexible cantilever and pointing towards the sample, ii) a piezoelectric scanner moving the sample with sub Ångstrom resolution iii) a photo-detection system composed of a laser reflecting at the end of the cantilever towards a photosensitive diode and iv) a controller analyzing the measured signal and comparing it with the desired input signal, correcting the error and thereby acting as an error-feedback loop (see Fig. 1A).

For a calibrated system the deflection of the cantilever measured by the photo detector can be converted into a force using Hooke's law ($F = k_c \cdot d_{\text{deflection}}$), whereas $F$ is the force, $k_c$ and $d_{\text{deflection}}$ are the spring constant and deflection of the cantilever, respectively.

In the system used for this work the sample holder is moving in $x$, $y$ and $z$ direction, thereby approaching and retracting the sample from the cantilever while scanning in $x$ and $y$ (see Fig. 1B).

**Figure 1: Schematic of an AFM setup** (A). Close-up of a moving sample holder that approaches and retracts from the cantilever in the $z$-direction (B).

**AFM as an imaging tool**

The AFM has become an ideal method to study the topography of biological membranes. Briefly, membranes are adsorbed onto flat supports and raster-
scanned in physiological conditions. AFM is mostly operated in contact mode, i.e. the tip is pressed with a low but constant force onto the biological sample while it contours its surface line by line. A topograph of the sample can be reconstructed by recording the $Z$ movement of the piezo-electric element adjusting for a constant deflection value between AFM cantilever and sample. For example, imaging membrane proteins AFM enables to contour the surface of native proteins at sub-nanometer resolution\textsuperscript{11,12}. The best attainable lateral and vertical resolutions of native membrane proteins approach 0.5-0.7nm and 0.1nm, respectively\textsuperscript{13,14}.

Working well on flat biological samples, contact mode AFM proved to be disadvantageous when working with more soft or corrugated biological samples such as single proteins or fibrils. Here, lateral forces between the AFM tip and the sample are hard to control and thus the sample is destroyed or removed from its support while being measured.

Furthermore, a contact-mode AFM is a pure imaging tool that it is somewhat limited in quantifying and mapping biological, chemical and physical parameters. In the following section, we discuss the use of AFM in the force spectroscopy-mode. This mode features only a short, intermittent contact between tip and sample, while approaching and retracting the cantilever from the sample. Additionally, this mode that enables to quantify the manifold interactions occurring between the AFM stylus and the biological sample under physiologically relevant conditions.

**AFM for force spectroscopy\textsuperscript{1}\textsuperscript{1}**

Shortly after the first applications of AFM to image biological systems, it was observed that the AFM stylus can act as a nanometer-sized probe and quantify electrostatic, van der Waals and hydrophobic interactions occurring between itself and the biological samples\textsuperscript{16,17,18,19,20,21}. To quantify these interaction forces, usually ranging from piconewtons to nanonewtons, AFM

\textsuperscript{1}This section contains excerpts from a previously published work\textsuperscript{15}.
is operated in force-spectroscopy mode, in which the AFM stylus vertically approaches to the sample, stays in contact with the surface for a defined time, and then retracts. During approach and retraction, the vertical displacement of the AFM stylus and sample and the deflection (e.g., force) of the AFM cantilever are recorded in so-called force-displacement (FZ) curves. Subtraction of the cantilever deflection \((d_c)\) from the vertical displacement \((Z)\) and a calculation of the contact point converts an FZ curve into a FD \((D=Z-d_c)\) curve that is used to quantify the distance dependence of the interaction forces between the AFM stylus and the sample \(^{22}\) (see Fig. 2). In addition to quantifying the inter- and intramolecular interaction forces of biological systems, the AFM stylus can be used to probe mechanical properties. Therefore, the AFM stylus is indented into and retracted from the sample while the mechanical response of the sample is being measured \(^{22}\). Such indentation-retraction experiments can provide insight into the deformation, elastic modulus, viscoelasticity, pressure, material fatigue, adhesion, electrostatic properties and energy dissipation of a biological sample. Shortly after the discovery that interactions can be detected between the AFM stylus and a sample, procedures were developed to use AFM-based force spectroscopy to characterize the specific binding of a ligand to its receptor. In this approach, the AFM stylus is functionalized with a receptor (or ligand) and a supporting surface with a corresponding ligand (or receptor) \(^{23,24,25}\). When the functionalized AFM stylus is brought into contact with the functionalized support, the receptor and ligand pair can bind. Retraction of the stylus from the support forces the specific bond formed between the receptor and ligand to break. The force required to break the receptor-ligand bond is detected by the deflecting AFM cantilever and referred to as a rupture force.
FD-based AFM combines AFM imaging and force spectroscopy².

AFM operated in the imaging mode and in the force-spectroscopy mode opened the door to the nanoworld¹⁰, and the idea was quickly born to combine both in the so-called FD-based AFM. Invented about 20 years ago, FD-based AFM imaging has since been optimized to the point that it can image the architecture of complex biological systems, such as living cells, cellular membranes, model membranes, protein complexes, and viruses and nucleic acids; at the same time, it can quantify and map their various properties to piconewton and nanometer resolution²⁶,²⁷,²⁸,²⁹.

While raster-scanning the native biological sample, FD-based AFM records an array of FD curves (see Fig. 2). For each pixel of the resulting AFM topography, the AFM records FD curves of the tip interacting with the sample with Ångstrom precision and piconewton sensitivity. These interaction forces are mapped, pixel by pixel, to the biological sample surface. From the resulting interaction map, a volume of forces is directly correlated to the sample topography²⁶.

The set point for each approach/retraction cycle is a fixed force (called imaging force or peak force) and corresponds to the desired maximal cantilever deflection when tip and sample are in contact. The relative z-movements between cantilever rest-position and sample needed to reach that peak force can be transformed into height values rendering the topographical structure of the protein. From each F-D curves the contact regime can also be analyzed to calculate contact mechanics of the tip-sample interaction such as elastic deformation and stiffness of the protein. Furthermore, the non-contact part of the F-D curves gives insight into attractive and repulsive interaction forces composed of weak van der Waals, electrostatic and hydration forces which manifest the molecular interaction between tip and sample (see section 1).

A chemically functionalized AFM stylus resembles a multifunctional nanoscopic toolbox. Molecular interaction forces can be specifically detected

²This section contains excerpts from a previously published work¹⁵
Figure 2: Force-distance curve-based (FD-based) AFM. (a) FD-based AFM approaches and retracts the tip of the AFM cantilever from the sample pixel-by-pixel. For each pixel the maximum cantilever deflection and thereby the imaging force ($F_i$) is controlled. (b) Force vs time curves of the approach (blue) and retraction (red) movements are recorded for each pixel. (c) The mechanical parameters such as the sample deformation can be extracted from the approach force vs distance (FD) curve (blue). (d) The adhesion force ($F_{adh}$), Young's modulus, and energy dissipation can be extracted from the retraction FD curve (red). The adhesion force provides insight into the attractive interactions between the AFM tip and the sample. (e) The topograph of the sample is reconstructed from the vertical AFM tip movement needed to reach the imaging force $F_i$ for each pixel. Furthermore, from the FD curves recorded for each pixel multiple parameters including adhesion and deformation can be determined and directly mapped to the topograph.
Detecting intermolecular forces by FD based AFM

With a typical nominal spring constants of 0.01 - 100 N/m and an instrumental sensitivity of 0.1 Å, minimal forces in the range of 0.1 pN - 10 nN are easily be detected. This makes this instrument able to detect van der Waals interactions (1 pN) till covalent bonds (10 nN). Intermolecular interactions can be categorized into two classes: short-ranged interactions and long-ranged interactions. These interaction energies (U) are generally modelled as power-law\textsuperscript{32}: $U \approx r^{-p}$ whereas $r$ is the distance between interacting particles and $p$ describes the magnitude of the power.

Electrostatic interactions belong to long-ranged interactions ($p \leq 3$), whereas van der Waals interactions fit into the short-ranged interactions ($p > 3$). Both weak non-covalent interactions have enthalpies of -1 to -10 kcal/mol, that reach from lower values in the range of thermal energy ($k_BT=0.6$ kcal/mol) to values that are significantly smaller than covalent bonds (100 kcal/mol). They belong to the class of electromagnetic forces and can result from steric repulsion, electrostatic- and dipole interactions, hydrogen bonding and hydrophobic effects.

Briefly, FD curves can be subdivided into three parts: the zero line, where tip and sample are in large distance and practically no interaction force is detected. The non-contact region, which describes the interactions before and after the tip comes into contact with the sample. The approach non-contact region describes attractive or repulsive forces forces before contact. When tip and sample are in contact and the force is continued to be exerted and the sample deforms. With the elastic potential of the cantilever ($U_c$) being in equilibrium with the elastic potential of the deformed sample, the sample deformation ($d_s$) is proportional to the cantilever deflection ($d_c$) following:
\[ d_s = \frac{k_c}{k_s} * d_c, \]
whereas \( k_c \) and \( k_s \) are the cantilever's and sample's spring constants, respectively. The retraction non-contact region treats the adhesion force. When tip and sample are separated from each other, a hysteresis between approach and retractions FD curves appears due to the adhesion between both parts. The adhesion force is needed for contact mechanic models describing the stiffness and elasticity of the samples.

The two main interaction forces in the biological world are the relatively short ranged van der Waals forces and the longer ranged electrostatic forces.

Van der Waals forces consist of three different forces containing the dipol-dipol (Keesom force), induced dipol-dipol (Debye force) and instantaneous dipol-dipol interactions (London dispersion force). The first one is proportional to \( \frac{1}{r^6} \), the latter ones are proportional to \( \frac{1}{r^8} \) \((r \text{ is the distance between atoms/molecules})\). The latter force is the predominant one since it acts between all molecules or atoms because temporarily fluctuating dipoles induce these forces. As singular unit van der Waals forces are comparatively small. However, in the world of biology, the additivity and non-directionality of these forces sum up to be the most important interaction forces between two molecules. In the scope of this PhD thesis, however, we turned our attention to the longer ranged electrostatic interactions, explaining interesting effects of protein interaction with charged particles/molecules.

Detection electrostatic interactions by AFM

One of the strongest physical interaction forces is the one between two charged particles \((q_1, q_2)\), the Coulomb force \((F_C)\).

\[ F_C = \frac{q_1 * q_2}{4\pi\varepsilon_0\varepsilon r^2} \]  
(1)
describes the force between two charged particles. Where the \( r \) is the distance between the charges and the proportionality factor \( 1/4(\pi\varepsilon_0\varepsilon) \) takes the electrical permittivity of the vacuum \((\varepsilon_0)\) and the relative permittivity of the
medium (ε, 80.1 for water), respectively, into account.

Here, each charged particle (q₁) generates an electric field \( E_{el} \) at a distance \( r \) away from the charge (q₂)

\[
E_{el} = \frac{F_C}{q_2} = \frac{q_1}{4\pi\varepsilon_0\varepsilon r^2}
\]  

In vacuum the energy of an ion pair would value about 100 \( k_BT \) per ion pair, equivalent to a covalent bond. However, when charged surfaces are immersed in water or buffered liquids these forces are much weaker\(^{35}\). Since van der Waals forces are always attractive one could presume that any particles in liquid would coagulate. Even our own body would be subject to this fate. Fortunately particles in liquid with a high dielectric constant are usually charged and hence exert repulsive electrostatic forces on similar-charged particles. In water and buffered liquids, these charges are considerably screened by counter-ions. Charged surfaces interaction in solution containing electrolytes is thereby described by the Electric Double Layer\(^{35,19}\). Any charged surface will generate a counter-ion and co-ion profile away from its surfaces. This property can be described by the Poisson-Boltzmann equation\(^{35}\).

\[
d^2\psi/dx^2 = -ze\rho/(\varepsilon_0\varepsilon) = -[ze\rho_0/(\varepsilon_0\varepsilon)]e^{-ze\psi/k_BT}
\]  

Here, \( z \) defines the valency of the charged particle, \( e \) the elementary charge and \( \rho_0 \) and \( \rho \) are the charge densities at the surface and the distance \( x \), respectively.

When solving this equation it gives the electrostatic potential (\( \psi \)), electric field (\( E_{el} \)) and counter ion density (\( \rho \)) at any point \( x \) between two like charged surfaces.

Interestingly, a repulsive force between two like charged surfaces surrounded by ions is originating from the pressure of counter-ions and co-ions confined in the space in between these two surfaces. This pressure originates from entropy (osmosis) rather than electrostatic reasons. Ions need to rearrange,
maximising their configurational entropy. While the electrostatic field describes forces, the electrostatic potential describes energies. In order to describe equilibria behaviour of proteins, the electrostatic potential is thereby the desired value to be determined. The energy \( w \) needed to move a charge in the presence of a fixed electrostatic field \( E_{el} \) by the infinitesimal distance \( dl \) is the product of the force \( F_{el} \cdot dl \). Hence, with equation 2 we attain\(^{36}\):

\[
W_{AB} = -q \int_{A}^{B} E_{el} \cdot dl
\]  

(4)

The difference of electrostatic potential \((\psi_A, \psi_B)\) is the work needed for a unit test charge \( q_{test} \) to move from A to B.

\[
\frac{W_{AB}}{q_{test}} = \psi_B - \psi_A = \Delta \psi
\]  

(5)

In other words, the electrostatic field is a measure of the gradient of the electrostatic potential and is defined as:

\[
E_{el}(x) = -\frac{\partial \psi}{\partial x}
\]  

(6)

Depending on the concentration of the ions and their valencies the surface charge of a protein is proportional to is emanating potential. For low surface potentials \((\leq 50 \text{ mV})\) this can be simplified by the so called Debye-Hückel equation

\[
\psi_x \approx \psi_0 e^{-\kappa x}
\]  

(7)

Whereas \( \psi_x \) the potential in distance \( x \) and \( \psi_0 \) is the surface potential of the surface charge and \( 1/\kappa \) is referred to as the Debye length, where

\[
\kappa = \sqrt{\sum_i p_{\infty i} e^2 z_i^2 / (\varepsilon_0 \varepsilon k_B T)} \text{ m}^{-1}
\]  

(8)

Here, \( p_{\infty i} \) is ionic bulk concentration, \( e \) is the elementary charge and \( z \)
the valency of the ions.

The Debye length describes the exponential decay of the potential from the surface and is described as

$$\kappa^{-1} = \frac{0.304}{\sqrt{[M]}} \, \text{nm}$$

for an 1:1 electrolyte concentration [M].

![Diffusive double layer of charged surfaces in electrolyte solution](image)

**Figure 3: Diffusive double layer of charged surfaces in electrolyte solution** A negatively charged surface will create counter-ion gradient of positive solvated cations away from the surface. The potential above the surface $\omega_x$ will decrease exponentially with the factor of the Debye length $\kappa$ (see equation 7).

For AFM measurements electrostatic interactions can be quantified if the charged AFM tip and the sample surface are repelling each other\(^\text{10}\).

In liquid systems these repulsive forces between tip and sample become apparent at tip sample-distances ranging from 3-10 nm. At smaller distances the continuum theories of the van der Waals force and double-layer forces are not valid anymore. Here other, much more complicated forces arise\(^\text{33}\). Interactions that take only van der Waals and double layer forces into account
are called DLVO (Derjaguin-Landau-Verwey-Overbeek) interactions\(^{22}\) and in the course of this thesis we extracted forces in the range of the above mentioned distances.

In a simplified way, in order to calculate the effective \(E_{el(x)}\) and \(\psi_x\) in a given electrolyte one can assume a charged AFM tip as a point charge that is experiencing a repulsive force at a distance \(x\) from the charged protein surface. Therefore, the electrical Field at a distance \(x\) form the sample is equal to the repulsive force divided by the tip charge (\(E_{el(x)} = F_{el}/q_{Tip}\), compare with equation 2) and the difference of electric potential between the protein surface and \(x\) is \(\Delta \psi = E_{el(x)} \cdot x\). Using the Debye-Hueckel theorem (see equation 7) one can then take the electrolyte concentration into account (via the Debye length) and deduce the surface potential, and theoretically also the surface charge of the protein. One problem, however, that this is relevant only for interacting point charges and the geometry of tip and sample has to be taken into account.

In the course of this PhD thesis these forces were analyzed at tip-sample distance between 3 and 5 nm to interpret the electrostatic interactions between an intrinsically negatively charged Silicon nitride AFM tip and the emanating electrostatic field of the outer membrane protein F (OmpF). This work was published in the journal *Nano letters* in 2013\(^{37}\) and is described in section 2.

**Detecting ligand receptor interactions by FD based AFM**

The previous paragraph was describing how we can exploit the FD based AFM in liquids to investigate basic interaction principles between a channel proteins and its charged solutes. Now we want to address the interaction of a specific ligand that interacts with a binding site of native proteins, preferably membrane receptors.

A great deal of the functionality of the aforementioned biological key elements depends on the molecular recognition of their interaction partners.
Molecular recognition plays a crucial role in biological systems. This recognition happens between ligand and receptor, antigen-antibody, DNA-protein, RNA-ribosome, sugar-lectin and so on.

AFM tips can be chemically functionalized with binding partners such as ligands, this will be described in detail further below.

When imaging biological samples with the functionalized AFM tip a successful interaction is described as an adhesion force that is correlating to the topography of the protein. Hence, the ligands binding site can be correlated to the structure of the protein. This mode is called molecular recognition force spectroscopy^{38,39,40}.

These interactions are attractive and the way to detect them by FD-based AFM is to allow a ligand tethered to a cantilever to come in contact with the receptor, so that the ligand can find its binding pocket. Upon withdrawal the interaction force is registered as a negative cantilever deflection if a specific ligand-receptor complex was formed (see Fig.4). AFM has recently emerged as a powerful tool to localize single receptor-ligand interactions and give insight into the dynamic association and dissociation of these bonds. Herefor, the AFM is applied as FD-based AFM.

**Tip functionalization**

Prior to the detection measurements an elaborated tip-surface modification chemistry that renders the AFM tip functional for molecular recognition is necessary to detect the ligand-receptor interactions^{24,41,38}. A second requirement to functionalize the AFM tip surface is to passivate the remaining areas of the tip to prevent it thereby from any unspecific interaction. Usually, the functional group is linked via a tether to the Si or Si₃N₄ tip. This gives the functional group a certain transitional and rotational freedom that enables it to find its binding pocket when in contact with the probed protein.

Since a dissociation or rupture event happens after the tether is fully extended it comes along with a rupture distance that is defined by the length
Figure 4: Schematic representation of the force spectroscopy experiment with a functionalized AFM tip and its corresponding FD curve. Approach and retraction of the functionalized tip towards the receptor protein. The functional molecule is terminating the linker molecule (red). During retraction the linker molecule is stretched and nearly no cantilever deflection is visible. When ligand and receptor are dissociated again the linker molecule is fully stretched and the interaction force pulls on the cantilever. Finally, the ligand-receptor complex ruptures visible in a significant rupture peak of the FD curve.
of that stretched molecular linker. Thus, one can differentiate between specific and unspecific interactions by looking at the rupture-lengths at which the ligand-receptor dissociation occurs. Unspecific interactions, that occur due to interactions with the AFM tip surface will show adhesion events at small tip-sample distances (≤5 nm).

Several surface modification protocols to functionalize the AFM tip have been established and in the course of this PhD thesis two main modification strategies have been pursued (see Fig. 5). In any case, a preferred route is to attach the ligand via a flexible polymer tether to the tip. Both approaches begin with a surface decontamination and activation by UV-Ozone radiation. Here, reactive ozone radicals are formed by the effect of UV light on atmospheric oxygen. These remove any organic contamination by oxidizing it to CO₂. Furthermore, the reactive oxygen species produce hydroxyl groups (-OH groups) on the metal surface that are needed for the subsequent functionalization step. The first functionalization strategy is followed by a bivalent metal coating performed through electron beam thermal evaporation that yields a 3 nm aluminum and a 10 nm thick gold layer on top of the SiO₂ layer of the AFM tip. The desired functional molecules can be covalently attached to the gold surface if they are bearing a terminal thiol (-SH) group that readily forms a covalent S-Au bond²² (see Fig. 5 A). The second strategy involves an amino functionalization of the activated AFM tip SiO₂ surface. Next, a heterobifunctional linker molecule specifically attaches to the newly formed amino groups and renders a reactive maleimide group at its proximal unreacted site. These react then in a separate reaction step with thiol-groups of the desired functional group/ligand. Thereby, for example also peptides can be readily covalently bound to the linker, if containing a terminal cysteine (see Fig. 5 B). Both approaches render functional tips that are covered with either a gold layer/functional group or amino-groups/linker/functional groups thereby increasing the tip-apex. The imaging capability, however, will consequently deteriorate since the AFM tip apex will become bigger.
Figure 5: Functionalization pathways of Si$_3$N$_4$ AFM tips. (A) Clean AFM cantilevers are coated with two subsequent layers of aluminium and gold (step 1), respectively. Thiol terminated functional groups are then covalently attached to the Au-surface via forming S-Au bonds (step 2). (B) Activated (Step 1), amino-functionalized cantilevers (step 2) are covalently linked to a hetero-bifunctional PEG linker (step 3). The final functionalization step happens between a thiol-containing functional group and the residing maleimid group of the linker.
Functionalization route A in figure 5 was applied to successfully detect Hexa-histidine (His$_6$) groups of native cartwheel like SAS-6 proteins at a resolution of $\approx$ 3 nm. This work is thoroughly described in section 2 and was published in the journal *Nano letters* in 2014\textsuperscript{42}.

The following paragraph will describe how collected data of detected rupture events between a ligand-receptor complex can not only determine their position but also lead to thermodynamic and kinetic informations of the ligand-receptor bond.

**AFM mediated dissociation of ligand-receptor bonds**

Investigating the dissociation behavior of a ligand-receptor complex under the application of an external force has been intensively studied in the past, known as single molecule force spectroscopy (SMFS)\textsuperscript{40,43}. Performing SMFS using the AFM, mostly the ligand is tethered to the tip, ligand and receptor are brought in close contact and are subsequently retracted from each other. This dissociation is a stochastic process where the ligand-receptor system is transferred from a bound state into an unbound state. Both states are Boltzmann distributed according to their energetic substrates and underlying interaction potential of ligand and receptor.

The chemical bond is generally stiffer than the AFM cantilever, thus in the simplest case, we can represent the tip-sample interaction with a single-well potential, and assume a parabolic potential of a Hookean spring for the cantilever. As the scanner moves the cantilever away from the biological surface the secondary minimum emerges on the potential energy surface. A force-induced bond rupture in the AFM is simply a thermally driven transition from the bound state into an unbound state over a potential energy surface that is constantly modified by the time dependent potential of the loading spring. At the moment of this transition the measured rupture strength of the bond is defined\textsuperscript{45}. This fundamentally kinetic view of the unbinding process, formulated in the pioneering works by Bell and Evans is the key to
Figure 6: Energy landscape of ligand receptor complex dissociation with and without external force (A) A ligand-receptor complex dissociation can be described a single step Markov process. The ligand-receptor bond must overcome an activation barrier to reach its transition state from where reached the the final dissociated products. The diagram below indicates the populations of bound (red) and unbound (blue) states correlating to their equilibrium constant. (B) When an external force is applied the activation barrier is lowered by $E_F = E_0 - Fx$ and hence the dissociation process is accelerated. (C) Representation of the microscopic view of a SMSF experiment during the dissociation of a ligand-receptor bond transferring from bound (red) via a transition state to an unbound (blue) state.
understanding the physics of force spectroscopy experiments.

It was found by Bell and co-workers in 1978\textsuperscript{46}, that the kinetic barriers of this bond can be calculated when observing the dissociation times (life times) over a range of applied forces during the mechanical unbinding. Or, in other terms, by investigating the unbinding forces at different loading rates. This referred to dynamic forces spectroscopy (DFS).

Evans and Ritchie discussed that unbinding behavior based upon the model of Bell in 1997\textsuperscript{47}. They described this process as a thermally activated decay of a bound state into it's unbound state.

The dissociation kinetics of a ligand-receptor bond is described by an exponential increase of the dissociation rate with a growing unbinding force.

The unbinding rate ($k_{off}$) is the product of a natural vibration frequency of the bond ($\omega_0$) and the probability of reaching the transition state energy barrier $E_b$ whereas $E_b$ is discounted by the external applied force over a distance.

At the point the ligand-receptor complex dissociates the pulling-force is referred to as the rupture force. The transition can be modelled as a rate process with the rate coefficient $k_{off}$ affected by the time-dependent pulling force $F(t)$.

$$k_{off} = \omega_0 e^{-\frac{(E_b - F(t)x_B)}{k_BT}}$$  \hspace{1cm} (10)

Where $x_B$ describes the distance to the transition-state and $k_BT$ is the Boltzmann constant times the temperature.

During retraction, the ligand-receptor bond is usually loaded at a constant speed. This implies that during time $(t)$ a force is build up that equals $v_{Tip} \cdot k_c \cdot t$, where $v_{Tip}$ is the tip-velocity (in nm/sec) and $k_c$ is the cantilever spring constant. Depending on the life time of a bond, it will rupture at the built-up force.

Since bond life times are distributed according to their energetic states, this unbinding process is stochastic. To find the most probable unbinding
force the maximum of the probability distribution \( P(F) \) of unbinding forces must be found \(^{48} \):

\[
P(F) = \frac{k_{off}(0)}{r} \cdot \exp[Fx/k_BT + k_{off}(0) r^{-1}k_BT/x(1 - e^{Fx/k_BT})] \tag{11}
\]

Where \( r \) is the loading rate (e.g. pulling velocity \( \times \) spring constant), and \( x \) pulling distance.

Under these conditions the most probable unbinding force is described by:

\[
<F> = \frac{k_BT}{x_B} \cdot \ln \left( \frac{r x_B}{k_{off} k_BT} \right) \tag{12}
\]

Thus, measuring rupture events at a broad range of loading range, defining the most probable unbinding force for each loading rate and plotting rupture force vs the natural logarithm of the loading rate lets one unlock the kinetic parameters of the ligand-receptor interaction under zero force. The slope of that plot determines the position of the transition state, \( x_B \), the y-intercept the dissociation rate \( k_{off} \) and thereby the \( \Delta G^t \), the Gibb’s energy of activation be calculated

\[- \Delta G^t = k_BT \ln \left( \frac{k_{off} h}{k_BT} \right) \tag{13}\]

Although a generally accepted model to interpret force-spectroscopy experiments the Bell-Evans model has a few draw-backs.

For example, these models neglected possible re-binding events after the ligand-receptor bond is stressed by an external force. However, at low loading rates the ligand-receptor complex is in a near equilibrium state. The Friddle-De Yorero model\(^{49} \) allows for a rebinding probability at very slow
loading rates, thereby describing an thermodynamic equilibrium behavior of the ligand-receptor complex. The binding transition-rate ($k_b$) is defined by the natural frequency of the transducer, which is scaled by the Boltzmann-weighted energy of an extended spring with distances ranging from the spring minimum and the barrier location (see Eq. 14). The unbinding rate ($k_u$) still follows the Bell model, that predicts a linear force dependence on the barrier (see Eq. 15). We get

$$k_b(f) = k_b^0 \exp \left[ -\beta \left( \frac{f}{k_c} - x_t \right)^2 \right]$$  \hspace{1cm} (14)

$$k_u(f) = k_u^0 \exp \left[ \beta \left( f x_t - \frac{1}{2} k_c x_t^2 \right) \right]$$  \hspace{1cm} (15)

$$= k_u(f) \exp \left[ \beta \left( \Delta G_{bu} - \frac{f^2}{2k_c} \right) \right]$$  \hspace{1cm} (16)

Where $\beta^{-1}$ is $k_B T$ and $k_c$ is the cantilever spring-constant.

The unbinding force is then approximated as:

$$F(r) = f_{eq} + f_\beta \ln \left( 1 + \frac{re^{-\gamma}}{k_\text{off}(f_{eq}) f_\beta} \right)$$  \hspace{1cm} (17)

Here, $\gamma$ is the Euler’s constant, $f_\beta$ is $(k_B T)/x_u$ and $f_{eq}$ is the force at which dissociation- and association-rate are equal given by the equilibrium equation:

$$f_{eq} = \sqrt{2k_c \Delta G_{bu}}$$  \hspace{1cm} (18)

This eventually lets one determine the true free binding energy $\Delta G_{bu}$ of the bond.

$k_{\text{off}}(eq)$ is the dissociation rate at $f_{eq}$. 
This model predicts an equilibrium regime at low loading rates and a kinetic regime at high loading rates. Performing the experiment at a wide range of loading rates one can plot again the rupture forces vs the logarithm of loading rates.

Fitting the equation 17 to the plot lets one extract the parameters \( f_{eq} \), \( f_\beta \) and \( k_{off}(f_{eq}) \). From equation 18 \( \Delta G_{bu} \) can be calculated, \( x_u \), the distance to the transition state is equal to \( (k_B T)/f_\beta \).

A further aspect that has to be considered is that a tether doesn’t behave like a harmonic oscillator, which is the prerequisite for describing the pulling potential as a quadratic one (see equation 15). Therefore, we need to correct the \( kc \) in equation 18 with an effective spring constant \( k_{eff} \). This can be done by describing the visco-elastic properties of the linker molecule as a worm-like-chain model and combining both, the cantilever spring constant and the linker spring constant. For example a PEG spacer molecule would be described as:

\[
\frac{1}{k_{eff}} = \frac{1}{k_c} + \frac{2\beta L_c l_p(1 + \beta F l_p)}{3 + 5\beta F l_p + 8(\beta F l_p)^{5/2}} \tag{19}
\]

Whereas, \( k_c \) is the nominal cantilever spring constant, \( L_c \) is the contour length of, \( \beta = (k_B T)^{-1} \) and \( l_p \) is the persistence length of the linker-functional molecule system.

In the context of this PhD thesis native PAR1 proteins could be imaged and several specific interaction events were detected between an AFM tip that was functionalized with the native binding peptide of PAR1. The Friddle-de Yoyer model was applied on the data set of rupture force-loading rate pairs. This made it possible not only to assess the kinetic but also the thermodynamic properties of the ligand receptor bond. A manuscript has been written so summarize this work and was submitted to the Journal Nature Protocols. This manuscript is reprinted in section 2.

Additionally, in the course of this PhD thesis a technique on how to detect several different ligand-receptor interaction simultaneously using FD-based
AFM was developed. This technique allows, similar to multi-color fluorescence imaging, to detect and separate an extra- and intracellular interaction site of reconstituted PAR1. This work was summarized in a manuscript which is currently submitted for publication and reprinted in section 2.

References


1 INTRODUCTION


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2 Results

Quantitative imaging of the electrostatic field and potential generated by a transmembrane protein pore at subnanometer resolution

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Abstract

Elucidating the mechanisms by which proteins translocate small molecules and ions through transmembrane pores and channels is of great interest in biology, medicine and nanotechnology. However, the characterization of pore forming proteins in their native state lacks suitable methods that are capable of high-resolution imaging (∼1 nm) while simultaneously mapping physical and chemical properties. Here we report how force-distance (FD) curve based atomic force microscopy (AFM) imaging can be applied to image the native pore forming outer membrane protein F (OmpF) at sub-nanometer resolution and to quantify the electrostatic field and potential generated by the transmembrane pore. We further observed the electrostatic field and potential of the OmpF pore switching ‘on’ and ‘off’ in dependence of the electrolyte concentration. Because electrostatic field and potential select for charged molecules and ions and guide them to the transmembrane pore the insights are of fundamental importance to understand the pore function. These experimental results establish FD-based AFM as unique tool to image biological systems to sub-nanometer resolution and to quantify their electrostatic properties.

Keywords: Force-volume AFM, electrostatic interactions, multiparametric imaging, nanopore, surface charge,
The transport of ions and molecules across cellular membranes is essential for many processes of life. Therefore, understanding of this transport finds broad interest in biology and medicine and is required to engineer synthetic nanopores. From atomic-resolution structures determined for proteins forming transmembrane pores, channels and pumps we begin to understand the architecture and mechanism of selective molecular transport. To be specifically transported across the cellular membrane ions and molecules have to undergo a subset of physical and chemical interactions. Among these electrostatic interactions take essential roles. The electrostatic properties of transmembrane proteins can be calculated and visualized using three-dimensional (3D) structural models. However, so far no experimental method exists that can image native proteins at sub-nanometer resolution and at the same time probe their electrostatic properties in vitro. Such a method would be of particular interest to image transmembrane pores or channels and to structurally localize and to quantify the electrostatic fields that translocate molecules, ions, ligands, or proteins.

Since its invention, atomic force microscopy (AFM) has been applied to image the surface of native membrane proteins at sub-nanometer resolution. Because AFM can record topographs in buffer solution and at ambient temperature it enables the observation of single membrane proteins at work. Examples comprise the surface-layer from Deinococcus radiodurans, connexins forming animal communication channels in epithelial cells, the light-driven proton pump bacteriorhodopsin, the cyclic nucleotide-regulated and the pH-gated potassium channel, the ATP-gated purinergic receptor P2X4, and the outer membrane proteins (Omps) OmpG and OmpF from Escherichia coli. Although such AFM imaging studies bring exciting insight into the structure-function relationship of membrane proteins, the understanding of how membrane proteins work requires gathering additional information. Interestingly, within the past decade AFM has been developed towards a multifunctional nanotool that allows quantify-
ing structural, biophysical and chemical properties of biological systems\textsuperscript{10,26}. The so-called force-distance (FD) curve based AFM (FD-based AFM; also called force-volume AFM (FV-AFM)) contours a sample surface while approaching pixel-by-pixel AFM tip and sample to record FD curves (Supporting Information Figure S1a,b)\textsuperscript{27-30}. Analyzing such FD curves allows the mechanical and electrostatic properties of the sample to be determined and mapped to the sample topography\textsuperscript{29,31}. For more than a decade FD-based AFM has suffered from technical limitations such as slow imaging speed, limited number of pixels, poor force resolution ($\approx$0.1–1 nN), and poor spatial resolution ($\approx$10–100 nm)\textsuperscript{29}. Recent advances pushed the limits of FD-based AFM to detect biological forces in the pN regime, at a spatial resolution approaching 1 nm, and at imaging times approaching that of conventional AFMs ($\approx$8 min for 512x512 pixels)\textsuperscript{32-36}. Owing to these developments FD-based AFM is increasingly used to characterize the mechanical properties of biological samples\textsuperscript{30}. In this work we introduce FD-based AFM to image single membrane proteins and at the same time to structurally map the electrostatic field and potential generated by their transmembrane pore. The experiments demonstrate that FD-based AFM can be used to quantify the electrostatic properties of native protein surfaces at sub-nanometer resolution.
Quantitative imaging of the electrostatic field and potential generated by a transmembrane protein pore at subnanometer resolution

Figure 1: FD-based AFM topographs of native OmpF trimers reconstituted into lipid membranes. (a) FD-based AFM topograph showing an OmpF membrane adsorbed onto mica. OmpF membranes protruded 7.0±1.0 nm (n=60) from the supporting mica. (b) Higher magnification topograph revealing OmpF trimers assembled into two-dimensional crystalline arrays. (c) Cross-correlation averaged and symmetrized OmpF trimers recorded in (b). OmpF trimers exposing their extracellular (purple line) and periplasmic (green line) surface are outlined. Full color ranges correlate to vertical scales of 15 nm (a), 2.0 nm (b) and 1.7 nm (c). FV-AFM topographs were recorded in buffer solution (300 mM KCl, 10 mM Tris-HCl, pH 7.4) applying an imaging force of 150 pN. (d) Model showing the packing of OmpF trimers imaged in (c). Purple OmpF trimers expose the extracellular surface and green trimers expose the periplasmic surface. OmpF trimers (PDB file 1OPF) were arranged using Pymol. OmpF trimers have been reconstituted into lipid (DMPC) membranes (Materials and Methods).

Results & Discussions

FD-curves detect mechanical and electrostatic properties of biological samples. FD curves recorded by AFM describe a mechanical process (Supporting Information Figure S1a,b). For this reason, in the majority of cases, FD curves are taken to measure mechanical properties of a sample. For example, indenting an AFM tip into a soft biological sample repels the tip in a distinct way. Measuring this repulsion allows to quantify the deformation of the sample (Supporting Information Figure 1c). Depending on
the analysis of FD curves many other mechanical properties of the sample can be quantified\textsuperscript{29,31}. In the aqueous solution the surfaces of the AFM tip and of most biological systems are charged\textsuperscript{7,37}. As both surfaces approach they electrostatically repel each other if their surface charges are of the same sign. More than two decades ago it was shown that the electrostatic repulsion between AFM tip and sample can be measured by recording FD curves (Supporting Information Figure 1d). This repulsion can be used to quantify the surface charge of the AFM tip or of a point on the biological sample\textsuperscript{31,38,39}. In principle this electrostatic repulsion could be used to structurally localize the electrostatic properties of native proteins using FD-based AFM\textsuperscript{29,31,39}. Moreover, FD-based AFM can quantify and map this repulsion directly in the so-called deformation channel (Supporting Information Figure 1d). However, until now quantifying the electrostatic interactions of complex biological samples at sub-nanometer resolution has been hampered by technological and methodological limitations\textsuperscript{30}. In the following we apply FD-based AFM to image native membrane proteins at high-resolution and to quantify and visualize their electrostatic properties.

**FD-based AFM imaging of native OmpF.** As sample we have chosen the OmpF from \textit{E. coli} because its structure and function has been well studied and its transmembrane pore displays unique electrostatic features\textsuperscript{40-42}. OmpF is a transmembrane $\beta$-barrel protein that naturally exists as trimer. Each $\beta$-barrel, folded from 16 antiparallel $\beta$-strands, forms a transmembrane pore through which hydrophilic solutes up to $\approx$600 Da can freely diffuse\textsuperscript{42,43}. The translocation rate of the pore is linearly related to the solute concentration. However, the cation selectivity of the transmembrane pore increases with decreasing electrolyte concentration\textsuperscript{44}. This selectivity originates from charged amino acids, which line the inner constriction of the transmembrane pore and generate an electrostatic potential the strength of which depends on the electrolyte concentration of the aqueous solution\textsuperscript{43,45-47}. Whereas the negative electrostatic potential generated by the OmpF pore is greatest in
the absence of electrolyte it becomes increasingly screened as the electrolyte concentration rises.

To characterize OmpF by FD-based AFM the trimeric membrane proteins were purified (Supporting Information Figure S2), reconstituted into lipid membranes and adsorbed onto freshly cleaved mica in buffer solution\textsuperscript{48}. The OmpF membranes were then imaged in buffer solution by FD-based AFM (Figure 1). To suppress most of the electrostatic repulsion between the AFM tip and the OmpF membrane the pH and electrolyte concentration of the buffer solution were chosen pH 7.4 (10 mM Tris-HCl) and 300 mM KC\textsubscript{1}\textsuperscript{49}. At low magnification membrane patches of densely packed OmpFs were observed (Figure 1). At higher magnification the supramolecular assembly of the OmpF trimers became visible (Figure 1b-c) showing the alternating rows of trimers exposing either their periplasmic or extracellular surface (Figure 1d).

**Sub-nanometer imaging and mapping mechanical properties of OmpF.** Next, we increased the lateral and vertical resolution of the FD-based AFM topographs. The imaging feedback parameters (Materials and Methods) were iteratively optimized until the surface of the native OmpF trimers could be contoured at a force sensitivity of ±7 pN, the AFM topographs revealed finest structural details, and the error force signal was minimized. The pixel size of the AFM topographs was \(\approx 0.3 \times 0.3 \text{ nm}^2\) to enable a lateral resolution <1 nm. At the optimized imaging force of \(\approx 150\) pN the AFM tip contoured in great detail the alternating rows of OmpF trimers exposing either their periplasmic or extracellular surface (Figure 2a). The long extracellular polypeptide loops of the OmpF trimer protruded 1.3 ± 0.2 nm (average ± SD; \(n=50\)) from the lipid membrane, whereas the doughnut-like entrances surrounding the transmembrane pores protruded 0.5 ± 0.1 nm (\(n=30\)). Cross-correlation averaged topographs showed the long extracellular loops of the OmpF trimers and the periplasmic doughnut-like entrance of the transmembrane pores more clearly (Figure 2a). The resolution of these FD-based AFM topographs, as estimated from structural details of single
OmpF trimers, approached ≤1 nm.

To optimize the signal-to-noise ratio of the deformation map, deformation values were recorded applying forces ranging from 45 pN to 150 pN to the OmpF membrane. The lower force threshold of 45 pN was chosen to clearly lie above the noise (~10–20 pN) of the FD curves. Consequently, the deformation value represented the difference of the tip-sample distances recorded at 45 pN and at the imaging force of 150 pN (Supporting Information Figure S1). Correlation of the FD-based AFM topograph and the simultaneously recorded deformation map revealed a slightly enhanced deformation for OmpF trimers exposing their extracellular surface (~0.4 ± 0.1 nm, n=40) compared to OmpF trimers exposing their periplasmic surface (~0.2 ± 0.1 nm, n=40, Figure 2b). The maximum deformation of 0.4 nm localized at the long and flexible extracellular polypeptide loops that protruded 1.3 nm from the lipid bilayer.

The high-resolution FD-based AFM topographs were in excellent agreement with topographs determined earlier by high-resolution contact-mode AFM and with the atomic structure of the OmpF trimer. The heights detected for the extracellular and periplasmic loops, each of which connecting two β-strands forming the transmembrane β-barrel, were within ±0.2 nm of those measured from high-resolution contact mode AFM topographs and the atomic structure of the OmpF trimer. This demonstrates that providing the imaging parameters are adjusted appropriately the structurally flexible domains of native membrane proteins can be contoured at sub-nanometer resolution using FD-based AFM. However, the periplasmic loops deformed to a lesser extent (~0.2 nm) than the long extracellular polypeptide loops (~0.4 nm). These deformation values suggest that the protein was only slightly deformed. Averaged topograph and deformation map of the OmpF trimer (Figure 2a,b) showed that the slightly enhanced deformation at the extracellular surface was localized at the inner vestibule formed by the three extracellular loops of the OmpF trimer. Previous AFM measurements showed
that these extracellular loops exhibit an enhanced structural flexibility that allows them to reversibly collapse onto the OmpF pore. This collapse has been suggested to close and gate the transmembrane OmpF pore. A similar closing mechanism induced by extracellular loops was observed to gate the transmembrane pore formed by the β-barrel forming outer membrane proteins OmpG and maltoporin (LamB) from *E. coli*. These insights lead to the speculation that the deformation map recorded of the extracellular OmpF trimer surface locates the functionally relevant structural flexibility of the extracellular loops.

In summary, the high-resolution FD-based AFM topographs showed that the flexible polypeptide loops of the extracellular surface of the OmpF trimer can be contoured in their fully protruding state (Figure 2a). In addition the doughnut-like periplasmic entrance of the transmembrane pore was contoured in great detail. This demonstrated that the conditions used to record high-resolution FD-based AFM topographs allow the observation of native OmpF trimers in the minimally perturbed state.

**Detecting electrostatic interactions of OmpF at sub-nanometer resolution.** Amino acid residues lining the constriction of the transmembrane OmpF pore can generate a predominantly negative electrostatic potential that promotes the transport of cations and of positively charged molecules. At sufficiently high electrolyte concentration, for example at 300 mM KCl at neutral pH, this electrostatic potential is largely screened and the charge selectivity of the pore disappears. However, at an electrolyte concentration of 20 mM KCl the negative electrostatic potential established by the transmembrane pore is sufficiently strong to repel anions and to preferentially transport cations. As with an anion, the silicon AFM tip carries a negative charge at neutral pH and if the transmembrane pore of OmpF establishes a sufficiently strong negative electrostatic potential the AFM tip will be electrostatically repelled. To see whether this electrostatic repulsion can be detected by FD-based AFM we recorded the same OmpF trimers,
which been before imaged at 300 mM KCl, at 20 mM KCl (Figure 2c). The high-resolution FD-based AFM topograph recorded at 20 mM KCl (Figure 2c) showed very similar structural details to the topograph recorded at higher electrolyte concentration (Figure 2a). However, the deformation map recorded at low electrolyte concentration showed significantly increased values of $1.2 \pm 0.1$ nm ($n=50$) at the periplasmic pores of the OmpF trimer (Figure 2d), whereas the deformation detected at the long and flexible polypeptide loops of the extracellular surface did not change ($0.4 \pm 0.1$ nm; $n=50$).

In summary, the correlation of FD-based AFM topographs with deformation maps showed that at low electrolyte concentration the OmpF trimer did not change structure but that the transmembrane pores at their periplasmic surface generated considerable deformation maxima of 1.2 nm. These deformation maxima quantify the repulsion between the AFM tip and the transmembrane OmpF pore (Figure 1d). The atomic model of the OmpF trimer shows that the periplasmic pores are open and cannot be structurally occluded. In addition, the high-resolution topographs of the OmpF trimers do not indicate any deformation in the range of $\approx 1.2$ nm. Therefore, we conclude that the electrolyte dependent repulsion detected at the transmembrane pore is not related to structural changes.

Characterizing the electrostatic repulsion of the transmembrane pore.

To better understand the origin of the electrolyte dependent deformation detected at the transmembrane OmpF pore we calculated the electrostatic potential generated by the OmpF trimer. For this we have set up an atomistic model of the OmpF trimer embedded into a DMPC bilayer (Supporting Information Figure S3) and computed the equipotential surfaces of the electrostatic potential by solving the Poisson-Boltzmann equations (Figure 3 and Supporting Information Figure S4). At 300 mM monovalent electrolyte the
Quantitative imaging of the electrostatic field and potential generated by a transmembrane protein pore at subnanometer resolution.

Figure 2: High-resolution FD-based AFM of native OmpF trimers recorded at different electrolyte concentrations. Topograph (a) and deformation map (b) of OmpF trimers recorded in ‘high’ electrolyte concentration (300 mM KCl, 10 mM Tris-HCl, pH 7.4). OmpF trimers reconstituted in a lipid (DMPC) membrane exposed their extracellular (purple line) and periplasmic (green line) surface. Topograph (c) and deformation maps (d) of the same OmpF membrane but recorded in ‘low’ electrolyte concentration (20 mM KCl, 10 mM Tris-HCl, pH 7.4). Insets show two-fold symmetrized cross-correlation averages (n=60) of OmpF trimers. FD-based AFM topographs were recorded applying an imaging force of 150 pN. Full color ranges correspond to vertical ranges of 1.6 nm (a and c), 0.5 nm (b) and 1.1 nm (d). Deformation values give the difference of the tip-sample-distances recorded at 150 pN and 45 pN.

The negative electrostatic potential generated by the pore is rather weak and remains localized to the protein surface (Figure 3a). However at 20 mM mo-
novalent electrolyte the negative electrostatic potential generated by the pore became much stronger and protruded into the aqueous solution (Figure 3c). The electrostatic potential extended into the solution much further from the periplasmic surface than from the extracellular surface. In contrast, the electrostatic potential at the extracellular surface remained inside the vestibule formed by the long extracellular loops (Supporting Information Figure S4).

A negatively charged AFM tip interacting with a negative electrostatic potential such as generated by the OmpF trimer will be electrostatically repelled\textsuperscript{31,38,39,47,49}. If sufficiently large, this repulsive interaction can be detected by recording FD curves between AFM tip and sample. As FD-based AFM records these FD curves for every pixel of the sample topograph we extracted FD curves from our high-resolution topographs for further analysis (Figure 3). Because single FD curves can vary considerably we averaged 100 FD curves recorded at the periplasmic pore and 100 FD curves recorded at the extracellular loops (Supporting Information Figure S5). At high electrolyte concentration (300 mM KCl) FD curves recorded at the extracellular surface showed a slightly enhanced repulsion compared FD curves recorded at the periplasmic surface (Figure 3b). This was expected from high-resolution deformation maps that detected enhanced values at the structurally flexible extracellular loops (Figure 2b). When lowering the electrolyte concentration to 20 mM KCl the FD curves recorded on the extracellular surface of the OmpF trimer showed no significant change (Figure 3d). In contrast, FD curves recorded at the periplasmic pore detected a significantly enhanced repulsion. This repulsion was fully reversible and only depended on the electrolyte concentration of the buffer solution (at pH 7.4). At low electrolyte concentration (20 mM KCl) the repulsion detected at the periplasmic pore was maximal whereas at high electrolyte concentration (300 mM KCl) the repulsion disappeared.

In a nutshell, the combination of simulations and FD curves revealed that at low electrolyte concentration the OmpF trimer generates significant elec-
Quantitative imaging of the electrostatic field and potential generated by a transmembrane protein pore at subnanometer resolution

trostatic repulsion at the periplasmic pore. In FD-based AFM this repulsion was detected as deformation maximum above the periplasmic OmpF pore (Figure 2c, 2d).

**Visualizing and quantifying the electrostatic field and potential generated by the transmembrane pore.**

Superimposing the deformation map and AFM topograph of the OmpF trimers shows clearly that with decreasing electrolyte the electrostatic repulsion contributes to the deformation value (Figure 4). This electrostatic repulsion maximizes above the periplasmic OmpF pore (Figure 4d-f). The atomistic model of the OmpF trimer (Supporting Information Figure S3) shows that the periplasmic pore lacks structural features that could contribute to the increased deformation value detected at low electrolyte concentration and highlight the electrostatic nature of the repulsion quantified. The electrostatic nature of this repulsion is nicely detected by FD curves recorded at the OmpF pore (Figure 3d). In the following we analyze the repulsive electrostatic force detected at the OmpF pore to quantify the electrostatic field and potential generated by the transmembrane pore.

Using Coulomb's law we can roughly estimate the electrostatic field by dividing the force of the electrostatic repulsion through the net charge $Q_{\text{tip}}$ of the AFM tip. $Q_{\text{tip}}$ is the surface charge density multiplied with the surface area of the AFM tip. We quantified the surface charge density of the silicon AFM tip by recording FD curves of freshly cleaved mica in aqueous solution containing 3 mM KCl (Supporting Information Figure S6). At such low electrolyte concentration the electrostatic double layer repulsion between AFM tip and mica can be clearly detected, which allowed to estimate the surface charge density of the AFM tip to $-0.032 \pm 0.010 \, \text{C/m}^2$ (n=5)$^{31,39}$. This value corresponds to the surface charge density ($-0.032 \, \text{C/m}^2$) previously determined for Si$_3$N$_4$ tips$^{38}$. From the spatial resolution of our AFM topographs we estimated the radius of the AFM tip to be $\approx 2 \, \text{nm}^{52}$. Approximating the shape of the AFM tip interacting at high-resolution with
the sample to be a half-sphere this yields a total charge of the AFM tip $Q_{\text{tip}}=(-8.04\pm2.51) \times 10^{-19}$ C\textsuperscript{52,53}. With the deformation threshold force of $\approx 45$ pN used (Figure 3) we can conclude that at low electrolyte concentration (20 mM KCl) the AFM tip contoured the electrostatic field at $(-56.0\pm25.4) \times 10^6$ V/m. Because FD-based AFM quantifies the distance at which this field is detected from the pore entrance ($\approx 1.2$ nm) we can calculate the electrostatic potential generated at the pore entrance to be $-67 \pm 35$ mV. This experimentally approached potential is very similar compared to the negative electrostatic potential calculated from the atomistic OmpF model (Figure 3, Figure 4 and Supporting Information Figure S4). Although the experimental and calculated values agree remarkably well improved data analysis and theoretical approaches may in the future provide even more accurate values.

Electrostatic potentials across cellular membranes, including the outer membrane from \textit{E. coli}, typically range from $-30$ to $-100$ mV\textsuperscript{42,54,55}. With a membrane thickness ranging from 5 to 8 nm such potentials generate electrostatic fields ranging from (-4 to -20) $\times 10^6$ V/m. Thus, the electrostatic field and potential generated by the OmpF pore is of similar range as typically generated across cellular membranes. However, in contrast to the electrostatic field/potential generated across cellular membranes, OmpF generates a structurally localized electrostatic field/potential by charged residues lining the constriction of the transmembrane pore\textsuperscript{43-47}. By acting over longer distances, the electrostatic field/potential of every OmpF pore generates electrostatic forces that are able to guide diffusing charged molecules and ions into the vicinity of the transmembrane pore, where short range interactions (van der Waals, hydrogen, or steric) are able to take hold\textsuperscript{56-58}. With decreasing electrolyte concentration this electrostatic field/potential generated by the OmpF pore increases strength, protrudes further from the membrane and interacts over an extended distance/volume with charged solutes. According to the Boltzmann law, an electric potential of $-67$ mV at the OmpF pore will
increase the local concentration of cations by a factor of \( \approx 14 \) compared to the cation concentration of the bulk solution. This increased electrostatic potential may help \( E. \ coli \) to accumulate positively charged solutes (including cations) at the OmpF pore for transport across the outer membrane at low electrolyte concentrations (\( \leq 20 \text{ mM} \)), which occur for example in the lower part of large intestines.
Figure 3: Electrostatic potential calculated and electrostatic repulsion measured of OmpF trimers embedded into the lipid membrane. (a, c) Electrostatic potential calculated based on the atomistic model of the OmpF trimer (PDB 1OPF)\(^\text{40}\) embedded in a lipid (DMPC) membrane (Supporting Information Figure S3). For clarity lipids are not shown. The electrostatic potentials calculated for aqueous solutions containing 300 mM (a) and 20 mM (c) monomeric electrolyte are displayed as equipotential surfaces at \(\approx 77\) mV. Side views of OmpF trimers and other equipotential values are displayed in Supporting Information Figure S4. Green and purple circles indicate the regions from which FD-curves were extracted from FD-based AFM topographs (Figure 2). (b, d) FD curves recorded either on the periplasmic or the extracellular surface of OmpF trimers imaged in Figure 2. FD curves have been recorded in buffer solution containing either 300 mM KCl (b) or 20 mM KCl (c) at pH 7.4. Each FD curve represents the average of 100 FD curves (Supporting Information Figure S5). At low electrolyte concentration the increasing electrostatic repulsion between the AFM tip and the periplasmic surface of OmpF porin significantly alters the shape of the FD curve (comp. Supporting Information Figure S1d). FD-AFM records this electrostatic repulsion as ‘deformation’. ‘Deformation’ values indicated by blue (b) and red (d) arrows give the difference of the tip sample-distances recorded at 150 pN and at 45 pN, which are mapped in the deformation maps (Figure 2).
Figure 4: FD-based AFM topographs and deformation maps recorded of the native OmpF trimer localize and quantify the electrostatic field and potential generated by the transmembrane pore. (a) Periplasmic (green arrows) and extracellular (purple arrows) surface of OmpF trimers recorded at high electrolyte concentration (300 mM KCl). Individual periplasmic channel entrances of OmpF are visible (green arrows) whereas the extracellular surface of OmpF protrudes long characteristic polypeptide loops above (≈1.3 nm) the surface of the lipid membrane (purple arrows). (b) The deformation map recorded at high electrolyte concentration shows maxima at the structurally flexible extracellular loops (purple arrows) and minima at periplasmic pores (green rows). (c) Superimposition of topograph and deformation map recorded at high electrolyte concentration. (d) Periplasmic (green arrows) and extracellular (purple arrows) surface of OmpF trimers recorded at low electrolyte concentration (20 mM KCl). (e) The deformation map recorded at low electrolyte concentration shows maxima at the periplasmic entrance of the pores (green arrows) whereas the deformation of the structurally flexible extracellular loops remained as detected at 300 mM KCl (purple arrows). The enhanced deformation values recorded at low electrolyte concentration are based on the electrostatic repulsion (Figure 3). (f) Deformation values recorded in the presence of electrostatic repulsion contour the electrostatic field (red) and potential (blue) generated by the OmpF pore. Full color ranges correspond to vertical ranges of 1.7 nm (a and d), 1.2 nm (b, c and e), and −56 x 10⁶ V/m (red, f) and −67 mV (blue, f).
Conclusion

We have visualized and quantified the electrostatic field and potential generated by the transmembrane pore forming protein OmpF using FD-based AFM. The imaging process is sufficiently sensitive to reproducibly image the native surface of OmpF in the lipid membrane at physiological conditions and at sub-nanometer resolution. The deformation of the structurally flexible OmpF surfaces could be reduced to a minimum of \( \approx 0.2 \) nm. FD-curves are particularly well suited to detect the electrostatic interaction between the AFM tip and a sample. The transmembrane pore of OmpF generates a negative electrostatic field and potential, which is screened at increased electrolyte concentrations but becomes strong at low (or none) electrolyte concentration. Because the AFM tip is also negatively charged an electrostatic repulsion between AFM tip and pore is recorded in FD-curves. Conveniently, FD-based AFM can directly contour the electrostatic field by recording deformation maps. The deformation value quantifies the distance from the protein surface at which the electrostatic field is sufficiently strong to repel the charged AFM tip with a given force (here \( \approx 45 \) pN). Hence the deformation map contours the electrostatic field generated by the transmembrane pore at a given strength. Using the Coulomb's law the electrostatic field strength can be approximated from the electrostatic force and the surface charge of the AFM tip. Furthermore, multiplying the electrostatic field strength by the distance from the pore at which this field strength was detected approximates the electrostatic potential generated by the pore. In principle the technological approach described here can be applied to structurally map and quantify the electrostatic properties of other biological systems at high resolution. However, its utility might be further explored by using a conductive AFM tip which electric potential can be modulated\(^59\). The utility of the approach may be also expanded by chemically modifying the AFM tip to render its surface charge (and chemical properties)\(^60,61\).

Using chemical groups that change charge upon photoactivation would for
example allow to switch the electrostatic properties of the AFM tip and to
discern mechanical (uncharged groups) from electrostatic (charged groups)
interactions. With such functionalized tips FD-based AFM could be ap­
plied in the future to spectroscopically characterize and structurally map the
electrostatic properties of single native membrane proteins at sub-nanometer
resolution. Such structurally resolved quantification of electrostatic proper­
ties is not only important to understand transmembrane ion channels and
pores but also to characterize many other biomolecular processes, such as cell
adhesion, macromolecular assembly, molecular recognition, transport, and si­
gnaling.

Materials and Methods

**Purification and reconstitution of OmpF.** OmpF from *E. coli* was puri­
ified by detergent extraction and solubilized in n-octylpolyoxyethylene (Octyl
POE, 1% w/v, Enzo LifeSciences) as described. Purity of OmpF was ve­
rified by SDS-PAGE and silver-staining gels (Supporting Information Figu­
re S2). Solubilized OmpF was reconstituted into 1,2-dimyristoyl-sn-glycero-
3-phosphorylcholine (DMPC, Avanti Lipids) by dialysis-driven detergent removal. Briefly, OmpF
was mixed with n-dodecyl-N,N-dimethylamine-N-oxide (LDAO)-solubilized
DMPC (1% w/v, Affymetrix) at a lipid-to-protein ratio of 0.5 (w/w), pla­
ced into dialysis buttons and dialyzed against detergent-free buffer (100 mM
NaCl, 20 mM HEPES, 10 mM MgCl₂, 0.01 % [w/v] NaN₃, 0.2 mM dithioth­
reitol (DTT), pH 7.4) for 6 days at 30 °C. All chemicals used were analytical
grade and the water used was ultrapure (>18 MΩm/cm).

**High-resolution FD-based AFM imaging.** Native OmpF reconstituted
in into lipid membranes was prepared for AFM as described. Briefly, OmpF membranes were adsorbed onto freshly cleaved mica in buffer solu­
tion (300 mM KCl, 10 mM Tris-HCl, pH 7.6). After 50 minutes of adsorption
the sample was rinsed with the imaging buffer (20 mM KCl or 300 mM,
10 mM Tris-HCl, pH 7.4) and imaged in imaging buffer at room temperature (\(\approx 22 ^{\circ} \text{C}\)). FD-based AFM imaging was performed using a Bruker Nanoscope Multimode 8 (Bruker, Santa Barbara, USA) equipped with a 120 \(\mu\)m piezoelectric scanner (J-scanner). Rectangular shaped silicon cantilevers (38 \(\mu\)m long) had a sharpened silicon tip with a nominal radius of 8-10 nm, a nominal spring constant of 0.1 N/nm, and a resonance frequency of \(\approx 110 \text{ kHz}\) in liquid (Biolever mini, Olympus). During AFM imaging FD curves were recorded at a frequency of 2 kHz applying a sinusoidal amplitude of 24 nm to the piezoelectric element moving the sample vertically. High-resolution AFM images were taken at a scan rate of 0.65 Hz and a pixel size of 3 x 3 \(\text{Å}^2\) (768 x 768 pixel at 230 nm x 230 nm scan size). The maximum deflection for each FD curve (imaging force) was preset to 150 pN with an accuracy of 5-7 pN\(^3\), 64. Feedback-loop parameters of the AFM were adjusted to minimize the imaging force error and maximize the topographic resolution. FD-based AFM images were simultaneously recorded in trace and retrace scanning direction and saved in separate channels for the topograph, deformation, elastic modulus (DMT) and imaging force error. Deformation values were mapped at 70% (45 pN) of the imaging force (150 pN). Before and after recording FD curves of the OmpF membrane we recorded FD curves on the supporting mica to see whether the AFM tip contaminated. In case of contaminations FD curves recorded on mica changed significantly in shape and reproducibility. To brush of contaminations from the AFM tip the mica was scanned at high speed and modulating the forces applied to the AFM cantilever\(^48\).

**Averaging of FD-based AFM images.** High-resolution FD-based AFM topographs and deformation channels simultaneously recorded of densely packed OmpF trimers were cross-correlation averaged using the SEMPER image processing system as described\(^3\), \(^6\). Briefly, unit cells of individual OmpF trimers were localized and extracted from high-resolution topographs, cross-correlated, averaged and symmetrized. Position and orientation of every OmpF trimer as revealed from analyzing the FD-based AFM topographs
were taken to extract and orient unit cells from the deformation channel. For each channel the unit cells were then averaged and symmetrized.

**Averaging of FD curves.** Individual FD curves recorded from the extracellular and the periplasmic surface of OmpF were exported as text file using the FD-based AFM data processing software (NanoScope Analysis, Bruker). FD curve text files were transferred to MatLab (MathWorks Corporation). The baseline of each FD curve was brought to zero force (offset correction) and the tip-sample distance was approximated from the vertical piezo movement minus the cantilever deflection. FD curves recorded of the extracellular and periplasmic OmpF surface were separately aligned at their first data point and superimposed. From the superimposed FD curves a density map was calculated using MatLab with data points being colored according to their density.

**Computation of the electrostatic potential.** To compute the electrostatic potential of the OmpF trimer, the X-ray structure of the trimer (PDB identifier 1OPF) was embedded into a DMPC bilayer consisting of 512 DMPC molecules and equilibrated at 303 K (Supporting Information Figure S3). Embedding was carried out using the g__membed module of the GROMACS simulation suite version 4.5.467, and the Amber99-ildn-sb* extensions to the Amber99 force field. Amber99 force field parameters of DMPC lipids were taken as published. Electrostatics computations were carried out using the adaptive Poisson-Boltzmann solver (APBS) interface of the visual molecular dynamics software VMD on 2,146,689 grid points. Mobile ion concentrations were considered implicitly. The APBS pqr input files were generated with editconf (www.gromacs.org). Solutions were obtained using the FEtk finite element solver. Equipotential surfaces were obtained by interpolating between grid points having the same electrostatic potential and overlayed to the solvent accessible surface of the trimers (Figures 3 and Supporting Information Figure S4).
References


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Quantitative imaging of the electrostatic field and potential generated by a transmembrane protein pore at subnanometer resolution


Quantitative imaging of the electrostatic field and potential generated by a transmembrane protein pore at subnanometer resolution
Quantitative imaging of the electrostatic field and potential generated by a transmembrane protein pore at subnanometer resolution - Supplementary Information

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Supplementary Information. Schematics showing the principles of FD-based AFM (Figure S1), SDS gel of OmpF trimers purified from *Escherichia coli* (Figure S2), all-atom setup used to calculate the electrostatic potential generated by the OmpF trimer (Figure S3), electrostatic potential calculated of the OmpF trimer at different electrolyte concentrations (Figures S4), FD curves recorded on the extracellular and the periplasmic surface of the OmpF trimer (Figures S5) and determination of the surface charge density of the silicon AFM tip (Figures S6). This material is available free of charge via the Internet at http://pubs.acs.org.
Supplementary Figure S1: Principles of force-distance (FD) curve based AFM. FD-based AFM contours the sample surface (a) and pixel-by-pixel approaches (black arrow) and retracts (red arrow) AFM tip and sample to record FD curves (b). The AFM cantilever deflection measures the force interacting between AFM tip and sample. Attractive forces pull the AFM tip toward the sample and the cantilever deflects downward (negative). Repulsive forces between AFM tip and sample deflect the cantilever upward (positive). To record the sample topography at a preset force, the AFM tip is pressed onto the sample until a preset maximum contact force (imaging force) is reached, which triggers the reverse movement (retraction) of the tip. Finally, the sample topography is contoured at the imaging force. (c) The mechanical deformation of a soft biological sample is described by the indentation of the much stiffer AFM tip. This indentation is detected as repulsive force. Conventionally, deformation values give the distance recorded at the imaging force (here 150 pN) minus the distance recorded at a given threshold force (here 45 pN). '0 nm' approximates the contact between AFM tip and sample. (d) Exemplified FD curves recorded between a stiff silicon nitride AFM tip and stiff freshly cleaved mica in aqueous solution containing 0.02, 0.3 or 3 M monovalent electrolyte. The force applied to record the FD curves was too small to mechanically deform mica. The shapes of the FD curves demonstrate that the electrostatic repulsion between AFM tip and sample (mica) depends on the electrolyte concentration. In the case shown, increasing the electrolyte concentration decreases the electrostatic double layer repulsion. Thus, the 'deformation value' of FD curves can measure electrostatic repulsion.
Supplementary Figure S 2: SDS gel of OmpF trimers purified from *Escherichia coli*. SDS-PAGE gel of purified and solubilized OmpF (in 1% (w/v) Octyl POW) before (lanes 1-4) and after (lanes 5-8) treatment with 100 mM EDTA in 1% (w/v) LDAO for 4h at room temperature. Electrophoresis was performed at room temperature using 12% separating and 5% stacking gels. Gels were stained with coomassie brilliant blue. Purified OmpF migrates at a mass of \( \approx 80 \) kDa, which corresponds to the calculated mass of 117 kDa of the transmembrane OmpF trimer\(^4\). The ladder-like pattern of OmpF (lane 1-4) corresponds to increasing numbers of lipopolysaccharides (LPS) bound to the outer membrane protein\(^8\). Applied OmpF concentrations were between 0.5 and 4.0 µg per lane.
**Supplementary Figure S 3:** All-atom setup used to calculate the electrostatic potential generated by the OmpF trimer at different electrolyte concentrations. (a) Side view of the OmpF trimer (PDB entry file 1OPF) embedded into a lipid (DMPC) membrane. The top shows the extracellular and the bottom the periplasmic surface of the OmpF trimer. All atoms of the lipids and the backbone of the OmpF trimer are displayed. (b) Top view on the periplasmic surface. All atoms of the lipids and the surface of the OmpF trimer are displayed.
Supplementary Figure S 4: Electrostatic potential calculated of the OmpF trimer at different electrolyte concentrations. The negative electrostatic potential of the OmpF trimer calculated for 300 mM and 20 mM monovalent electrolyte is displayed using equipotential surfaces at (a) −35 mV, (b) −67 mV, and (c) −101 mV. Shown are the top and tilted views of the periplasmic and extracellular surface of the OmpF trimer.
Supplementary Figure S 5: FD curves recorded on the extracellular and the periplasmic surface of the OmpF trimer. 100 FD curves were extracted on the periplasmic and extracellular surface of OmpF from high-resolution FD-based AFM images recorded at high (a-c) and low (d-f) electrolyte. (a) and (d) show the FD curves as density plot derived from the extracellular surface, (b) and (e) from the periplasmic surface, respectively. (c) and (f) show the averages of the FD curves recorded at the periplasmic (green) and extracellular (purple) surface of the OmpF trimer.
Supplementary Figure S 6: Determining the surface charge density of the silicon AFM tip by recording FD curves on mica in electrolyte solution. Figure caption are followed on next page.
Supplementary Figure S. 6: Determining the surface charge density of the silicon AFM tip by recording FD curves on mica in electrolyte solution. Shown is an average FD curve that has been calculated from 1,000 superimposed FD curves. The data points of the superimposed 1,000 FD curves are shown in black and their average in blue. Every single FD curve has been recorded approaching the silicon tip of the AFM cantilever (BioLever mini) to freshly cleaved mica in aqueous solution (ultrapure water, >18 MOhm/cm) containing 3 mM KCl electrolyte. The 1,000 FD curves have been recorded on five different locations on the mica surface at 0.5 Hz acquisition rate, 44 data points/nm, and applying a maximal force of 0.4 nN. The electrostatic double layer force interacting between the AFM tip and mica in electrolyte solution can be described as following\textsuperscript{7,8}:

\[ F_{el}(D) = \frac{4 \pi \varepsilon_0 R \sigma_m \sigma_T \lambda}{\varepsilon_0 \varepsilon_r \sigma_T} \cdot e^{-D/\lambda} \]  

Equation S1

with the nominal tip radius \( R \approx 10 \) nm\textsuperscript{*} (BioLever mini), the Debye length \( \lambda = 0.304/(0.0031^{1/2}) \) nm, the surface charge density of mica in 3 mM KCl is \( \sigma_m = -0.009 \) C/m\textsuperscript{2} [ref.\textsuperscript{9}] the distance between AFM tip and mica \( D \), the permittivity of vacuum \( \varepsilon_0 = 8.854 \times 10^{-12} \) C/Vm, the relative permittivity of water \( \varepsilon_r = 80.1 \), and the surface charge density of the AFM tip \( \sigma_T \). Accordingly, the charge density of the AFM tip can be determined at \( D = 0 \) nm plotting \( \sigma_T = \left( \frac{F_{el}(0) \varepsilon_0 \varepsilon_r \sigma_T}{4 \pi R \sigma_m \lambda} \right). \) However, because the absolute distance between AFM tip and mica surface is difficult to determine,\textsuperscript{2} we fitted the FD curve using Eq. S1 using the parameters given above to determine \( \sigma_T \). The fitted curve is shown in red dashes. Measurements and analysis were repeated five times using five different AFM cantilevers. The fits revealed a surface charge density of the silicon AFM tips (Biolever mini) of \(-0.032 \pm 0.010 \) C/m\textsuperscript{2} (\( n = 5 \)).

\* The nominal tip radius taken for determining the surface charge density of the AFM tip was larger compared to the tip radius used for high-resolution AFM imaging (see main manuscript). The reason was that the electrostatic double layer interaction extends over several nanometers, whereas the interaction contributing to high-resolution AFM images must be much more localized to be able to contour the sample surface a sub-nanometer resolution\textsuperscript{10,11}. 

Quantitative imaging of the electrostatic field and potential generated by a transmembrane protein pore at subnanometer resolution - Supplementary Information
Supporting References


Localizing chemical groups while imaging single native proteins by high-resolution AFM

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Abstract

Simultaneous high-resolution imaging and localization of chemical interaction sites on single native proteins is a pertinent biophysical, biochemical and nanotechnological challenge. Such structural mapping and characterization of binding sites is of importance in understanding how proteins interact with their environment and in manipulating such interactions in a plethora of biotechnological applications. Thus far this challenge remains to be tackled. Here, we introduce force-distance curve based atomic force microscopy (FD-based AFM) for the high-resolution imaging of SAS-6, a protein that self-assembles into cartwheel-like structures. Using functionalized AFM tips bearing Ni²⁺-N-nitrilotriacetate groups, we locate specific interaction sites on SAS-6 at nanometer resolution and quantify the binding strength of the Ni²⁺-NTA groups to histidine residues. The FD-based AFM approach can readily be applied to image any other native protein and to locate and structurally map histidine residues. Moreover, the surface chemistry used to functionalize the AFM tip can be modified to map other chemical interaction sites.

Keywords: Biomolecular bonds, chemical recognition imaging, multiparametric imaging, force spectroscopy, ligand-receptor interaction, single-molecule imaging and spectroscopy
Introduction

Originally conceived as an imaging tool, atomic force microscopy (AFM) rapidly developed into a multifunctional toolbox enabling researchers to observe biological systems including tissues, cells, membranes and single molecules, and to manipulate these systems with unprecedented precision. More recently, the AFM has matured into a multiparametric tool that allows the imaging of biological systems while simultaneously mapping their mechanical properties to sub-nanometer resolution. The imaging and quantification of mechanical properties of biological samples has been much simplified by force-distance (FD) curve based AFM (FD-based AFM), which records FD curves for every pixel of the AFM topograph. For each FD curve, the AFM tip is approached to contact and is withdrawn from the biological sample. The deflection of the AFM cantilever detects the interaction forces between tip and sample. These interaction forces enable the quantification of adhesion, deformation, Young’s Modulus, energy dissipation, and other parameters that mechanically characterize the biological sample. Because FD-based AFM records one or more FD curves for every pixel of the sample topograph these properties can be directly correlated to the structure of the biological system under investigation. Recently, FD-based AFM has been applied to measure mechanical properties of cells, viruses, protein membranes, lipid membranes, proteins, and fibrils.

Interestingly, FD-based AFM can also detect specific interactions that are exposed on biological samples. To measure these interactions, FD-based AFM records the interaction forces between a chemically functionalized AFM tip and a sample. This approach allows the direct correlation of a sample topography with structurally localized mechanical properties and a specific interaction map. Recent examples have used AFM tips functionalized with specific bioligands to image live cells and at the same time to locate cell surface receptors functionalized with chemical groups to...
map the chemical properties of cell surfaces\textsuperscript{31}, and functionalized with specific lectin to map N-acetylgalactosamine-terminated glycolipids on red blood cells\textsuperscript{28}. In these examples, FD-based AFM was also used to quantify the force required to rupture particular biochemical interactions. Remarkably, analysis of the force required to separate biochemical bonds (e.g., receptor-ligand bonds) can provide insight into their kinetic, energetic and mechanical properties\textsuperscript{32,33}. However, although powerful, FD-based methods using functionalized AFM tips to locate and characterize specific interactions have been impaired by poor temporal and lateral resolution, which has limited their use in high-resolution imaging of single proteins.

In this work, we introduce FD-based AFM for the combined high-resolution imaging and quantitative high-resolution mapping of specific interaction sites in single native proteins. The experiments demonstrate that FD-based AFM equipped with Ni\textsuperscript{2+}-N-nitrilotriacetate (Ni\textsuperscript{2+}-NTA) functionalized AFM tips can be employed to specifically locate His\textsubscript{6}-tags on native proteins at nanometer resolution and to characterize the strength of the chemical bond formed between NTA and histidine residues of the protein.
Figure 1: Locating and quantifying specific interactions using FD-based AFM. a) Pixel-for-pixel FD-based AFM approaches and retracts the tip of an AFM cantilever from the sample to record interaction forces, F, over the tip-sample distance in FD curves. The high precision of the approach allows detection of forces with piconewton sensitivity and pixel sizes <1 nm with a positional accuracy of ≈0.2 nm. b) Approach (blue) and retraction (red) FD curves. The upper graph shows the force recorded over time. The lower graph shows FD curves recorded upon approach and retraction. The AFM tip is approached until a maximal contact force (Ft) is reached. Zero distance indicates the contact point of tip and sample. Following parameters can be extracted from FD curves: distance (sample height) at maximum contact force, deformation, elasticity (Young’s modulus), energy dissipation and adhesion. c) AFM tip functionalized by NTA-terminated polyethylene glycol (PEG)-alkanethiols to detect the adhesion forces to CGA-His6 peptides derivatized to a ≈10 nm thick gold (Au) layer coating the cantilever. To prevent the AFM tip from unspecific interactions it was additionally covered by non-terminated PEG-alkanethiols. d) FD curves recording i) no interactions of NTA-terminated AFM tip and Au surface derivatized with unspecific CGSL-PETGG peptides, ii) specific interactions between Ni^{2+}-NTA-terminated AFM tip and Au surface derivatized with CGA-His6 peptides in the presence of Ni^{2+}, and iii) unspecific interactions of NTA-terminated AFM tip and Au surface derivatized with CGA-His6 peptides in the absence of Ni^{2+}. 
Results & Discussion

Functionalizing the AFM tip and optimizing the experimental parameters for detecting histidine residues

In order to locate histidine residues, we modified AFM tips to present NTA groups at their surfaces. Therefore, Au-coated AFM tips were functionalized with a mixture of NTA-terminated polyethylene glycol (PEG)–alkanethiols and PEG-alkanethiols in order to dilute NTA groups in a PEG matrix (Figure 1c). This PEG matrix limits unspecific interactions between the AFM tip and the biological sample or sample support and minimizes contamination of the tip.\(^{34-36}\) NTA is a tetradental ligand that forms a hexagonal complex with divalent metal ions, such as Ni\(^{2+}\), occupying four of the six binding sites. The remaining two binding sites are accessible to electron donor groups such as free electron-pair carrying nitrogen atoms of histidine side-chains. To ensure specific interactions, histidine side chains have to be oriented to fit the coordination sphere of the hexagonal Ni\(^{2+}\)-NTA complex. These specific interactions are frequently used to purify proteins engineered to carry a short polypeptide stretch of five to eight histidine residues, termed histidine-tag (His\(_{5-8}\)), at defined structural positions.

As a proof of concept, we measured the interaction between NTA-Ni\(^{2+}\) functionalized AFM tips and Au-coated mica supports, which were covalently functionalized with either i) ‘unspecific’ Cys-Gly-Ser-Pro-Glu-Thr-Gly-Gly (CGSLPETGG) peptides or ii) Cys-Gly-Ala-His\(_6\) (CGA-His\(_6\)) peptides that can specifically bind to NTA groups (Figure 1c). Using FD-based AFM, the functionalized tip and support were repeatedly brought into contact and separated. Every approach and retraction cycle was recorded as FD curves (Figure 1d). The contact time of the AFM tip with the support (≈220 ± 60 µs) and the frequency of periodic approach and retraction (0.5 kHz) were then optimized to be able to detect chemical interactions (Materials and Methods). In the case of Au-coated supports functionalized with peptides lacking a histidine-tag, the FD curves showed no significant adhesion events.
Localizing chemical groups while imaging single native proteins by high-resolution AFM

(Figure 1d, top FD curve). In the case of Au-coated supports functionalized with CGA-His\textsubscript{6} peptides, the FD curves recorded in the presence of the coordinating Ni\textsuperscript{2+} showed force peaks indicating adhesion events (Figure 1d, middle FD curve). On average, the adhesion force peaks amounted 172 ± 56 pN (ave ± SD; n=85) and were detected at a distance of 5.7 ± 1.5 nm from the support (Supporting Information Figure S1). These adhesion forces are in accordance with those measured for the rupture of single NTA-His\textsubscript{6} bonds in vitro, which range from 50 to 250 pN\textsuperscript{36-38}. The FD curves of measurements taken with Au-coated supports functionalized with CGA-His\textsubscript{6} peptides in absence of coordinating Ni\textsuperscript{2+} showed very low adhesion force peaks indicating weak adhesion events (Figure 1d, bottom FD curve). On average, these lower adhesion force peaks amounted 45 ± 8 pN (n=200) and were detected in the tip-sample contact region. These control experiments showed that the stronger adhesion between Ni\textsuperscript{2+}-NTA and histidines residues was abolished after removing Ni\textsuperscript{2+} from the AFM tip (Figure 1d). In summary, these results show that functionalization of an AFM tip with NTA-terminated PEG-alkanethiols enables the detection of specific interactions between the chemically functionalized AFM tip and histidine residues covalently tethered to a Au-coated support. Unspecific interactions were largely suppressed by functionalizing the AFM tip with chemically inert non-terminated PEG-alkanethiols. Although similar procedures to functionalize AFM tips with Ni\textsuperscript{2+}-NTA groups to detect histidine residues have been reported previously\textsuperscript{34-36}, we here adapted and optimized the system for our experimental FD-based AFM setup.

**High-resolution FD-based AFM imaging**

After having demonstrated that we can functionalize AFM tips to detect specific interactions established between Ni\textsuperscript{2+}-NTA and histidine groups we wanted to apply these tips to image native proteins and to localize their histidine residues. As a model system we used the spindle assembly abnormal protein 6 homolog (SAS-6), a protein that is implicated in centriole duplication. Each SAS-6 molecule encompasses two independent dimerizati-
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on interfaces. Whereas the N-terminal domains dimerize head-to-head, the C-terminal domains form $\approx 40$ nm long parallel two-stranded coiled-coil rods (Figure 2a, inset). This arrangement of dimerization interfaces leads to the self-assembly of SAS-6 molecules into a cartwheel-like structure in which the coiled-coil rod domains emanate radially from a central ring (Figure 2a)\textsuperscript{39,40}. For our AFM experiments we fused a His\textsubscript{6}-tag to the C-terminal end of \textit{C. reinhardtii} SAS-6 (Materials and Methods, Supporting Information Figure S2). Before imaging the engineered SAS-6 cartwheels using Ni\textsuperscript{2+}-NTA functionalized AFM tips we wanted to image SAS-6 using untreated AFM tips. These FD-based AFM images were to be used as reference points for the subsequent functional AFM imaging.

For FD-based AFM, the SAS-6 specimens were adsorbed onto freshly cleaved mica in adsorption buffer (300 mM KCl, 20 mM Tris, pH 7.2)\textsuperscript{41}. The sample was subsequently imaged in imaging buffer (150 mM KCl, 20 mM Tris, pH 7.2) by FD-based AFM using untreated AFM tips and applying imaging forces of $\approx 50$ pN (Figure 2b). It has previously been shown that AFM imaging forces of $\approx 50$ pN are sufficiently low to prevent the deformation of fragile biological samples such as lipids, proteins and even single polypeptide loops connecting transmembrane $\alpha$-helices of single proteins\textsuperscript{16,42}. AFM topographs revealed that the mica support was densely covered with a network of SAS-6 cartwheels (Figure 2b,c). At the distal end of each coiled-coil rod domain we observed a single protrusion that was wider and higher than the rest of the rod. Each of these protrusions localizes the C-terminal ends of a SAS-6 dimer. The structural details were in agreement with the well-known cartwheel of SAS-6 (Figure 2a)\textsuperscript{39,40,43}. However, single coiled-coil rods emanating from the central ring of SAS-6 differed in orientation, which suggests elevated flexible properties.

**Localizing His\textsubscript{6}-tags on SAS-6 cartwheels**

After having characterized the structure of SAS-6 oligomers, we used FD-
Localizing chemical groups while imaging single native proteins by high-resolution AFM

Figure 2: High-resolution FD-based AFM of SAS-6 cartwheel structures. a) Structural model of the SAS-6 cartwheel with coiled-coil rod domains radially emanating from the central ring formed by the N-terminal domains. At the distal end of each coiled-coil rod the SAS-6 subunit exposes its C-terminus to which six histidine residues were fused (C-terminal His₆-tag). b) FD-based AFM topograph illustrating the SAS-6 specimens adsorbed onto mica. c) High-resolution topograph of SAS-6 oligomers clearly reveals their cartwheel structures. The C-terminal domains at the distal ends of the coiled-coil rods are clearly visible. AFM topographs were recorded in imaging buffer (150 mM KCl, 20 Tris, pH 7.2) at ≈27 °C.

Based AFM to detect the His₆-tags that had been genetically engineered to the C-terminal end of each coiled-coil rod. The high-resolution AFM topographs recorded using functionalized Ni²⁺-NTA AFM tips show SAS-6 cartwheels distributed on mica supports (Figure 3a). Single protrusions located at the end of the coiled-coil rods were observed. The resolution of the AFM topographs recorded with the functionalized AFM tips was slightly lower (≈3-4 nm) compared to those recorded with the untreated tips (≈2-3 nm). The reason for this slight reduction in resolution is that functionalization of the AFM tip included the deposition of a 13 nm thick Al-Au layer onto the tip and its coating with a PEG matrix (Figure 1c). This treatment increased the AFM tip radius and thus contributed to the reduced resolution of the AFM topographs.

Adhesion maps corresponding to the AFM topographs localized the adhesive forces detected by the functionalized Ni²⁺-NTA-AFM tip (Figure 3b). The specific adhesion forces ranged between 80 and 220 pN (Supporting Information Figure S3a). For each adhesion event detected we extracted single FD
results curves for further analysis (Figure 3c). Most FD curves revealed an adhesive event at a distance (e.g., rupture length) that was clearly separated from the tip-sample contact region (>4 nm). On average the corresponding rupture forces were 124 ± 33 pN (Supporting Information Figure S3b). However, the rupture lengths ranged from 4 to 22 nm with an average of 11.7 ± 4.8 nm (n=85). This result suggests that the ≈40 nm long flexible coiled-coil rod domains bearing the His6-tag lifted up at their ends when stressed by the withdrawal of the AFM tip. These adhesion events, which were clearly unlike those measured on the mica support, were judged to be specific. Rarely we detected comparable adhesion forces in the contact region between tip and sample (<4 nm). We considered these adhesion events to be unspecific.

After classifying adhesion events as either specific or unspecific, the adhesion maps were directly compared to the corresponding AFM topographs (Figure 3a, b). The specific events were localized at the His6-tag containing protrusions at the distal ends of the coiled-coil rods of SAS-6 cartwheels, whereas unspecific adhesion events were not related to SAS-6. However, correlation of AFM topographs and adhesion maps showed that only a small number of the His6-tagged C-terminal ends of SAS-6 were detected by the NTA-Ni2+ functionalized AFM tip. Several effects may have contributed to this low detection frequency: i) the side chains of the histidine residues may not always be accessible to the Ni2+-NTA complex attached to the AFM tip. For example, some histidine residues may face the supporting mica or be structurally oriented in a way that does not complement the coordination sphere of the hexagonal Ni2+-NTA complex. It could also be that some His6-tags are not detected because they apparently form contacts with the coiled-coil rods of neighboring SAS-6 dimers (Figure 2, 3). ii) the contact time between the functionalized AFM tip and the histidine residues could be too short to allow binding in every instance45. iii) during FD-based AFM imaging the PEG-Ni2+-NTA complex attached to the AFM tip could adopt transient conformations some of which may not be suitable to bind histidine
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residues.

To further investigate whether the detected binding events were indeed specific, we performed two independent controls. In the first control, we used the same FD-based AFM setup used to map specific interactions and to image SAS-6 cartwheels in the absence of Ni$^{2+}$ (Figure 3d-f). In the absence of Ni$^{2+}$ the NTA-groups attached to the AFM tip cannot interact specifically with histidine residues$^{38}$. The AFM topographs recorded in the absence of Ni$^{2+}$ revealed similar SAS-6 cartwheel structures as observed in the presence of Ni$^{2+}$ (Figure 3d). However, the adhesion maps recorded low interaction forces at positions where no protein was observed (Figure 3d,e). On average this low adhesion force was 65 ± 17 pN (n=98). Analysis of the FD curves showed that these adhesion force peaks occurred in the contact region of the AFM tip and the sample and so these forces were judged to be unspecific (<4 nm) (Figure 3f). In the second control experiment, the AFM tip was functionalized with only PEG groups (Figure 3g). Again the high-resolution AFM topograph showed individual SAS-6 cartwheels with their emanating coiled-coil rods and the adhesion map detected low adhesion forces (<70 pN) in regions areas without protein (Figure 3g,h). On average this low adhesion force was 55 ± 11 pN (n=200). Furthermore, the force curves indicated that these adhesion events occurred in the proximate, 'unspecific' contact region of the AFM tip and sample. These two control experiments both showed significantly fewer adhesion events with lower adhesion forces. Most importantly no adhesion events occurred with rupture length of >4 nm indicative of the stretching of the PEG linker and of the coiled-coil rods bearing the His$_6$-tags. Additionally, these adhesive events were not associated with SAS-6 cartwheels. This result confirms that the stronger adhesive interaction forces shown in Figure 3a-c are specific and originate from interactions between the Ni$^{2+}$-NTA groups attached to the AFM tip and the His$_6$-tags located at the C-terminal end of the SAS-6 coiled-coil rod domains.
Figure 3: Figure captions on following page.
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Figure 3: Mapping chemical interactions of SAS-6 cartwheels using high-resolution FD-based AFM. a-c) Mapping specific interaction forces between the Ni$^{2+}$-NTA-modified AFM tip and His$_6$-tag containing SAS-6 oligomers. a) High-resolution AFM topograph of SAS-6 specimens adsorbed onto a freshly cleaved mica support. Some of the SAS-6 molecules assembled to partial or full cartwheels. b) Map of specific (red circles) and unspecific (grey circles) adhesion forces ranging between 100 and 200 pN. c) FD curve of each pixel detecting an adhesion event in the adhesion map. Red colored FD curves (S1-S4) detect specific adhesion events that are clearly separated from the tip-support contact region (<5 nm). The black colored FD curve detects an unspecific adhesion event within the tip-support contact region (U1). d-f) In the absence of the coordinating Ni$^{2+}$ the NTA-modified AFM tip cannot detect specific interaction events. d) High-resolution topograph of SAS-6 cartwheels. e) Map of adhesion forces ranging between 100 and 200 pN. f) FD curve of each pixel detecting an adhesion event in the adhesion map. The FD curves detect unspecific adhesion events within the tip-support contact region (U2-U6). g-i) In the absence of NTA PEG-modified AFM tips cannot detect specific interaction events. g) High-resolution topograph of SAS-6. h) Map of adhesion forces ranging between 50 and 80 pN. i) FD curve of each pixel detecting an adhesion event in the adhesion map. The FD curves detect unspecific adhesion events within the tip-support contact region (U7, U8). AFM topographs were taken in buffer solution (150 mM KCl, 20 mM Tris, pH 7.2 while including 1 mM NiCl$_2$ for a-c)) at ~27 °C. Dashed circles help correlating topography and adhesion events.

Improving the adhesion maps of His$_6$-tags fused to SAS-6 molecules

After having demonstrated that AFM tip functionalized with Ni$^{2+}$-NTA groups can be employed to detect His$_6$-tags in SAS-6, we sought to improve the recording of adhesion maps. One disadvantage of these maps is that the number of specific adhesion events detected is rather low (see discussion above). The simplest way to increase the number of adhesion events and thereby to improve the detection of histidine residues is to increase the number of FD curves recorded. In addition, we also wanted to investigate whether the histidine residues of SAS-6 can be repeatedly detected. Therefore we decided to record multiple high-resolution adhesion maps of SAS-6 cartwheels (Figure 4). The adhesion events from three consecutive adhesion maps show that, in most cases (88.2% (31 of 34)), adhesion was repeatedly detected (at
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least twice) at the same structural regions (Figure 4b). Only a few distal ends of the coiled-coil rods showed no adhesive interaction. The mapping of these adhesion events to the high-resolution topograph reveals their localization to the C-terminal ends of the SAS-6 coiled-coil rods (Figure 4c). This experiment demonstrates that interactions can be detected reproducibly. It further indicates that some histidine groups of SAS-6 are not accessible to bind to the functionalized AFM tip whereas a few histidine groups were accessible only occasionally. We know from biochemical characterization that all SAS-6 cartwheels carry a functional His$_6$-tag at the C-terminal end of their coiled-coil rods (Supporting Information Figure S2) and so failure to detect binding events using a functionalized AFM tip does not exclude the presence of histidines as binding partner.

![Figure 4: Improving the mapping of chemical interactions of SAS-6 cartwheels by repetitive imaging.](image)

**Figure 4:** Improving the mapping of chemical interactions of SAS-6 cartwheels by repetitive imaging. a) High-resolution FD-based AFM topograph of the SAS-6 cartwheels as shown in Figure 3a. b) Adhesion maps showing the adhesion peaks measured in three consecutive scans indicated by three different colors. Shown are specific adhesion events >100 pN. c) Mapping of all adhesion peaks to the topograph localizes the C-terminal ends of the SAS-6 coiled-coil rod domains with improved accuracy. AFM images were taken in imaging buffer containing 1 mM NiCl$_2$ using a Ni$^{2+}$-NTA modified AFM tip at $\approx$27 °C. AFM topographs show color a) and gray c) scales corresponding to a vertical range of 6 nm. Dashed circles highlight topographic regions at which specific adhesion events were detected.
Analyzing the specific interactions detected on SAS-6 cartwheels

We analyzed the adhesion maps collected in our FD-based AFM experiments, with each experiment using different Ni\textsuperscript{2+}-NTA functionalized AFM tips, to characterize the specific adhesion to SAS-6 cartwheels. In this analysis, the FD curves were extracted and specific rupture forces and lengths plotted in histograms (Supporting Information Figure S3; n=75). The force distribution ranges from 70 to 220 pN with an average force of 124 ± 33 pN required to rupture Ni\textsuperscript{2+}-NTA-His\textsubscript{6} bonds at an average loading rate of 1.0 ± 0.2 mN/s. The magnitude of these rupture forces is similar to those previously reported for other His-tagged systems at similar loading rates (~50 to 250 pN).\textsuperscript{36-38} The average force measured to rupture Ni\textsuperscript{2+}-NTA-His\textsubscript{6} bonds of His\textsubscript{6}-tags directly attached via short peptides to the Au-surface and of His\textsubscript{6}-tags engineered in the coiled-coil rods of SAS-6 differed by ~48 ± 65 pN. One reason for this discrepancy may be that in the first case the short peptides anchoring the histidine residues to Au provides a rather stiff linker and in the second case the coiled-coil rods provide a softer and more flexible linker. A more flexible linker lowers the loading rate applied to the bond (at unchanged pulling velocity) and so such an explanation seems thus plausible.\textsuperscript{33,46} Indeed the slope of the rupture force peak is steeper in case of the short linker (Supporting Information Figure S1) compared to the slope measured during the rupture of bonds in SAS-6 (Supporting Information Figure S3). An additional explanation may be that the His\textsubscript{6}-tags engineered in SAS-6 are less accessible to NTA functionalized AFM tips than the tags attached with short linkers and thus bond strength is rarely maximized.\textsuperscript{47}

Conclusion

In its current state of the art, FD-based AFM can readily be applied to image single proteins, protein complexes and nucleic acids to sub-nanometer resolution and to simultaneously map various mechanical properties to the
AFM topograph\textsuperscript{11,16,21-24}. Excitingly, this enables the characterization of mechanical, structural and functional properties of biological samples at high resolution. One remaining important challenge was to be able to image single native proteins and at the same time map their specific interaction groups. In this work we have introduced FD-based AFM to image native proteins, to structurally localize their histidine residues, and to characterize the interactions of these residues. The FD-based AFM setup can be modified to detect any other specific interactions that occur on native proteins in physiologically relevant environments. This approach may open a new avenue for the chemical imaging of native proteins and nucleic acids.

It has recently been shown that an electrostatically charged AFM tip can be used to image native proteins at sub-nanometer resolution and at the same time map the electrostatic field and potential generated by transmembrane pores formed by single proteins\textsuperscript{20}. In principle, electrostatic and chemical interactions are detected via approach (e.g., deformation channel) and retraction (e.g., adhesion channel) FD curves, respectively. Thus, in the near future it may even become possible to use FD-based AFM to image individual proteins in physiological relevant environments and to simultaneously map their electrostatic and chemical properties.

Materials and Methods

\textbf{SAS-6 cloning and purification.} The DNA fragment coding for \textit{C. reinhardtii} SAS-6 (residues 1-503) (Uniprot ID A9CQL4) was cloned into the pET-based bacterial expression vector NSKnl that encodes for a C-terminal His\textsubscript{6}-tag using a negative selection method\textsuperscript{48}. The protein was expressed in the \textit{Escherichia coli} strain BL21(DE3) (Stratagene) in auto induction medium\textsuperscript{49} at 20 °C for 24 h. Cell pellets were resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, and complete protease inhibitors (Roche) and lysed by sonication. After initial clearance of cell de-
bris by centrifugation, the protein was purified by metal affinity purification using a His-Trap HP column (GE Healthcare) and step elution by imidazole. Subsequently the protein samples were purified by size exclusion chromatography using a HiLoad Superdex 200 16/60 column (GE Healthcare). Untagged SAS-6 protein was prepared as described\textsuperscript{39,40}. The final buffer was 50 mM Tris, pH 7.0, 150 mM NaCl, and 2 mM β-mercaptoethanol. Proteins were concentrated by centrifugal ultrafiltration. The homogeneity of protein samples was assessed by SDS-PAGE and their identity confirmed by Western Blot (Supporting Information Figure S2). The protein concentration was determined by absorption at 280 nm.

**Functionalization of AFM tips.** High-resonance rectangular-shaped Si\textsubscript{3}N\textsubscript{4} cantilevers (0.04 N/nm, 35 kHz in water, Olympus, AC40) were immersed in ethanol for 10 min, rinsed with ethanol, and dried with a stream of filtered N\textsubscript{2} and cleaned for 10 min in a ultraviolet radiation and ozone (UV-O) cleaner (Jetlight, CA, USA). AFM cantilevers and tips were coated by a 3 nm thick base coating of Al followed by a 10 nm thick Au layer. Both metal layers were deposited without breaking the vacuum using an electron beam evaporator (EVA 300, Alliance Concept) at an evaporation rate of 0.1 nm/s. Coated cantilevers could be stored for weeks in air. For functionalizing with thiol terminated peptides, the Au coated tips were rinsed with ethanol and dried in a filtered N\textsubscript{2} stream, subjected to UV-O cleaning for 10 min and immediately immersed overnight in ethanol containing a mixture of 0.05 mM of nitrilotriacetate-terminated and 0.05 mM triethylene-glycol-terminated alkane thiols (ProChimia). As a negative control some tips were functionalized with 100% of triethylene-glycol-terminated alkanethiols. The day after thiol functionalization, tips were incubated in ethanol (2x 10 min), then rinsed with ethanol and dried in a stream of filtered N\textsubscript{2}. The functionalized tips were then immersed in 1 mM NaOH for 1 min to completely deprotonate the acetate groups. Finally, the tips were immersed in 40 mM NiSO\textsubscript{4} solution for 30 min\textsuperscript{50} and directly transferred to the cantilever holding glass block, which
was mounted to the AFM.

**Functionalization of supports.** Mica discs (diameter 6 mm) where cleaved and coated by a 3 nm thick Al layer followed by 10 nm thick Au layer using electron beam thermal evaporation (EVA 300). These Au supports were rinsed with EtOH, dried in a filtered N₂ stream and exposed to UV-Ozone (UV-O cleaner, 42 SERIES, Jelight Company) for 10 min to remove organic contaminations. Immediately after UV-Ozone cleaning the Au supports were incubated with buffer solution (0.1 mM, 50 mM KCl, 10 mM Tris, pH 7.0) containing Cys-AGHHHHHHH (specific His₆-tag) and/or Cys-GSLPETGG (unspecific) peptides (Genscript, Piscataway, NJ, USA). To prevent unspecific interactions of the Ni²⁺-NTA functionalized AFM tip with the Au support, the Au support was incubated with buffer solutions containing only the unspecific Cys-GSLPETGG peptide. To detect specific interactions with the Ni²⁺-NTA functionalized AFM tip and to prevent the AFM tip from unspecific interactions with the Au support, the Au support was incubated with buffer solutions containing specific and unspecific peptides (ratio 1:10). Au supports were incubated with peptide solutions for 2 h at 4 °C and rinsed 10 times with imaging buffer prior to FD-based AFM experiments.

**Sample preparation.** A stock solution of SAS-6 proteins (1.6 mg/mL) was diluted to 3.3 µg/mL in adsorption buffer (300 mM KCl, 20 mM Tris pH 7.2). 30 µL of the diluted sample was adsorbed onto a freshly cleaved mica surface for 10 min. The sample was rinsed with imaging buffer (150 mM KCl, 20 mM Tris pH 7.2) 5 times prior to AFM.

**FD-based AFM.** A Nanoscope Multimode 8 (Bruker, Santa Barbara, California) was operated in the PeakForce mode to conduct FD-based AFM. The AFM was equipped with a 120 µm piezoelectric scanner. Rectangular shaped silicon cantilevers having a spring constant of ≈0.05 N/m and 35 kHz resonance frequency in liquid (AC40, Olympus) were used. Overview images (2 x 2 µm²) were recorded at imaging forces of ≈70 pN, the AFM tip was
oscillated vertically at 2 kHz applying a 24 nm amplitude, the sample was scanned using a line frequency of 1 Hz and 512 pixels were scanned per line (512 lines). Best high-resolution AFM topographs and interaction maps were found at imaging forces ≥50 pN, oscillation frequency ≤0.5 kHz, oscillation amplitudes between 30 and 60 nm, and line scanning frequencies ≤0.25 Hz. All FD-based AFM images were recorded in imaging buffer at ≈27 °C. In order to detect specific Ni-NTA-His6-tag interactions the imaging buffer was supplemented with 1 mM NiSO4.

**Data analysis.** Adhesion maps were saved as arrays of FD curves and the analysis software tool ‘Nanoscope Analysis’ was used to display adhesive interaction forces by selecting for adhesion forces in the range indicated in the figures. FD curves of topographic areas within this range of adhesion forces were exported to Matlab as ascii files and individually analyzed to differentiate between unspecific and specific interactions (Figure 3 c,f,i). Matlab was used to select and filter specific and unspecific interactions FD curves out of ≈10,000.

**References**


[4] Fotiadis, D.; Scheuring, S.; Muller, S. A.; Engel, A.; Muller, D. J. Imaging and manipulation of biological structures with the AFM. Micron


RESULTS


Localizing chemical groups while imaging single native proteins by high-resolution AFM - Supplementary Information

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Supporting Information. Histograms of rupture forces and lengths detected between a Ni²⁺-NTA functionalized tip and Au surfaces derivatized with CGA-His₆ peptides (Figure S1). Quality control of SAS-6 purity and of the specificity of the His₆-tag engineered to SAS-6 (Figure S2). Histograms of rupture forces and lengths detected between a Ni²⁺-NTA functionalized tip and SAS-6 carrying a His₆-tag (Figure S3).
Supporting Information Figure S 1: Specific rupture forces and lengths detected between the Ni\(^{2+}\)-NTA-modified AFM tip and the His\(_6\)-tag functionalized Au-support. Distribution of rupture forces (a) and lengths (b) detected in 85 FD curves (c). (c) Superimposition of all FD curves analyzed. Histograms have a bin size of 20 pN (a) and 1.5 nm (b). The average rupture force was 172 ± 56 pN and the average rupture length was 5.7 ± 1.5 nm. FD curves were recorded in imaging buffer supplemented with 1 mM NiSO\(_4\) at ≈ 27 °C.
Supporting Information Figure S 2: Quality control of SAS-6 proteins used for FD-based AFM imaging. Coomassie stained SDS-PAGE confirms the purity of the proteins (left). Shown are SAS-6 proteins without (-) and with (+) a C-terminal His$_6$-tag. Western blot using an anti His$_6$ antibody confirms the presence and functionality of the His$_6$-tag fused to the C-terminus (right). The absence of crossreactivity with the untagged protein proves the antibody specificity.
Supplementary Information Figure S 3: Specific rupture forces and lengths detected between the Ni$^{2+}$-NTA-modified AFM tip and the His$_6$-tag functionalized SAS-6. Distribution of rupture forces (a) and lengths (b) detected in 75 FD curves (c). (c) Superimposition of all FD curves analyzed. Histograms have a bin size of 20 pN (a) and 1.5 nm (b). The average rupture force was 124 ± 33 pN and the average rupture length was 11.7 ± 4.8 nm. FD curves were recorded in imaging buffer supplemented with 1 mM NiSO$_4$ at ≈27 °C.
Imaging of G-protein coupled receptors while quantifying their ligand-binding free energy landscape

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Abstract

The state of membrane receptors depends on their heterogeneous assembly in the cellular membrane. Here, we introduce experimental and theoretical developments that allow force-distance curve-based atomic force microscopy (FD-based AFM) to simultaneously image native human protease-activated receptors (PAR) in membranes and to quantify their dynamic binding strength to native and synthetic ligands. These binding strengths provide kinetic and thermodynamic parameters of individual ligand-receptor complexes. We report distances to the transition state barrier extending from 0.2 to 0.7 Å, free binding energy differences from -4.4 to -9.8 kcal/mol, and dissociation constants from 75 nM to 600 µM. Recorded in the absence and presence of antagonists, the values describe the ligand-binding free energy landscape of native and synthetic ligands to the G-protein-coupled receptor (GPCR) with remarkable accuracy. Excitingly, our nanoscopic method opens an exciting avenue to directly image and characterize ligand-binding of native membrane receptors.

Keywords
Agonist, affinity, antagonist, binding sites, free energy barrier, G-protein-coupled receptor, high-resolution imaging, single-molecule imaging and force spectroscopy, ligand-receptor binding, free energy landscape.
Introduction

Protease-activated receptors (PARs) belong to the class A subfamily of G protein-coupled receptors (GPCRs), which are characterized by seven-transmembrane domains. PAR1 serves as a cell surface receptor for thrombin and is expressed by platelets, endothelial cells, smooth muscle cells and fibroblasts, and a variety of other cell types. Together with the coagulation cascade, this receptor links tissue injury to cellular responses that mediate hemostasis, inflammation, and repair. Vorapaxar, a PAR1 antagonist, attenuates thrombin-induced platelet activation and was recently approved for secondary prevention of heart attacks and strokes. PAR1 is activated by an unusual proteolytic mechanism. Thrombin cleaves the N-terminal exodomain of the receptor at a specific site. The proteolytic cleavage of the N-terminus exposes the thrombin receptor-activating peptide (TRAP). This hexa-amino acid sequence, SFLLRN, works as a tethered ligand that binds, presumably intramolecularly, to the receptor’s heptahelical bundle to affect transmembrane movement and to activate G-proteins on the cytoplasmic face of the plasma membrane. Once activated, PAR1 signals through multiple G-proteins, including $G_{i/o}$, $G_{q11}$ and $G_{12/13}$, to modulate various downstream signaling pathways. However, how tethered ligands bind PAR1 has hardly been quantitatively described. Understanding this process is likely to illuminate peptide hormone-receptor interaction as well as tethered ligand mechanisms in general, which operate in multiple GPCR types and other biological regulators.

Recently, force-distance curve-based atomic force microscopy (FD-based AFM) matured into a nanoscopic tool that allows imaging of biological systems and simultaneous mapping of their multiple properties at (sub-)nanometer resolution. In order to achieve this, FD-based AFM approaches and retracts the AFM tip from the sample for every pixel of the AFM topograph. To decrease the image acquisition time, the tip is oscillated following a sinusoidal trajectory in the kHz
range (Fig. 1b,d). The deflection of the AFM cantilever corresponds to the interaction forces between tip and sample (Fig. 1d)\(^{12}\), which can be displayed as force vs time or force vs distance (FD) curves. Analysis of these force curves allows the extraction of parameters such as adhesion, applied contact force, sample deformation, energy dissipation and Young’s modulus (Supplementary Fig. 1)\(^8\). Determined pixel-for-pixel, these parameters are used to create parametric maps complementing the sample topography (Supplementary Fig. 1). In principle functionalization of the AFM tip with, for example, a ligand allows determination of both the topography and the interaction map of the ligand with a biological sample. However, so far FD-based AFM could not be applied to quantify the forces of a ligand binding to a membrane receptor and to correlate these specific binding events to the topography of individual receptors. Furthermore, it has not been possible to measure the kinetic and thermodynamic properties of ligands binding to receptors imaged by AFM.

With these fundamental challenges as motivation, we developed a FD-based AFM approach to image single native human PAR1 in proteoliposomes at high-resolution (<5 nm) and to simultaneously map their ligand-binding energy landscape under physiologically relevant conditions. Our nanoscopic approach directly characterizes the bond strength from near- to far-from-equilibrium regimes and, combined with a new theoretical model, quantifies kinetic and thermodynamic parameters that describe the binding of PAR1 to the native TRAP ligand (Fig. 1a-c) and to other synthetic ligands.
Figure 1: Figure captions on following page
Figure 1: Principle of FD-based AFM to detect ligand-binding to PAR1. (a) (i) PAR1 reconstituted in a lipid bilayer. Thrombin cleaves the N-terminal domain of the GPCR and exposes the SFLLRN sequence of the cleaved N-terminus. This SFLLRN sequence functions as a tethered ligand and binds to PAR1 (ii). Once the ligand has bound to PAR1 the receptor is activated and initiates transmembrane signaling. (iii) Using an AFM tip derivatized with the N-terminal SFLLRN sequence to detect interaction forces with PAR1. PAR1 structure (pdb id 3VW7) taken from3. (b) Pixel-for-pixel FD-based AFM approaches and retracts the tip of an AFM cantilever from the sample to record interaction forces F over the tip-sample distance in FD curves (see also Supplementary Fig. 1). Therefore the cantilever is oscillated in the kHz range (green curve). (c) AFM tip functionalized with a PEG-linker terminated by the N-terminal SFLLRN peptide. (d) The sinu­sooidal movement of the cantilever allows intermittent contact of the tip with the sample. The recorded tip-sample interactions are displayed as force vs time or force vs distance curves. Sample properties (adhesion, deformation) can be extracted from individual force curves9.

Results

FD-based AFM imaging of native PAR1 in proteoliposomes Human PAR1 was purified and reconstituted at 10 µM in liposomes made of 0.5 mg/mL phospholipids (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)) and 0.05 mg/mL of the cholesterol analog cholesteryl hemisuccinate (CHS) (On­line Methods). The presence of PAR1 in liposomes was confirmed by SDS-PAGE (Supplementary Fig. 2) and by single-molecule force spectroscopy (SMFS) (Supplementary Fig. 3). SDS-PAGE revealed a band at ≈40 kDa, which is in good agreement with the expected size of 43.9 kDa calculated from the PAR1 sequence. Unfolding single PAR1 molecules from proteoliposomes by SMFS revealed FD curves with characteristic force patterns. The reproducible force patterns indicate that all PAR1s in the proteoliposomes show the same fold. Because the FD curves recorded upon unfolding of PAR1 look similar to those previously recorded for the human β2-adrenergic receptor (β2AR)13, a class A GPCR showing high structural homology to PAR114, we conclude that PAR1 folded correctly into the proteoliposomes15.
RESULTS

Figure 2: Mapping receptor-ligand interactions on human PAR1 using FD-based AFM. (a) Overview topography (height image) of human PAR1 reconstituted in proteoliposomes taken with the SFLLRN ligand functionalized AFM tip oscillated at 0.25 kHz and amplitudes of 30-50 nm (b). (c) Representative force-distance (FD) curves recorded between the tip and PAR1 proteoliposome. Topograph (d) and adhesion map (e) of the area highlighted in (a). To increase visibility adhesion pixels were enlarged by a factor four (e). (f) Overlay of adhesive interactions (red colored) with AFM topograph (gray). Dashed circles and numbers localize force curves recorded in (c).

After confirming that native PAR1 had been reconstituted, we imaged the PAR1 proteoliposomes by FD-based AFM. To this end, the proteoliposomes were adsorbed onto freshly cleaved mica in buffer solution at 37 °C. The AFM topographs revealed membrane patches protruding 4.5 ± 0.7 nm (average ± SD, n=10), from the mica consistent with the height of a DOPC lipid bilayer (Fig. 2a and Supplementary Fig. 4)16. Most of the membrane patches showed sparsely distributed protrusions, which originated from single and clustered PAR1s (Fig. 2a and Supplementary Fig. 5).

Simultaneous imaging and detecting ligand-specific interactions of PAR1

To characterize ligand binding to PAR1 we used FD-based AFM to measure adhesive forces of the SFLLRN sequence, which is the native ligand of
PAR1 (Fig. 1a). Therefore we covalently linked the sequence of the native PAR1 terminus ending with the SFLLRN sequence to a PEG spacer which has been chemically attached to the AFM tip (Online Methods) (Fig. 1c). This tethering mimicking the thrombin cleaved N-terminus of PAR1 ensured that our experiment describes a physiologically relevant setup. Using the functionalized AFM tip we then imaged PAR1 proteoliposomes and recorded pixel-by-pixel FD curves (Fig. 2b,c), from which we reconstructed sample topography (Fig. 2d) and adhesion map (Fig. 2e and Supplementary Fig. 6). Generally, the FD curves (Fig. 2c) showed one of three characteristics: (i) no adhesion events (Fig. 2c, curve 1), (ii) unspecific adhesion events detected in the contact region (<4-5 nm) of tip and sample (Fig. 2c, curve 2), or (iii) specific adhesion events that were distant (>5 nm) from the contact region (Fig. 2c, curves 3 and 4). The overlay of topography and adhesion map of the PAR1 proteoliposome structurally correlated unspecific and specific adhesion events (Fig. 2f). Mostly, FD curves detected no adhesion on membrane areas devoid of PAR1. This lack of adhesive interactions demonstrates that the SFLLRN peptide-functionalized AFM tip shows no affinity to the lipid bilayer. However, we also observed that some PAR1s did not interact with the functionalized AFM tip. As the ligand binds to the extracellular surface of PAR1 (Fig. 1a), this lack of specific binding events could result from PAR1s exposing their intracellular surface to the AFM tip. Alternatively, one can speculate that the contact time (~1 ms) between AFM tip and proteoliposome is too short to allow ligand-binding to PAR1 or that some PAR1s resided in an inactive state. Very rarely (<0.1%, n>10'000), FD curves detected unspecific adhesion events on the lipid bilayer, which probably resulted from nanoscopic defects in the bilayer. These force curves showed adhesion events at distances within the unspecific contact region between tip and sample.

Finally, some force curves detected specific adhesion events ranging from 40-150 pN, at rupture distances >5 nm and closely localized to receptors (<10 nm). Small divergences in co-localizing PAR1 and specific interaction
are due to the fact that the specific ligand is tethered to the AFM stylus via a $\approx 8$ nm long flexible linker (Fig. 1c). To determine whether these binding events were specific, three independent controls were performed using i) a bare (not functionalized) AFM tip, ii) a tip functionalized with an unspecific hexa-Gly peptide, and iii) the SFLLRN ligand-functionalized tip after blocking PAR1 with the peptide mimetic antagonist BMS-200261 (BMS) (Supplementary Fig. 7)\textsuperscript{19}. These controls showed that bare or hexa-Gly functionalized AFM tips do not detect specific interactions with PAR1 and that the antagonist (1 µM BMS) abolished any specific interactions of the SFLLRN-functionalized AFM tip with PAR1.

In our FD-based AFM experiments the oscillating AFM tip touches the proteoliposomes at the end of every downward movement. Optimal conditions to detect ligand-specific interactions were found at oscillation frequencies of 0.25 kHz and amplitudes of 30-50 nm. Under these conditions, the contact time between the AFM tip carrying the ligand and the membrane was very short ($\approx 1$ ms). This gave rise to the question of how the SFLLRN ligand can bind PAR1 in such short time ranges? In FD-based AFM the ligand is repeatedly brought in close proximity (binding radius limited by linker length) to PAR1 where binding is allowed for a certain time period. During this short time period the effective concentration of the ligand increases from infinitively low (ligand kept separated from PAR1) to molar range (ligand brought close to PAR1). At such a high concentration ligand binding to the receptor is not in equilibrium thereby forcing the association of the ligand-receptor pair. This explains why we can measure ligand binding to PAR1 in relatively short contact times, but also suggests that our approach could in principle force even low affinity ligands to bind the receptor.
Imaging of G-protein coupled receptors while quantifying their ligand-binding free energy landscape.

Figure 3: Figure captions on following page.
Figure 3: Extracting energetic, thermodynamic and kinetic parameters from force curves describes the ligand-binding free energy landscape. (a) According to the Bell-Evans model\textsuperscript{22}, a receptor-ligand bond can be described using a simple two-state model. The bound state resides in an energy valley and is separated by an energy barrier from the unbound state. The transition state \( \dagger \) must be overcome to separate ligand and receptor. \( x_u \) represents the distance between bound state and transition state, \( k_{off0} \) and \( k_{off} \) are transition rates for crossing the energy barrier under zero force and applied force \( F \), respectively. \( \Delta G^\ddagger \) gives the activation free energy to cross the transition state and \( \Delta G_{bu} \) the free energy difference between bound and unbound state. (b) A force-distance curve (upper curve) can be displayed as a force vs time curve from which the loading rate \( LR \) can be extracted from the slope of the curve just before bond rupture. (c) 3D plot of representative force-distance curves recorded at different loading rates.

A fast approach to probe the free energy landscape of the receptor-ligand complex

In the previous section we have described imaging of native PAR1 in proteoliposomes and probing of their specific interaction forces with the native PAR1 ligand. Generally, force-probing methods such as FD-based AFM measure the strength of single bonds under an externally applied force. As described by the Bell-Evans model\textsuperscript{20,21}, an external force stressing a bond reduces the activation energy barrier towards dissociation and, hence, reduces the lifetime of the ligand-receptor pair (Fig. 3a)\textsuperscript{22,23}. The model predicts that far-from-equilibrium, the rupture force (e.g., strength) of the ligand-receptor bond is proportional to the logarithm of the loading rate \( LR \) (i.e., \( F \sim \ln LR \)), with the loading rate \( LR=\frac{dF}{dt} \) being the force applied over time. Thus, measuring a bond’s strength depends on the LR’s applied. To accurately describe the properties of a ligand-receptor bond requires determining its energy landscape (Fig. 3a). Here, the Bell-Evans model nicely provides a way to approximate free energy landscape parameters of the bond by measuring the bond’s strength over a wide range of LR’s. These parameters include the dissociation rate at zero force (e.g., off-rate) \( k_{off} \) and thermodynamic parameters such as the distance separating the bond from transition state \( x_u \).
and the height of the transition state free energy barrier $\Delta G^\ddagger$. More recently, Friddle-Noy-Yorero, introduced a model to interpret the non-linearity of the rupture forces measured over the LR suggesting that this non-linearity arises through the re-formation of bonds\textsuperscript{24}. Such bond re-formation is supported by the confining potential of the force transducer. Excitingly, this model provides direct access to the equilibrium free energy $\Delta G_0$ between the bound and unbound states (Fig. 3a). Based on these theoretical considerations we questioned how we could use FD-based AFM to extract free energy landscape parameters of ligand-receptor bonds.

Conventionally, to approach the free energy landscape parameters of a ligand binding to a receptor requires the acquisition of force spectra over a wide range of LRs\textsuperscript{22,25,26}. Thus, one would have to record many FD-based AFM images of PAR1 at different velocities separating the functionalized AFM tip from the sample. However, our FD-based AFM oscillates the AFM cantilever at a fixed frequency in a sinusoidal manner to approach and separate AFM tip and sample\textsuperscript{9}. Therefore, depending on the tip-sample distance at which the ligand-receptor bond ruptures, the velocity of the tip, and thus the LR applied to the bond, is different. This tip-sample distance of the rupture event is influenced by different factors such as the localization of the individual ligands on the apex of the tip, the protruding height of the receptor from the membrane, and the relative position of the ligand tethered to the tip and the receptor. To determine the LR for each bond rupture force we displayed the FD curve as a force vs time curve (Fig. 3b). From this force-time curve, the LR was calculated from the slope of the force peak detecting a bond rupture. Fig. 3c shows representative force curves recorded at different loading rates.

**Determining the free energy landscape parameters**

To quantify the free energy landscape of the SFLLRN ligand binding to native PAR1 we analyzed the specific rupture events detected in FD-based AFM topographs. The resulting DFS plot showed that SFLLRN-PAR1 bonds ruptured at forces ranging from 40-150 pN and at LRs ranging from 4'000-
RESULTS

1'100'000 pN/s (Fig. 4a). The resulting plot nicely demonstrates that the force required to rupture the ligand-receptor bond depends on the LR applied (Fig. 4a).

To fit the data of the DFS plot, we used the analytical approximation of the Friddle-Noy-Yoreo model$^{24}$:

\[
< F > \approx F_{eq} + F_\beta \ln (1 + e^{-\gamma R(F_{eq})}) \]  
[Eq. 1]

with:

\[
F_\beta = \frac{k_B T}{\chi_a}; \quad R(F_{eq}) = \frac{r}{k_{eff}(F_{eq})F_\beta}; \quad \text{and} \quad F_{eq} = \sqrt{2k_{eff}\Delta G_{bu}} \]  
[Eq. 2]

where $F_{eq}$ is the equilibrium force for the bond/transducer system, $F_\beta$ the thermal force, $\gamma$ the Euler's constant, $k_B$ the Boltzmann constant, $T$ the temperature and $k_{eff}$ the effective spring constant of cantilever and linker. $k_{eff}$ is calculated using the cantilever stiffness $k_c$ and the linker stiffness $k_L$.

The $k_L$ of the 27-segment PEG linker tethering the PAR1 ligand is estimated by the stretching of PEG chains in liquid as described (Supplementary Fig. 9)$^{27}$. $F_{eq}$, representing the lowest force required to break a bond for a given force transducer stiffness $k_{eff}$, has been determined by theoretical and experimental studies$^{28-30}$.

The fit of equation [2] to the DFS plot is excellent, and we obtained reasonable values for all free energy landscape parameters describing the ligand-receptor bond (Fig. 4a). We found a distance from the bound state to the transition state barrier of 0.6 ± 0.2 Å, which is in good agreement with values determined for cell surface receptors bound to peptide-based ligands (e.g., $\alpha_2\beta_1$-integrin binding RGD peptides)$^{31}$. The SFLLRN ligand showed a rapid dissociation rate of 3672 ± 1677 s$^{-1}$ from PAR1. Such high dissociation rates were predicted for other ligands binding to GPCRs in the absence of G-proteins like procaterol binding to human $\beta_2$AR and are thought to hinder the crystallization of agonist-bound GPCRs$^{32}$. Molecular dynamics simulations showed that in the absence of a G-protein an agonist-bound active GPCR spontaneously relaxes to an inactive-like conformation$^{32}$. Furthermore, the binding equilibrium free energy $\Delta G_{bu}$ of -8.76 kcal/mol corresponds
to a dissociation constant $K_d \approx 425 \text{ nM}$ which is of the same order as the $EC_{50}$ of $\approx 800 \text{ nM}$ found in platelet aggregation assays$^{33}$. A $K_d$ in this range is expected for high-affinity interactions.

Next, we wanted to see whether the FD-based AFM experiment had an influence on the determined energy landscape parameters. Therefore, we changed the drive frequency oscillating the tip from 0.25 kHz to 0.5 kHz (Supplementary Fig. 9). As expected the forces required to rupture the ligand-receptor bond shifted towards higher LRs. This shift, however, did not change the kinetic and thermodynamic parameters estimated from fitting the rupture forces. In summary, these results highlight that the FD-based AFM method, applied in combination with a new theoretical approach, is suitable to quantify the kinetic and thermodynamic binding of a set of ligands with the native PAR1.

Quantifying subtle differences among native and synthetic ligands

The previous section highlights that FD-based AFM can be used to quantify thermodynamic and kinetic parameters of a ligand binding to a native membrane receptor. Next, we wanted to test whether our nanoscopic approach is sufficiently sensitive to detect differences in the free energy landscape of different ligands binding to PAR1. As observed in previous studies, PAR1 activation strongly depends on the sequence of the SFLLRN peptide, with the Phe and the Arg being important for affinity$^{33-35}$. We therefore analyzed the influence of these two residues in the SFLLRN peptide on ligand-receptor binding by replacing them individually by Ala. Interestingly, substituting the Arg residue of the native SFLLRN ligand by Ala dropped the equilibrium free energy of ligand-binding to -6.86 kcal/mol and reduced affinity to $K_d \approx 10 \text{ µM}$ (Fig. 4b). The DFS plot recorded using the SALLRN peptide revealed a reduced binding equilibrium free energy of -4.4 kcal/mol and low affinity of $K_d \approx 600 \text{ µM}$ for PAR1 (Fig. 4c). This low affinity approaches the typical mM range for non-affinity ligands and agrees well with platelet aggregation assays detecting no response at 200 µM SALLRN$^{33}$. In summary, these results show
that substitution of Phe or of Arg by Ala in the SFLLRN sequence on the AFM tip abolished high-affinity interaction with PAR1, consistent with previous functional studies of PAR1 activation by SFLLRN-based peptides and mutational studies of the SFLLRN native tethered ligand.

Through a systematic approach towards developing high-affinity TRAPs, Ala-Phe(p-F)-Arg-Cha-H-Arg-Tyr was found to have the greatest potency to bind PAR1. To quantify the binding of this synthetic ligand, we attached it to the AFM tip as described (Fig. 2) and conducted FD-based AFM of PAR1 proteoliposomes. The DFS plot showed much higher binding strengths of the synthetic ligand compared to all other ligands probed (Fig. 4d). Fitting the plot with the Friddle-Noy-Yoreo model revealed that the equilibrium free energy of this synthetic ligand binding to PAR1 significantly enhanced to $\Delta G_{\text{bu}} \approx -9.77$ kcal/mol corresponding to an affinity of $K_d \approx 75$ nM. These values were in very good agreement with the higher potency of this ligand vs SFLLRN in functional studies.

Taken together, the results show that our FD-based AFM approach reveals subtle differences in the energy landscape of different ligands binding to native PAR1. Altered ligands known to have reduced function at PAR1 showed reduced binding free energy and affinity, while a ligand known to have greater potency showed higher binding free energy and affinity compared to the native TRAP ligand.

**Thermodynamic insights into vorapaxar inhibition of PAR1** Recently approved by the FDA to protect patients against recurrent myocardial infarction, vorapaxar binds PAR1 close to the extracellular surface. The binding pocket formed between vorapaxar and PAR1 is composed of residues from transmembrane helices 3, 4, 5, 6 and 7 as well as extracellular loops 2 and 3. Although the binding site of vorapaxar is known, details of how vorapaxar affects TRAP binding are not understood. To this end, we measured the binding strength of the SFLLRN and the SFLLAN peptide to PAR1 blocked by vorapaxar (Fig. 4e,f; Online Methods).
Figure 4: Loading rate-dependent interaction forces of single ligand-receptor bonds quantitatively describe the ligand-binding energy landscape of PAR1. For four different peptides SFLLRN (a), SFLLAN (b), SALLRN (c), and A(pF-F)ChahRY (d) the force required to separate the ligand from PAR1 is plotted against the loading rate. For SFLLRN (e) and SFLLAN (f) the force required to separate the ligand from PAR1 complexed with the antagonist vorapaxar is given. Each DFS plot contains more than 300 (a-d) or 150 (e,f) measurements. Fitting the data using the Friddle-Noy-Yoreo model provides average $F_{eq}$, $x_u$, $\Delta G_{bs}$, $k_{off}$ and $\zeta_{0.5}$ with errors representing the S.E. (inset). Values showing a two times higher standard error than the average value are not given. Shaded areas represent 95% confidence intervals of prediction.
Using the SFLLRN modified tip, specific interaction forces were recorded and plotted vs the LR (Fig. 4e). Surprisingly, while the antagonist BMS completely abolished interactions of PAR1 with SFLLRN (Supplementary Fig. 7e,f), vorapaxar did not fully block these interactions. The DFS data allowed us to extract kinetic and thermodynamic parameters of the native SFLLRN ligand binding to PAR1 complexed with vorapaxar. As the strength of the specific ligand-receptor bond dropped (Fig. 4e), the binding free energy of the ligand to the inhibited receptor dropped by \( \approx 1.78 \text{ kcal/mol} \) to \( \approx 7.09 \text{ kcal/mol} \) and the affinity reduced from \( \approx 425 \text{ nM} \) to \( \approx 10 \text{ µM} \). This observation suggests that the antagonist vorapaxar perturbs but does not completely inhibit the binding of the SFLLRN peptide to PAR1. Because PAR1 complexed with vorapaxar is inhibited, this result suggests that high-affinity binding of the native ligand is required to fully activate PAR 1. The binding strength of the SFLLAN peptide to PAR1 remained unchanged in the presence of vorapaxar (Fig. 4b,f).

**Free energy landscapes of ligands binding to free and vorapaxar inhibited PAR1**

We have quantified the free energy landscape parameters of different ligands binding to PAR1 (Fig. 4). In our experiments the entire extracellular PAR1 surface can interact with a ligand tethered to the AFM tip (Fig. 5a). Using these parameters we reconstructed the free energy landscape for each of the ligands tested (Fig. 5b). The landscapes highlight that the native SFLLRN ligand binds PAR1 at highest affinity, while ligands with substitutions known to decrease potency bind at lower affinity. Furthermore, the free energy barrier separating the ligand-bound state from the unbound state is much more distant for the native ligand and, thus, forms a wider free energy valley stabilizing the ligand-bound state. The lower the free energy (or affinity) of the peptide-ligand the smaller this distance to the transition state and thus the narrower the energy valley became (Fig. 5b). From a structural point of view a wider energy valley can host more conformational substates compa-
red to a narrower valley\textsuperscript{37-39}. The native ligand, thus, energetically maximizes the bound state and allows the receptor to adopt higher conformational variability than the other ligands. One may speculate that this conformational variability allows the receptor to undergo structural changes to initiate signal transduction without unbinding of the ligand.

The full antagonist vorapaxar blocks PAR1 activation by SFLLRN. Whether it physically occludes the binding site for SFLLRN or prevents PAR1 from accessing a conformation capable of binding SFLLRN is unknown. Regardless, our results suggest that vorapaxar prevents SFLLRN access to the high-affinity ligand-binding site/state of PAR1 to inhibit receptor activation (Fig. 5c)\textsuperscript{3}. However, FD-based AFM detected the native SFLLRN ligand binding to vorapaxar-blocked PAR1 at significantly altered energy landscape parameters. The energy landscape reconstructed from the parameters shows that the native ligand bound vorapaxar-PAR1 at lower free energy and reduced transition state distance (Fig. 5d). Intriguingly, the affinity of the interaction between SFLLRN and vorapaxar-PAR1 was similar to that of SFLLAN for free PAR1. Furthermore, within the accuracy of our method, SFLLAN showed no change in binding affinity to vorapaxar-blocked and free PAR1. These findings suggest that the alternative low-affinity ligand-binding site is not blocked by vorapaxar. Because vorapaxar blocks high-affinity but not low-affinity binding of the native ligand to PAR1, this result suggests that PAR1 has at least two ligand-binding sites or states, with the high-affinity ligand-binding site/state that is blocked by vorapaxar required for functional activation of the GPCR.
Figure 5: Figure captions on following page.
Imaging of G-protein coupled receptors while quantifying their ligand-binding free energy landscape

Figure 5: Free energy landscape describing the thermodynamic ($\Delta G_{bu}$) and kinetic ($x_u$) parameters of peptide-based ligands binding to PAR1. (a) Cartoon showing a peptide-based ligand binding to PAR1 through a high-affinity binding site. (b) Free energy binding landscape of three different peptide-based ligands depends on the sequence of the peptide. $x_u$ represents the distance to the transition state separating the ligand-bound and -unbound state. $\Delta G_{bu}$ gives the free energy difference between the ligand-bound and -unbound state. (c) Cartoon showing a peptide-based ligand interacting to vorapaxar-bound PAR1 through a low-affinity binding site. (d) Free energy landscape of ligands binding to vorapaxar-bound PAR1. PAR1 structures are shown in the vorapaxar-bound state (pdb id 3VW7). (e) Binding model of the native SFLLRN ligand (red) to PAR1. For both the vorapaxar inhibited and unbound states of PAR1 the ligand binds at low-affinity to the extracellular PAR1 surface, from which extracellular loops 2 and 3 have been proposed to bind the ligand$^{3,44}$. In the presence of the antagonist vorapaxar the native ligand cannot bind to the high-affinity binding site/state. In the absence of vorapaxar the native ligand can bind the high-affinity state/site, which functionally activates PAR1.

Discussion

Although the crystal structures of PAR1 and of many other GPCRs have been solved$^{3,14,18,32}$, quantifying ligand-binding to membrane receptors in physiological conditions remains challenging. Here we introduced FD-based AFM to image human PAR1s in proteoliposomes at high-resolution and to simultaneously quantify their dynamic binding strength to ligands at the single-molecule level. We further developed our single-molecule method to efficiently characterize the free energy landscape of ligands binding to PAR1 (Fig. 4). The native SFLLRN ligand showed high-affinity for PAR1 and our FD-based AFM measurements confirmed that Phe and Arg residues contribute considerably to this affinity. The energy landscape parameters determined show remarkably good agreement with what was known from bulk assays. We also map the free energy landscape of a synthetic ligand that has a much higher affinity for PAR1 compared to the native TRAP SFLLRN.

Characterizing ligand-binding to PAR1 inhibited by vorapaxar provided in-
sight into the ligand-binding mechanisms (Fig. 5). Surprisingly, the SFLLRN peptide binds to PAR1 even after inhibition of the receptor by vorapaxar. The affinity dramatically decreases to a low-affinity level confirming that the vorapaxar-binding site sufficiently blocks the high-affinity binding site or state leading to receptor activation. This suggests that PAR1 exposes alternative ligand-binding site(s) with lower affinity. While the high-affinity site is sensitive to vorapaxar and to changes in the SFLLRN sequence, the alternative binding site(s) appears to bind SFLLRN and SFLLAN equally well, with SFLLAN binding being insensitive to vorapaxar. The low affinity site(s) is therefore less specific for the structure of the SFLLRN ligand and, since vorapaxar did not alter SFLLAN binding, is not masked or occluded by the unbound and antagonist-bound conformations of the receptor. Whether the low-affinity site is involved in the physiological receptor activation is unknown. Previous studies demonstrating roles for the extracellular loops of PAR1 in tethered ligand function have led to a speculative model in which initial binding of the peptide-ligand occurs to the extracellular loops before it penetrates into the core of the receptor through a sequence of conformational intermediates. Our observation supports such two-step binding mechanism, where the TRAP first binds to the superficial low-affinity binding site/state at the extracellular surface and then to the central high-affinity binding site/state towards activating PAR1.

Although not yet applicable to characterize ligand-binding to many different membrane receptors, we see FD-based AFM as poised to become a valuable tool for the multiparametric characterization of membrane receptors. As FD-based AFM has proven capable of imaging native membrane proteins at sub-nanometer resolution, the next challenge will be to image membrane receptors at sub-nanometer resolution and to precisely detect where and how a ligand interacts with the ligand-binding pocket. Another challenge is to characterize the free energy landscape of ligands binding to a single receptor and to map the energy landscape of receptors showing heterogeneous
distributions and functional states in native membranes. Ultimately this will guide us towards mapping the ligand-binding free energy landscape of single membrane receptors in cells and help us understand how receptors are regulated.

Online Methods

Cloning, purification, overexpression, and reconstitution of PAR1
wtPAR1 was generated with an N-terminal FLAG epitope and a C-terminal octa-histidine tag to facilitate protein purification. The carboxyl terminus of PAR1 was truncated after residue Tyr397, and the amino terminus starts from residue Ala36. Such construct was tested in cell-based assays to show the ability of signaling in response to thrombin activation. PAR1 expression was done in Sf9 cells using the pFastBac baculovirus system (Invitrogen). To purify PAR1, infected cells were lysed by osmotic shock in low-salt buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 nM vorapaxar derivative and 100 µM TCEP. The vorapaxar derivative generated by reducing the non-aromatic carbon-carbon double bond in vorapaxar, showed much faster dissociation rate than vorapaxar in cell-based assays. PAR1 was further extracted from cell membranes with buffer 20 mM Heps 7.5, pH 7.5, 500 mM NaCl, 1% dodecyl maltoside (DDM), 0.03% cholesterol hemisuccinate (CHS), 0.2% sodium cholate, 15% glycerol, 100 nM vorapaxar derivative and 100 µM TCEP. Cell debris were removed by high-speed centrifugation. From this point, 1 µM vorapaxar derivative was added to all following buffers used for purification except for the buffer used in size exclusion chromatography. Nickel-NTA agarose resin was added to the supernatant after homogenization and stirred for 1h at 4 °C. The resin was then washed three times in batch with buffer comprised of 20 mM HEPES, pH 7.5, 500 mM NaCl, 0.1% DDM, 0.02% CHS and 1 µM vorapaxar derivative, and transferred to a glass column. The bound receptor was eluted with buffer containing
300 mM imidazole and loaded onto an anti-Flag M1 affinity column. After extensive washing with buffer comprised of 20 mM HEPES, pH 7.5, 500 mM NaCl, 0.1% DDM, 0.02% CHS, 1 mM vorapaxar derivative and 2 mM Ca\textsuperscript{2+}, the receptor was eluted from M1 resin using the same buffer without Ca\textsuperscript{2+} but with 200 µg/ml FLAG peptide and 5 mM EDTA. Size exclusion chromatography was used to obtain the final monodisperse receptor preparation. The running buffer contained 20 mM HEPES, pH 7.5, 100 mM NaCl, 0.1% DDM and 0.02% CHS. The flow rate was set at 0.2 ml/min to give enough time to allow the vorapaxar derivative dissociating from the receptor. The purified unliganded PAR1 was reconstituted into DOPC/CHS liposomes as described\textsuperscript{41}. Vorapaxar-bound PAR1 was prepared in a same way as described above except that 100 nM vorapaxar, not its derivative, was used for lysis and solubilization of cell membranes and 1 µM vorapaxar was used in the following Nickel-NTA, anti-FLAG M1 affinity and size exclusion chromatography steps.

**Functionalization of AFM tips.** To functionalize AFM tips we used NHS-PEG\textsubscript{27}-maleimide linkers\textsuperscript{42}. High-resonance rectangular-shaped Si\textsubscript{3}N\textsubscript{4} cantilevers (≈0.04-0.08 N/nm, 35 kHz in water, Olympus, AC40) were immersed in chloroform for 10 min, rinsed with ethanol, dried with a stream of filtered N2, cleaned for 10 min in a ultraviolet radiation and ozone (UV-O) cleaner (Jetlight, CA, USA) and immersed overnight into an ethanolamine solution (3.3 g of ethanolamine into 6.6 mL of DMSO). The cantilevers were then washed three times with DMSO and two times with ethanol, and dried with N\textsubscript{2}. Ethanolamine-coated cantilevers were immersed for 2 h in a solution prepared by mixing 1 mg of maleimide-PEG-NHS dissolved in 0.5 mL of chloroform with 30 µL of triethylamine, then washed with chloroform, and dried with N\textsubscript{2}. 100 µL of 1 mM of peptide of interest carrying a Cys at the C-terminal end was pre-mixed with 2 µL of EDTA (100 mM, pH 7.5), 5 µL of Hpes (1 M, pH 7.5), 2 µL TCEP hydrochloride (100 mM) and 2 µL Hpes (1 M, pH 9.6). This mixture was pipetted onto the cantilevers. After 4 h of
reaction, cantilevers were washed in PBS and used within few days.

**PAR1 preparation for AFM.** A 300-fold diluted solution in buffer (300 mM NaCl, 20 mM Hepes, 25 mM ceMgCl2) of PAR1 reconstituted in liposomes was adsorbed onto freshly cleaved mica for 1 h. The sample was rinsed with the same buffer 5 times prior to AFM measurements.

**FD-based AFM.** A Nanoscope Multimode 8 (Bruker, Santa Barbara, California) was operated in the PeakForce mode to conduct FD-based AFM. The AFM was equipped with a 120 μm piezoelectric scanner. Overview images (10 x 10 μm²) were recorded at imaging forces of ≈150 pN, the AFM tip was oscillated vertically at 2 kHz applying a 30-50 nm amplitude, the sample was scanned using a line frequency of 1 Hz and 512 pixels were scanned per line (512 lines). Best high-resolution AFM topographs and interaction maps were found at imaging forces of ≈150 pN, oscillation frequency of 0.25 kHz, oscillation amplitudes between 30 and 50 nm, and line scanning frequencies ≤0.125 Hz. All FD-based AFM images were recorded in imaging buffer at ≈27 °C.

**SMFS.** SMFS was carried out using automated AFM-based SMFS (Force-Robot 300; JPK Instruments, Berlin)⁴³. SMFS data of β2AR and PAR1 were recorded at pulling velocities of 300-900 nm/s as described previously¹³.

**References**


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Imaging of G-protein coupled receptors while quantifying their ligand-binding free energy landscape
Imaging of G-protein coupled receptors while quantifying their ligand-binding free energy landscape - Supplementary Information

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Figure 1: Principle of force-distance (FD) curve-based AFM. (a) FD-based AFM contours the sample surface while oscillating the AFM tip with a sine wave at a frequency of 0.25 kHz. Pixel-by-pixel the AFM tip is approached (blue curve) and retracted (red curve) from the sample. The AFM cantilever deflection measures the force interacting between AFM tip and sample. During these approach and retraction cycles the force vs time (b) and force vs distance (c) is recorded. Thereby, the maximal force (imaging force) used to touch the sample ($F_i$) is kept constant using a feedback loop. (c) The mechanical deformation or distance of deformation ($D_{Deff}$) of a soft biological sample is described by the indentation of a much stiffer AFM tip. This indentation is detected at a certain repulsive force. (d) During retraction, adhesive force ($F_{Adh}$) is recorded between the tip and the sample. Using a functionalized cantilever $F_{Adh}$ can detect the rupture of specific interactions between for example a functionalized tip and sample. (e) The parameters extracted from individual force curves can be displayed as maps such as the sample topography contoured at a given imaging force, the adhesion force or sample deformation.
Supplementary Figure 2: SDS-PAGE of PAR1 liposomes. SDS-PAGE was performed on a 12% acrylamide gel with 150 V and 60 min in a loading buffer (50 mM Tris, pH 7, 6% SDS, 50 mM DTT, 8% glycerol, 0.1% bromphenolblue) and stained with Coomassie blue. Lane 1, molecular weight marker; Lane 2, 10 µL of empty lipid vesicles (liposomes made of 0.5 mg/mL DOPC and 0.05 mg/mL CHS); Lane 3, 10 µL of PAR1 proteoliposomes (10 µM PAR1 reconstituted in liposomes made of 0.5 mg/mL DOPC and 0.05 mg/mL CHS).
Supplementary Figure 3: SMFS of human PAR1 and human β2AR reconstituted in liposomes. Selection of FD curves of recorded upon unfolding of single PAR1 (a) and β2AR (b) embedded in lipid membranes. Superimpositions of FD curves recorded of PAR1 (c) and β2AR (d). PAR1 proteoliposomes were adsorbed for 1 h at room temperature to freshly cleaved mica and SMFS was recorded in buffer solution (300 mM NaCl, 25 mM MgCl₂, 25 mM Tris, pH 7.0) at room temperature. β2AR proteoliposomes were adsorbed over night at 4°C to freshly cleaved mica and SMFS was recorded in buffer solution (300 mM NaCl, 25 mM MgCl₂, 25 mM Tris, pH 7.0) at room temperature. SMFS of both GPCRs was conducted as described previously². n gives the number of FD curves superimposed.
Supplementary Figure 4: Overview AFM topograph of PAR1 proteoliposome. (a) Topography (height image, 2.5 x 2.5 µm²) showing membrane patches on mica. Proteoliposomes were adsorbed to freshly cleaved mica in buffer solution. To remove weakly attached membrane patches, the sample was rinsed several times with the buffer (see Online Methods). After adsorption to mica the proteoliposomes break open so that they showed single-layered membrane patches. (b) Cross-section (white dashed line in (a)) showing a lipid membrane protruding 4.5 ± 0.7 nm (average ± SD, n=10) from the supporting mica. The sparsely distributed single protrusions originating from single or clustered PAR1. The FD-based AFM topograph was recorded in imaging buffer (300 mM NaCl, 20 mM Hepes, 25 mM MgCl₂, pH 7.0) at room temperature.
Supplementary Figure 5: Structural analysis of PAR1 reconstituted in proteoliposomes. (a) Topography of PAR1s sparsely distributed in lipid membranes made of 0.5 mg/mL DOPC and 0.05 mg/mL CHS. Histogram of diameter (b) and height (c) of PAR1 particles imaged in (a). (b) The diameter distribution showed two peaks centered at 8.1 ± 1.3 nm (average ± SD) and 14.7 ± 0.6 nm. Diameters were measured at full-width half maximum of particle heights. (c) The height distribution showed two peaks centered at 1.2 ± 0.2 nm (average ± SD) and 2.0 ± 0.3 nm, which could correspond to the height of the extracellular or intracellular surface emerging from the DOPS/CHS membrane, respectively (d). The FD-based AFM topograph was recorded in imaging buffer (300 mM NaCl, 20 mM Hepes, 25 mM MgCl2, pH 7.0) at room temperature. n gives the number of PAR1 particles analyzed.
Supplementary Figure 6: AFM topograph and multiparametric maps of PAR1 reconstituted in liposomes. (a) Topograph showing single and clustered PAR1 molecules protruding from the lipid bilayer. (b) Applied force error map showing low errors in the applied force (<20 pN). (c) Adhesion map showing the SFLLRN functionalized AFM tip interacting sparsely with the lipid bilayer and mainly with PAR1 (see (a)). (d) Deformation map showing enhanced deformation values of PAR1 molecules. The FD-based AFM data was recorded as described (Online Methods).
Supplementary Figure 7: Control experiments demonstrating that ligand functionalized AFM tips specifically detect interactions with PAR1. Height images (a,c,e) and corresponding adhesion images (b,d,e) recorded with either (a,b) a bare AFM tip, (c,d) a hexa-glycine functionalized tip or (e,f) a SFLLRN functionalized tip in the presence of peptide mimetic antagonist (1 µM BMS). This supplementary figure correlates to Fig. 2, which maps receptor-ligand interactions on PAR1 using SFLLRN functionalized AFM tips. The FD-based AFM data was recorded as described (Online Methods).
RESULTS

Supplementary Figure 8: Determination of the effective spring constant ($k_{\text{eff}}$). The effective spring constant $k_{\text{eff}}$ is the combination of the cantilever stiffness $k_c$ and the linker stiffness $k_L$. (b) The length of a PEG polymer, $L$, containing $n_m$ monomers, under stretching force, $F$, in water is described by a force extension relationship $^3$ where $L_k$ is Kuhn length, $K$ is the chain stiffness, $L_c(F)$ is a force-dependent contour length, $L_{\text{planar}}$ and $L_{\text{helical}}$ are the monomer lengths in planar and helical configuration, $\Delta G$ is the free energy difference between the planar and the helical configuration state, and $\Delta G(F)$ is the energy difference under applied force. (c) Calculated force (red curve) vs extension and stiffness (blue curve) of a 27-monomer PEG linker.
Supplementary Figure 9: Influence of the AFM tip drive frequency on ligand-receptor unbinding force. DFS plot showing that the tip drive frequency (0.25 kHz, red or 0.5 kHz, blue) has no influence on the force required to separate SFFLRN ligand and PAR1. Although the higher oscillation frequency reduced the contact time between tip and sample and, thus, lowered the frequency of bonds formed between ligand and PAR1, we could record a sufficient amount of specific unbinding events. Data were recorded in imaging buffer (300 mM NaCl, 20 mM Hepes, 25 mM MgCl₂, pH 7.0) at room temperature and fitted using the Friddle-Noy-Yoreo model (solid red line). Shaded area represents 95% confidence interval of prediction.
 Supplementary References


Imaging of G-protein coupled receptors while quantifying their ligand-binding free energy landscape -
Supplementary Information
Imaging single G-protein coupled receptors while quantifying their binding to two different ligands

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Running title
Mapping interaction sites of membrane proteins using bi-functionalized AFM tips

Quantifying and localizing the extra- and intracellular interactions of membrane receptors in the native environment is essential to understand their function. Similar to multicolor fluorescence imaging, we here introduce multifunctional high-resolution AFM imaging to simultaneously detect one extracellular and one intracellular ligand-binding site of native human protease-activated receptors (PAR1). The nanoscopic method is applicable to simultaneously detect and localize specific sites of any biological system at single-molecule resolution.
Abstract

In living cells signaling processes are governed by specific extracellular and intracellular interactions. For example, extracellular ligands binding to cell surface receptors can induce conformational changes, so that they intracellularly transduce signals\(^1\). In the cellular membrane, however, hundreds of different, functionally highly versatile receptors are heterogeneously distributed. Furthermore, the functional state of many receptors depends on their dynamic assembly in the cellular membrane so that the same type of receptor can bind a ligand strongly, weakly or not at all\(^2,3\). Moreover, because cell surface receptors can often bind different ligands there is a pertinent need to image individual receptors distributed in cellular membranes and to simultaneously quantify their interaction with multiple ligands. However, so far there is no high-resolution (<3 nm) microscopic method that allows to image individual membrane receptors and at the same time maps their extracellular and/or intracellular interactions with one or more ligands. Such method could provide a valuable tool to characterize the diversity of ligands binding to native membrane receptors and to systematically investigate factors contributing to the functional diversity of receptors.
Introduction

The human protease-activated receptor 1 (PAR1) belongs to the class A subfamily of G-protein-coupled receptors (GPCRs). These seven transmembrane domain receptors interact with a broad range of extracellular ligands to induce intracellular signaling pathways. PAR1 is activated by thrombin, which cleaves part of its extracellular N-terminal domain. The newly formed NH2-SFLLRN sequence of the N-terminus acts as a tethered thrombin receptor-activating peptide (TRAP). This TRAP binds intramolecularly to PAR1, induces a cascade of conformational changes, which lead to the association of G-protein subunits. Such activation of a coagulation protease followed by the activation of PAR and the binding of G-proteins triggers signaling cascades which link tissue injury to cellular responses that help to orchestrate haemostasis, thrombosis, inflammation and perhaps tissue repair. Once activated GPCRs rapidly phosphorylate mainly by G-protein-receptor kinases on serine and threonine residues localized on the third intracellular loop and C-terminus. PAR1 phosphorylation triggers the recruitment of arrestins, which promote the dissociation and hence, inactivation of G-proteins, a process referred to as desensitization.

Results & Discussion

Force-distance curve based atomic force microscopy (FD-based AFM) combines high-resolution imaging and single-molecule force spectroscopy (SMFS). While imaging a biological sample FD-based AFM approaches and retracts the AFM tip pixel-for-pixel. Each approach and retraction cycle is recorded in a force vs distance (FD) curve. Analyzing the FD curves provides mechanical insight of the biological sample such as adhesion, stiffness, deformation, elastic modulus and energy dissipation. Excitingly, functionalization of the AFM tip allows to detect and structurally map specific (bio-) chemical interactions. FD-based AFM me-
thods, using the AFM tip as multifunctional toolbox, are rapidly progressing within the past few years. Nowadays, the technology is ready to image native membrane proteins at (sub-)nanometer resolution and to map their mechanical properties, electrostatic potential, and chemical groups. Recently, FD-based AFM has been introduced to image native water-soluble protein complexes at high-resolution (~3 nm) and to structurally map their ligand-binding sites. However, so far it was not possible to image individual membrane receptors and to simultaneously detect their specific interactions with more than one ligand.

Here we address this challenge and introduce FD-based AFM to image human PAR1s at physiological relevant conditions and to simultaneously detect their extracellular and intracellular interactions with ligands (Fig. 1a). Therefore, we developed a procedure to functionalize the AFM tip with two different ligands (Fig. 1b). One ligand was the TRAP of PAR1 and the other ligand was a tris-(Ni$^{2+}$-NTA) group to detect a His$_8$-tag fused to the intracellular C-terminus of the receptor (Fig. 1c). The tris-NTA group was chosen because it shows similar size and affinity (KD ~30 nM) as the TRAP ligand. To separate unspecific from specific interactions both ligands were covalently attached to the beforehand amino-functionalized AFM tip via a ~10 nm long PEG$_{27}$ spacer. Then, we adsorbed PAR1 proteoliposomes to freshly cleaved mica and imaged the sample in buffer solution by FD-based AFM using the functionalized tip (Fig. 2). First, we wanted to detect only the interaction of the SFLLRN ligand to PAR1. Therefore, the imaging buffer contained no Ni$^{2+}$-ions, which coordinate the tris-(Ni$^{2+}$-NTA) to His$_8$-tag bond. We recorded FD-based AFM topographs with 256x256 pixels (2.5x2.5 µm$^2$) and one approach and one retraction FD curve per pixel. The topographs nicely showed the lipid membrane with protrusions corresponding to single proteins and assemblies of PAR1s (Fig. 2a). The adhesion map, which was reconstructed from all FD curves, recorded unspecific interactions of the AFM tip with the supporting mica and specific interactions co-localizing with
Figure 1: Chemical functionalization of the AFM tip and FD-based AFM mapping of adhesive interaction forces. (a) Interaction mapping on membrane proteins using FD based AFM. When recording an AFM topograph an approach (blue) and retraction (red) cycle between AFM tip and biological sample is performed for every pixel. In each cycle the cantilever deflection (e.g. force) is monitored and transformed into an approach and distance FD curve. (b) Bi-functionalizing the AFM TIP. The amino-functionalized Si₃N₄ AFM tip is functionalized by a hetero-bi-functional NHS-PEG₂₁-maleimide linker to which the thiol bearing ligands TRAP and tris-(Ni²⁺-NTA) are bound. (c) The membrane receptor PAR1 reveals an intracellular C-terminal His₉-tag (green) and an extracellular TRAP binding pocket (red), which interact specifically with the tris-Ni²⁺ NTA and the TRAP ligands, respectively. When imaging the receptor the retraction FD curve can discern between non-specific and specific interactions. Non-specific interactions reveal adhesive forces at close distance (<3 nm) between tip and the biological sample. Specific interactions are detected at larger distances (>5 nm) that correspond to the stretching of the PEG-linker tethering the ligand to the tip.
receptors imaged in the topographs (Fig. 2b, Supplementary Fig. 2). Characteristic for specific interactions was that the adhesive forces were detected at tip-sample distances (>10 nm), which correlated to the length of the extended PEG-linker (≈10 nm) tethering the SFLLRN ligand to the tip (Fig. 2c). The adhesive forces of these interactions distributed from 35 to 80 pN (Fig. 2c), which is characteristic for forces required to separate ligand-receptor bonds.}\textsuperscript{10,21-23}

\textbf{Figure 2:} Figure captions on following page.
Figure 2: Imaging PAR1 proteoliposomes and mapping the binding of SFLLRN peptide or of tris-NTA ligands. (a-d) Imaging the proteoliposome and mapping the binding of the native SFLLRN ligand and the extracellular binding pocket of PAR1. (a) AFM topograph of a PAR1 proteoliposome recorded with the bi-functionalized (tris-NTA/TRAP) AFM tip. To prevent tris-NTA interacting with the C-terminal His8-tag of PAR1 the experiments were conducted in the absence of Ni2+. (b) Adhesion map showing specific rupture forces recorded on the proteoliposome (dashed). (c) FD curves recording specific interactions in (b) at distances corresponding to the PEG-linker tethering the ligand to the AFM tip. (d) Distribution of rupture forces ranging from 35 to 85 pN (e-h). Imaging the proteoliposome and mapping the binding of the tris-NTA ligand to the intracellular His8-tag of PAR1. (e) AFM topograph of the PAR1 proteoliposome recorded with the bi-functionalized AFM tip. To promote tris-NTA binding to the His8-tag and to prevent the SFLLRN ligand to bind PAR1 the experiments were conducted in the presence of Ni2+ and of the antagonist BMS, respectively. (f) Adhesion map showing specific rupture forces recorded on the proteoliposome (dashed). (g) FD curves recording specific interactions in (f) at distances corresponding to the PEG-linker tethering the ligand to the AFM tip. (h) Distribution of rupture forces ranging from 65 to 140 pN. Encircled positions of AFM topographs and adhesion maps indicate where specific interactions have been detected. Height of the topographs (a,e) are according to scaling bars. The bin size of the rupture force distributions (c,d,g,h) is 5 pN. Images were recorded in imaging buffer (300 mM NaCl, 20 mM HEPES, 25 mM MgCl2, pH 7.2) and as stated in the presence of 2 µM BMS, 5 mM NiCl2, and 10 mM EDTA.

After having detected the binding of the extracellular SFLLRN ligand we wanted to detect the intracellular His8-tags fused to the C-terminus of the receptor. Therefore, we used the same bi-functionalized AFM tip, but to promote binding of tris-NTA to the His8-tag we preincubated the tip in 10 mM NiCl2 and added 5 mM NiCl2 to the imaging buffer. To prevent binding of the SFLLRN peptide to PAR1 we added the high-affinity antagonist BMS-200261 (BMS)24 to the buffer solution. Then, we used FD-based AFM to image the PAR1 proteoliposome (Fig. 2e). The proteoliposome was the same as imaged before but slightly changed shape because of membrane mobility. Again, the adhesion map showed unspecific interactions of the
bi-functionalized AFM tip with the supporting mica and localized specific interactions to PAR1s imaged in the topograph (Fig. 2f). Specific interactions were detected at tip-sample distances correlating to the length (≈10 nm) of the stretched bi-functional PEG-linker (Fig. 2g). The adhesion forces distributed from 65 to 250 pN (Fig. 2h), which is characteristic for separating the bond formed between tris-NTA and His8-tag$^{18,25,26}$.

Next, we tested the specificity of our bi-functionalized AFM tip by blocking all specific interactions to the PAR1 proteoliposome. Consequently, we left the antagonist BMS but removed Ni$^{2+}$ from the buffer solution by adding 10 mM EDTA and imaged the proteoliposome using FD-based AFM (Supplementary Fig. 3). The functionalized AFM tip showed unspecific interactions with mica and few unspecific interactions with the proteoliposome. Very rarely force curves (≈1-2 per adhesion map of 256x256 (65'536) FD curves) showed the signature of a 'specific interaction'. We attributed these interaction events to rupture forces of tip-tethered TRAPs bound to single PAR1s. For these specific ligand binding events, one must assume that single PAR1s spontaneously dissociated from the BMS antagonist, having a very small dissociation constant of ≈20 nM$^{24}$. Alternatively, one may speculate that in rare cases BMS dissociated from PAR1 while the receptor is being mechanically probed by the AFM tip. In summary, these control experiments show that both ligands tethered to the AFM tip are successfully blocked by the antagonist and removal of Ni$^{2+}$. The experiments further confirm that the AFM tip is functionalized with both ligands.

After having shown that the bi-functionalized AFM tip can either detect the His8-tag or the ligand-binding site of PAR1, we wanted to investigate whether the tip could simultaneously detect both specific interactions. Therefore, FD-based AFM topographs were recorded in physiological buffer solution supplemented with 5 mM NiCl$_2$ (Fig. 3a), which allows both ligands of the bi-functionalized tip to detect specific interactions. The adhesion map
showed specific interactions with $\approx 45\%$ (n=172) of the PAR1s topographically imaged (Fig. 3b). After imaging the sample three consecutive times $\approx 80\%$ of the PAR1 imaged showed specific interactions with the tethered ligands. These interactions were detected at rupture lengths corresponding to the PEG-linkers (Fig. 3c). To distinguish between the rupture events of SFLLRN and tris-NTA ligands we set up a force filter. As measured above, the strength of the SFLLRN-PAR1 bond ranges from 35 to 80 pN, whereas that of the tris-NTA-His-tag bond ranged from 60 to 250 pN (Fig. 2, Supplementary Fig. 4). Thus, we designed the force filter to discard all rupture events between 60 and 80 pN from further analysis (Fig. 3d). Accordingly, adhesive forces $< 60$ pN allocated SFLLRN-PAR1 bonds and forces $> 80$ pN tris-NTA-His$_5$-tag bonds.

To further increase the probability of detecting ligand binding by all receptors we repeatedly imaged the same proteoliposome at a $\approx 3.5$ fold higher magnification thereby increasing the number of FD curves (pixels) probed per PAR1. After three consecutive recordings, we detected specific ligand-binding events for $\approx 90\%$ of PAR1s. Based on the strength of the unbinding events detected, we could assign which one of the two ligands bound to the extracellular or intracellular surface of individual PAR1s and thus determine the orientation of the reconstituted receptors in the membrane. In larger assemblies of PAR1s we could observe receptors having both orientations (Fig. 3e). In summary, our approach shows that compared to multi-color imaging, even two distinct functional groups/proteins can be detected and localized at proximity closer than $\leq 5$ nm. Such differential imaging of membrane receptors binding to different ligands can be a useful tool in order to investigate the composition and functional state of membrane-protein complexes in their native environment at unprecedented detail (resolution) and information (binding strength).

We here introduced the high-resolution ($\approx 3$ nm) imaging of native human
Figure 3: Imaging PAR1 proteoliposomes and simultaneously mapping the binding of two different ligands. (a) AFM topograph of a PAR1 proteoliposome recorded with the bi-functionalized AFM tip. Measurements performed in presence of Ni\(^{2+}\) ions allow both ligands tethered to the tip, the tris-NTA and the SFLLRN peptide, to bind PAR1. (b) Adhesion map simultaneously recorded with the topograph (a). Adhesion events detected on the proteoliposome (dashed boundary) were categorized into three different interaction force scales: 30-65 pN for the extracellular TRAP-PAR1 bond (red), 65-80 pN for the force filter applied (blue), and 85-200 pN for the intracellular Ni\(^{2+}\)-tris-NTA-His\(_8\)-tag bond (green). (c) Selected FD curves showing specific adhesion forces and recorded at areas encircled in (a) and (b). (d) Distribution of rupture forces characterizing the rupture of specific ligand-receptor bonds. Applying a force filter from 65 to 80 pN (blue) discerns between both types of specific interactions (red vs green). (e) PAR1 proteoliposome imaged at higher magnification. Interactions detected after three consecutive recordings are color-coded for extracellular (red circles) and intracellular (green circles) interactions. Numbers give interactions detected two or three times. Encircled positions of AFM topographs and adhesion maps indicate were specific interactions have been detected. (f) Distribution of rupture forces of SFLLRN-PAR1 (red) and of tris-NTA-His\(_8\)-tag (green) bonds recorded in 3 consecutive images of the PAR1 proteoliposome. Height of topographs (a,c) are according to scaling bars. The bin size of the rupture force distributions (c,d,g,h) is 5 pN. All images were recorded in imaging buffer (300 mM NaCl, 20 mM HEPES, 25 mM MgCl\(_2\), 5 mM NiCl\(_2\), pH 7.2).
GPCR embedded in the physiological relevant membrane and simultaneously detected the specific binding of two different ligands. To achieve this goal, we functionalized the AFM tip with two ligands interacting either with the extracellular or the intracellular binding site of PAR1. Using FD-based AFM we imaged the PAR1 proteoliposome and localized the specific ligand binding forces to PAR1s. Introducing a force filter discriminates between the two specific ligand-receptor bonds and enables to assign individual receptors binding to either one of both ligands. In principle our dual-functionalization imaging approach is applicable to force probe a wide variety of biological systems and to map at the same time their interactions with different ligands or chemical groups. However, given a better force resolution or by using for each ligand linkers having very different structural or mechanical properties one may even consider to increase the number of ligands that can be simultaneously characterized while imaging single receptors.

Methods

Cloning, purification, overexpression, and reconstitution of PAR1. wtPAR1 was generated with an N-terminal FLAG epitope and a C-terminal octa-histidine tag to facilitate protein purification. The C-terminus of PAR1 was truncated after residue Tyr397, and the N-terminus starts from residue Ala36. Such construct was tested in cell-based assays to show the ability of signaling in response to thrombin activation. PAR1 expression was done in Sf9 cells using the pFastBac baculovirus system (Invitrogen). To purify PAR1, infected cells were lysed by osmotic shock in low-salt buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 nM vorapaxar derivative and 100 µM TCEP. The vorapaxar derivative generated by reducing the non-aromatic carbon-carbon double bond in vorapaxar, showed much faster dissociation rate than vorapaxar in cell-based assays. PAR1 was further extracted from cell membranes with buffer 20 mM Hepes 7.5, pH 7.5, 500 mM NaCl, 1% dodecyl maltoside (DDM), 0.03% cholesterol hemisuccinate (CHS), 0.2% so-
dium cholate, 15% glycerol, 100 nM vorapaxar derivative and 100 µM TCEP. Cell debris was removed by high-speed centrifugation. From this point, 1 µM vorapaxar derivative was added to all following buffers used for purification except for the buffer used in size exclusion chromatography. Nickel-NTA agarose resin was added to the supernatant after homogenization and stirred for 1h at 4 °C. The resin was then washed three times in batch with buffer comprised of 20 mM HEPES, pH 7.5, 500 mM NaCl, 0.1% DDM, 0.02% CHS and 1 µM vorapaxar derivative, and transferred to a glass column. The bound receptor was eluted with buffer containing 300 mM imidazole and loaded onto an anti-Flag M1 affinity column. After extensive washing with buffer comprised of 20 mM HEPES, pH 7.5, 500 mM NaCl, 0.1% DDM, 0.02% CHS, 1 µM vorapaxar derivative and 2 mM Ca²⁺, the receptor was eluted from M1 resin using the same buffer without Ca²⁺ but with 200 µg/mL FLAG peptide and 5 mM EDTA. Size exclusion chromatography was used to obtain the final monodisperse receptor preparation. The running buffer contained 20 mM HEPES, pH 7.5, 100 mM NaCl, 0.1% DDM and 0.02% CHS. The flow rate was set at 0.2 mL/min to give enough time to allow the vorapaxar derivative dissociating from the receptor. The purified unliganded PAR1 was reconstituted into DOPC/CHS liposomes as described. Vorapaxar-bound PAR1 was prepared in a same way as described above except that 100 nM vorapaxar, not its derivative, was used for lysis and solubilization of cell membranes and 1 µM vorapaxar was used in the following Nickel-NTA, anti-FLAG M1 affinity and size exclusion chromatography steps.

**AFM tip functionalization.** AFM cantilevers were functionalized according to . Briefly, the Si₃N₄ tips (Biolever mini, Olympus) were cleaned with EtOH (10 min), dried with filtered N₂ and treated with an ultraviolet radiation and ozone (UV-O) cleaner (Jetlight, CA, USA) for 10 min. For the amino-functionalization the tips were immersed in an 8.09 M ethanamine solution (EtOH in DMSO) for 16 h. Tips were cleaned 3x1 min in DMSO and 2 min in EtOH, rinsed with EtOH and dried with filtered N₂.
RESULTS

For the linker attachment the tips were immersed in a solution of maleimide-PEG27-NHS (1 mg/0.5 mL in CHCl₃) and 30 µL triethylamine for 2 h. Then, tips were cleaned 3x10 min in ceCHCl₃, dried with filtered N₂. 50 µL of 100 µM of each of the SH-group bearing functional groups was premixed with 2 µL of 1 M EDTA, 5 µL of 1 M HEPES (pH 7.5), 2 µL of 100 mM TCEP hydrochloride and 2 µL of 1 M HEPES (pH 9.0). This mixture was pipetted onto the cantilevers. After a reaction of 4 h, the cantilevers were washed in in PBS (3x5 min) and used within 24 h. Tips were functionalized with the tris-NTA group were immersed in 5 mM NiCl₂ (in imaging buffer) for 10 min prior to use. The tris-NTA-thiol compound (Mercapto acetic acid tris-NTA) was synthesized as described²⁵,²⁹. The cysteine bearing peptide (NH₂-SFLLRNPNDDKYEPFWEDDEEKNESGLTEYRGGGGCG-OOH) was purchased (GenSript, USA).

PAR1 preparation for AFM imaging. A 300-fold diluted solution in buffer (300 mM NaCl, 20 mM Hepes, 25 mM MgCl₂) of PAR1 reconstituted in liposomes was adsorbed onto freshly cleaved mica for 1 h. The sample was rinsed with the same buffer 5 times prior to AFM measurements.

FD-based AFM. A Multimode8 AFM with a Nanoscope5 controller (Bruker, Santa Barbara, California, USA) was operated in the 'PeakForceTapping' mode. The AFM was equipped with a 120 µm piezoelectric scanner (J scanner). Rectangular Si₃N₄ cantilevers with nominal spring constants of ≈0.04-0.08 N/m and a resonance frequency in water of ≈35 kHz were chosen (Biolever Mini, Olympus). Overview images (2-25 µm) were recorded at 2 kHz oscillation frequency, applying an imaging force of 100-200 pN, scanned at 1 line/second, with an vertical oscillation amplitude of 40 nm and a resolution of 512x512 pixels. Adhesion maps were recorded oscillating the functionalized tip at 0.25 kHz with an amplitude of 60 nm, scanning 0.125 lines/second and applying an imaging force of 100 pN. Assuming that the contact time
between tip and sample constitutes about one quarter of the oscillation cycle
a 0.25 kHz oscillation frequency results in a tip-sample contact time of \( \approx 1 \) ms.

**Data analysis.** The force vs time curves of each interaction recognition experiment were saved and exported as ascii files. The software Matlab was used to translate these force vs time curves into FD curves. If FD curves showing specific adhesion events correlated with the topography of the PAR1 proteins within a radius of \( \leq 5 \) nm, they were further analyzed. When imaging proteoliposomes \( \approx 0.5\% \) (2.5\% if Ni\(^{2+} \) was present) of the FD curves showed non-specific interaction events, where the rupture force appeared a small tip-sample distances \(< 10 \) nm. The x,y positions of specific interactions were saved and plotted onto the topography. Comparing several recorded images one could address the appearances of specific interactions to the individual PAR1 proteins.

**Control experiments.** In order to test whether the functionalization was successful and if non-specific interactions were detected, we performed several control experiments. We imaged the proteoliposomes with an unmodified (Si\(_3\)N\(_4\)) AFM tip and recorded a recognition map as described above (Supplementary Fig. 2a-d). We detected higher adhesion forces (20-60 pN) on the negatively charged mica surface and very low adhesion forces on proteoliposomes (0-30 pN). No specific adhesion was detected on individual PAR1s. Secondly, we imaged the sample with a PEG\(_{27}\)-maleimide functionalized tip that had no functional groups attached to the terminal maleimide group (Supplementary Fig. 2e-h). On mica non-specific adhesive forces were detected ranging from 30-180 pN. On the proteoliposome low adhesive forces (0-40 pN) were detected originating from non-specific interactions.
References


Imaging single G-protein coupled receptors while quantifying their binding to two different ligands - Supplementary Information

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Supplementary Figure 1: Force-distance curve-based (FD-based) AFM.
(a) FD-based AFM approaches and retracts the tip of the AFM cantilever from the sample pixel-by-pixel. For each pixel the maximum cantilever deflection and thereby the imaging force \( F_i \) is controlled. (b) Force vs time curves of the approach (blue) and retraction (red) movements are recorded for each pixel. (c) The mechanical parameters such as the sample deformation can be extracted from the approach force vs distance (FD) curve (blue). (d) The adhesion force \( F_{\text{adh}} \), Young's modulus, and energy dissipation can be extracted from the retraction FD curve (red). The adhesion force provides insight into the attractive interactions between the AFM tip and the sample. (e) The topograph of the sample is reconstructed from the vertical AFM tip movement needed to reach the imaging force \( F_i \) for each pixel. Furthermore, from the FD curves recorded for each pixel multiple parameters including adhesion and deformation can be determined and directly mapped to the topograph.
Supplementary Figure 2: FD-based AFM imaging of PAR1 proteoliposomes using non-functionalized and PEG<sub>27</sub>-maleimide functionalized AFM tips. (a) AFM topograph of a PAR1 proteoliposome recorded using a non-functionalized AFM tip. (b) Adhesion map simultaneously recorded with the topograph (a) showing low unspecific adhesive forces (≈20-60 pN) between the AFM tip and the mica and even lower unspecific adhesive forces (<30 pN) between the AFM tip and the proteoliposome. (c) FD curves showing unspecific adhesion events on mica (FD curves 1-3 were taken at positions 1-3 marked in (a) and (b)). Characteristically, such unspecific interaction events showed adhesive forces at small tip-sample distances (<10 nm). (d) FD curves showing no adhesion events on the proteoliposome (FD curves 4-6 were taken at positions 4-6 marked in (a) and (b)). (e) AFM topograph of a PAR1 proteoliposome recorded using a PEG<sub>27</sub>-maleimide functionalized tip. (f) The adhesion map shows adhesive forces between the functionalized AFM tip and mica (30-180 pN) and no adhesive forces on the proteoliposome. Thus, the PEG<sub>27</sub>-maleimide functionalized tip shows no adhesive interactions with the PAR1 proteoliposome. (g) FD curves recorded on mica (FD curves 1-3 were taken at positions 1-3 marked in (e) and (f)). (f) FD curves recorded on the proteoliposome (FD curves 4-6 were taken at positions 4-6 marked in (e) and (f)).
Supplementary Figure 3: Blocking the specific ligand binding interactions of the bi-functionalized AFM tip using EDTA and BMS.

Figure captions on following page.
Supplementary Figure 3: Blocking the specific ligand binding interactions of the bi-functionalized AFM tip using EDTA and BMS. (a) FD-based AFM topograph of PAR1 proteoliposomes recorded with the AFM tip bi-functionalized with active tris-NTA (tris-NTA(+)) and SFLLRN (SFLLRN(+)) ligands. Measurements were performed in the presence of Ni\textsuperscript{2+}-ions to support the formation of the tris-Ni\textsuperscript{2+}-NTA-His\textsubscript{6}-tag bond. (b) Adhesion map generated from FD curves recorded while recording the topograph shown in (a). (c) Example of FD curves detecting unspecific interactions of the bi-functionalized AFM tip with mica (FD curves 1-3 were taken at positions 1-3 marked in (a) and (b)). (d) Example of an FD curve detecting specific interactions of the TRAP-PAR1 bond (FD curve 6 was taken at position 6 marked in (a) and (b)) and of the tris-Ni\textsuperscript{2+}-NTA-His\textsubscript{6}-tag bond (FD curves 4 and 5 were taken at positions 4-5 marked in (a) and (b)). Each of the positions 4-6 co-localized with PAR1s in proteoliposomes. (e) AFM topograph of the proteoliposome imaged in (a) recorded when the specific interactions of both ligands functionalizing the AFM tip were blocked. BMS (2 µM) was added to the imaging buffer to suppress specific interactions of the SFLLRN ligand (SFLLRN(-)), and EDTA (10 mM) was added to suppress specific interaction of the tris-NTA ligand ((tris-NTA(-)). (f) Adhesion map of the topograph recorded in (e). (g) Example of FD curves showing unspecific interactions recorded on mica (FD curves 1-3 were taken at positions 1-3 marked in (e) and (f)). (h) Example of FD curves showing unspecific adhesion events recorded on the proteoliposome (FD curves 4-6 were taken at positions 4-6 marked in (e) and (f)). Images were recorded in imaging buffer (300 mM NaCl, 20 mM HEPES, 25 mM MgCl\textsubscript{2}, pH 7.2) and as stated in the presence BMS (2 µM) and/or NiCl\textsubscript{2} (5 mM).
Supplementary Figure 4: Recording consecutive topographs of the same proteoliposome to increase the rupture events detected of single SFLLRN-PAR1 and tris-Ni\(^{2+}\)-NTA-His\(_{8}\)-tag bonds. The data was recorded using an AFM tip bi-functionalized with tris-NTA and SFLLRN ligands (a). Distribution of forces characterizing only the rupture of single SFLLRN-PAR1 bonds. The data was collected from eight topographs and adhesion maps recorded from the same PAR1 proteoliposome. To prevent formation of the tris-Ni\(^{2+}\)-NTA-His\(_{8}\)-tag bond, the FD-based AFM data was recorded in the absence of Ni\(^{2+}\). (b) Distribution of forces characterizing only the rupture of single tris-Ni\(^{2+}\)-NTA-His\(_{8}\)-tag bonds. The data was collected from seven different topographs and adhesion maps. To support formation of tris-Ni\(^{2+}\)-NTA-His\(_{8}\)-tag bonds, the FD-based AFM data was recorded in the presence of NiCl\(_{2}\) (5 mM) and to suppress the formation of SFLLRN-PAR1 bonds the antagonist BMS (2 µM) was added. Data was recorded in imaging buffer (300 mM NaCl, 20 mM HEPES, 25 mM MgCl\(_{2}\), pH 7.2) and as stated in the presence of BMS (of 2 µM) and/or NiCl\(_{2}\) (5 mM).
3 Conclusion

Molecular interactions play a crucial role in biology. Knowing how proteins interact with their environment helps us to understand their functionality. Maps of protein structures with the location of interaction sites identified provide even more information on the working mechanism and functional states of proteins.

In this PhD thesis, AFM based methods were used to image single native proteins at high resolution and to simultaneously define their mechanical, chemical and ligand-binding properties. Towards this end, we adopted a stepwise approach in which we incrementally introduced improvements and additional functionality to our imaging technique.

This project started with the measurement of electrostatic forces around a native membrane protein by recording a topographic map of its solvent-exposed surface at high resolution with an unmodified cantilever tip. Electrostatic forces are of great importance in certain protein interactions, for example in guiding charged molecules of particular sign towards binding pockets, and repelling others. The electrostatic repulsion between the negative field of a protein and a negatively charged AFM tip could be studied by analyzing the approach FD curves recorded during imaging. A map of electrostatic potential could then be superimposed on a high resolution topography of the membrane protein of interest.

After having demonstrated the utility of unmodified cantilevers in functional imaging, we decided to graft specialized ligands to the tips of cantilevers. We achieved our goal of detecting receptor sites on native proteins at high resolution (in the range of 3 nm). In our model system we detected His$_6$ tags on native SAS-6 cartwheel like proteins using a Ni$^{2+}$ NTA functionalized tip which had previously been coated with gold. Now confident that we had the tools to combine force spectroscopy with high resolution AFM imaging we began to investigate ligand-receptor dissociation processes. Using an aminofunctionalized tip on which a native peptide is terminally attached via a
PEG spacer, we could localize interaction sites on sparsely distributed native G-coupled protein receptor Par1 in lipid bilayers. By analyzing over 200 interaction events per ligand we could determine the kinetic properties as well as the binding free energy of the ligand-receptor interaction.

Furthermore, we demonstrated that two different ligands can be attached to a cantilever tip and the two corresponding interactions sites probed simultaneously.

We were able to verify that our instrument (AFM) was amenable to functionalization techniques necessary to answer the biological questions we posed. Signal detection and imaging resolution were also adequate and combined with sophisticated data analysis allowed the interactions of native (membrane) proteins to be studied.

Elaborate AFM tip functionalization chemistry and data analysis routines provided a tool of unprecedented capabilities. Electrostatic properties such as the surface potential of single native proteins have thus far only been calculated using theoretically parameters. We show that electrostatic properties of proteins can now be determined experimentally, either in their physiological environment or under artificial conditions imposed by the experimenter. This method could also be applied to detect changes in electrostatic properties when ligands or other small solutes bind membrane proteins.

Ligand-receptor interactions can be characterized by their binding affinities and energies. Here we determine both and localize them on maps of receptor proteins. With this information one might investigate the basic binding mechanisms, and thereby the functionality, of proteins. In addition, effects of mutations in membrane proteins or the effect of drug binding can be determined. It was shown in the here demonstrated studies that small changes such as the mutation of a single amino-acid in the ligand molecule created a detectable change in the kinetic and thermodynamic response towards its receptor protein. This makes this tool an excellent drug screening assay, where effects of small changes in the precursor's structure can be assessed in a close
to physiological environment.

It is of utmost interest to apply our method to a broad spectrum of trans­membrane proteins and thereby unravel the working mechanism of the bio­macromolecules. The work presented here proves that this goal is feasible. Together with the advances of crystallography, GPCRs, for example, can be investigated and interaction locations can be correlated to the atomistic structure of these important proteins.

Of course, not only isolated receptor proteins but also larger biological system constituted of many different proteins could be investigated. For example, larger protein complexes can be probed with functionalized tips that respond to the different binding sites of these proteins thereby assessing their functional properties.

However, AFM based techniques remain a time-consuming endeavor, the success of which is dependent on many factors such as sample stability, the ability to covalently bind ligands to cantilever tips and the technical limits of the AFM. A faster, more stable scanning device that records at higher data rates, combined with ultra-sharp functionalized tips that register interaction forces in the sub-nanometer regime, is clearly a desired goal in development of this multifunctional imaging approach.

Future improvements could involve grafting ligands whose functional states can be toggled. This could be done by the inclusion of photo-reactive groups within the ligands that induce conformational changes when excited by light of the appropriate wavelength. The possibility to attach multiple such ligands to the same tip opens the door for one to probe all manner of properties in a single experiment. Another future innovation would be the application of these techniques to living cells. Using live cells enables drug screening assays to be performed and single protein functionalities to be observed in a cellular context. Combined with optical imaging techniques the response of cell-surface receptors can be triggered and investigated by FD based AFM and the further signaling cascade can be monitored by performing optical
fluorescence microscopy on the labeled proteins that are involved in such a signaling cascade.

Overall, this thesis demonstrates that the appearance and functionality of a membrane protein really depends on how you ask. Different molecules will observe and interact with the same protein through different thermodynamic lenses. Using functional imaging by FD based AFM, one is able to adopt the point-of-view of whichever ligand one is interested in (assuming, the said ligand is willing to be tethered to a high frequency cantilever). The intricate regulatory mechanisms of membrane proteins, the workhorses of living cells, are perceived from thoroughly different perspectives by FD based AFM. There is no doubt that functional imaging techniques will play a major role in future research of membrane-embedded proteins, of living cells, and, indeed, of life.