Atomic force microscopy and spectroscopy of native membrane proteins

Daniel J Müller[1] & Andreas Engel[2]

[1]Center of Biotechnology, University of Technology, Tatzberg 47-51, Dresden, Germany. [2]M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland. Correspondence should be addressed to A.E. (andreas.engel@unibas.ch).

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Membrane proteins comprise 30% of the proteome of higher organisms. They mediate energy conversion, signal transduction, solute transport and secretion. Their native environment is a bilayer in a physiological buffer solution, hence their structure and function are preferably assessed in this environment. The surface structure of single membrane proteins can be determined in buffer solutions by atomic force microscopy (AFM) at a lateral resolution of less than 1 nm and a vertical resolution of 0.1–0.2 nm. Moreover, single proteins can be directly addressed, stuck to the AFM stylus and subsequently unfolded, revealing the molecular interactions of the protein studied. The examples discussed here illustrate the power of AFM in the structural analysis of membrane proteins in a native environment.

INTRODUCTION

Atomic force microscopy (AFM) contours the surface topography by raster scanning the sample below the stylus, which is attached to a flexible cantilever. Deflections of the latter can be resolved to better than 0.1 nm by an optical system. In contact mode imaging, the deflection signal activates a servo that displaces sample or cantilever vertically to maintain a constant cantilever deflection. Under appropriate conditions, stable contact mode imaging is possible in liquids at forces of ≈50 pN (ref. 1). In dynamic or oscillating mode imaging, the cantilever is oscillated at its resonance frequency during scanning. The deflection signal in the oscillating mode is derived either from the frequency shift[2] or the damping[3,4] of the oscillation resulting from the stylus–sample interaction. Images obtained in either mode not only exhibit a superb clarity[5] but also allow conformational changes to be monitored[5,6]. For membrane proteins that are tethered to the stylus, force–distance (*F–D*) curves can be recorded while increasing the stylus–support distance[7]. These *F–D* curves reveal the strength and location of the molecular interactions established within and between single membrane proteins.

In this protocol we introduce newcomers to the tricks for acquiring high-resolution AFM images and *F–D* curves of membrane proteins. For learning we recommend the imaging of purple membrane, since it is commercially available and structurally well studied by AFM and single-molecule force spectroscopy (SMFS)[7,8]. Comparing the high-resolution topographs and *F–D* curves with those published will allow new AFM and SMFS users to be trained to perfection.

Cantilever and stylus: the critical elements of an AFM

While modern instruments provide the stability for imaging at subnanometer resolution, cantilever and stylus remain the critical determinants for achieving such results. For contact mode imaging, cantilevers should have a spring constant k_L of 0.05–0.5 N m^{-1}. They should be much stiffer for the dynamic mode (k_L >10 N m^{-1}, depending on the resolution of the deflection sensor)[2]. In oscillating mode, the stylus contacts the sample once per oscillation cycle inducing a damping of the amplitude; ideally, this mode is executed with very small cantilevers of intermediate stiffness[9,10]. In stark contrast, cantilevers used to record *F–D* curves during unfolding of a protein must be much softer (k_L < 0.05 N m^{-1}).

Resonance frequencies for cantilevers used for contact mode imaging of biological samples range from 5 to 50 kHz in vacuum. A cantilever with l = 100 μm, w = 40 μm, t = 0.4 μm has a spring constant of k_L = 0.08 N m^{-1} and a resonance frequency in vacuum of f_0 = 40 kHz. When operating such a cantilever in fluid, the resonance frequency is reduced by a factor of 2–5. Importantly, the scan speed v needs to be adapted with respect to the spatial resolution d to be achieved: $v/d < f_0$. Another parameter characterizing the cantilever is the quality factor Q, which is defined by the stored energy W_0 and the total energy loss per oscillation cycle ΔW (ref. 11):

$$Q = \frac{2\pi W_0}{\Delta W} \quad (1)$$

In liquids, the hydrodynamic damping by the surrounding medium dominates the energy losses[11]. For suitable contact mode cantilevers, which have a quality factor of 10–100 in air, Q drops to 1–5 when operated in water. Resonance frequency and quality factor can be determined from the noise spectrum of the cantilever in water, which is obtained by recording the deflection signal at a sampling rate that is at least two times the resonance frequency of the cantilever[12,13]. For these measurements, AFM stylus and support should be separated by at least 20 μm.

The minimal force $F_{\min}$ that a cantilever can measure is ultimately limited by the thermal noise of the cantilever. In liquid, this can be expressed as a function of the spring constant, resonance frequency and quality factor:

$$F_{\min} = \sqrt{\frac{4 \cdot k_B \cdot T \cdot B}{2\pi} \cdot \frac{k_L}{f_0 \cdot Q}}, \quad (2)$$

where k_B is the Boltzmann constant, T the absolute temperature and B the measurement bandwidth[11]. For the cantilever mentioned earlier (k_L = 0.08 N m^{-1}, assuming f_0 = 10 kHz and Q = 2 in fluid), which is operated at room temperature (about 20 °C) and at a sampling rate equal to the resonance frequency (i.e., B = 10 kHz), Equation 2 gives a minimal detectable force of approximately 10 pN. Better force sensitivity and higher speed can be achieved by reducing

the cantilever dimensions[9]. A rectangular cantilever with $l = 20$ µm, $w = 5$ µm and $t = 0.16$ µm has ten times higher resonance frequency in vacuum compared with the 100-µm long, rectangular cantilever, while exhibiting the same spring constant (0.08 N m^{-1}). Hence, when keeping B fixed, the sensitivity is enhanced by a factor of 3 in contact mode or the scanning speed can be increased by a factor of 10 without an increase of the thermal noise.

Stylus sharpness and corrugation amplitude of the sample topography dictate the spatial resolution that can be achieved. Suppliers specify stylus radii worse than 10 nm, yet topographs of flat biological surfaces that exhibit a resolution better than 1 nm have been acquired routinely[1,14–16]. Hence, it is assumed that small protrusions having the size of 1–2 nm and being located at the apex of the stylus contour structural features at the subnanometer scale. However, to be able to visualize details of the biological sample at a resolution better than 1–2 nm, long-range interactions (e.g., electrostatic forces)[1] need to compensate both the short-range interactions (e.g., van der Waals forces) and the external force applied to the tip, so that localized stylus–sample interactions dictate the contouring process (see **Fig. 1**, panel **a**). Thus, only protrusions of the stylus nearest to the sample determine the lateral resolution, which is considerably better than would be expected based on the stylus radius.

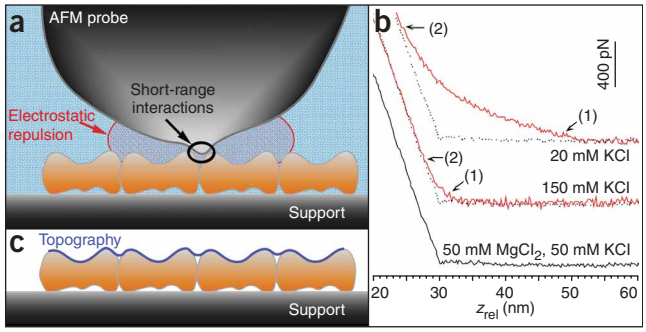

Figure 1 | High-resolution atomic force microscopy (AFM) imaging. (**a**) Small asperities of the AFM stylus contour the sample topography at high resolution. Interactions between AFM stylus and protein membrane can be divided in long- and short-range interaction forces. (**b**) Force–distance curves recorded reveal electrostatic contributions, ranging from (1) to (2). Increasing ion concentration and valency screens electrostatic interactions. This effect is used to precisely control the interaction forces to adsorb the sample onto the support, and to minimize the imaging forces applied between asperities of the AFM stylus and the protein. This allows minimizing the force for keeping the scanning stylus in contact with the membrane without inducing structural distortions (**c**, blue line).

Samples must be immobilized on a flat support

Biological membrane preparations are 5–10-nm thin layers that exhibit a lateral dimension of 0.5–10 µm. For observing them by AFM, these membranes must be spread-flattened onto a suitable support. Convoluted membranes that resist in spreading properly, or small liposomes, can actually be spread out using the AFM stylus as a tool. Mica and highly ordered pyrolytic graphite (HOPG) are the most widely used support materials, because they are atomically flat and very clean after stripping off the top layers. Mica is negatively charged (–0.0025 C m^{-2}, neutral pH) and thus rather hydrophilic, whereas HOPG exposes a hydrophobic surface. Mica is an insulator, and HOPG a good conductor[17]. For certain applications, glass slides have proven to be excellent supports, which may be particularly suitable when combining AFM or SMFS with light microscopy. Such supports are usually glued to a Teflon layer, which in turn is glued to the metal disc that is fastened to the scanner by a magnet, as detailed in the PROCEDURE. The hydrophobic Teflon spacer prevents the aqueous solution covering the sample support from flowing away.

Selecting buffer conditions and AFM operating parameter

To adsorb membrane patches, ionic strength and pH have to be adapted so that the membranes are pulled down onto the support and held tight by van der Waals interaction[18]. Sufficiently large patches will trap the salts in the gap formed with the support and thus allow the ionic strength of the buffer to be changed for optimized stylus–sample interactions as described later. In addition, each change of the frame size (magnification) requires the scan speed to be adapted for properly contouring sample corrugations by the AFM stylus and to achieve the best possible resolution. The latter is determined not only by the sharpness of the stylus and balanced tip–sample interactions but also by the Nyquist–Shannon sampling theorem and the theoretical scan speed limit[19]. Optimization of the scan speed and applied force is the key to achieve ultimate results.

Overview of the procedure

In the present procedure, we describe a precise protocol that can be applied to image purple membrane[7,8].

MATERIALS

REAGENTS

- Nanopure water (≈18 mOhm cm^{-1})
- Analytical grade buffers (Tris for purple membrane)
- Analytical grade electrolytes (HCl and KCl for purple membranes)
- Purple membranes (of *Halobacterium salinarum* or membranes of choice; Munich Innovative Biomaterials GmbH) (see REAGENT SETUP)
- Adsorption buffer for purple membrane: 300 mM KCl, 10 mM Tris/HCl (pH 7.8)
- Imaging buffer for purple membrane: 150 mM KCl, 10 mM Tris/HCl (pH 7.8)
- Ethanol and/or propanol

EQUIPMENT

- Mica punch set (e.g., 'Punch and die'; Precision Brand Products Inc.)
- Vibration isolation (active or passive), acoustic noise isolation (e.g., glass bell)
- AFM with fluid cell
- Oxide sharpened V-shaped Si_3N_4 cantilever (contact mode $k_L \approx 0.1$ N m^{-1}, dynamic mode $k_L > 10$ N m^{-1})
- Steel disc or other suitable AFM sample holder
- Teflon foil thickness less than 0.2 mm. Punch to diameter ≈1.5–2 cm
- Muscovite mica (Mica New York Corporation) cleave to thickness less than 0.5 mm, punch to diameter ≈0.5–1 cm
- Teflon compatible glue (Loctite 770) and chemically inert two-component epoxy glue (e.g., Araldite)

REAGENT SETUP

Purple membranes of *H. salinarum* Stock suspensions of purple membrane fragments (0.25 mg ml^{-1}) in double-distilled water containing 0.01% NaN_3 should be stored at 4 °C.

EQUIPMENT SETUP

Experimental setup Glue Teflon onto AFM sample holder (Loctite) and mica onto Teflon (epoxy glue). After hardening of all glues (over night), cleave the mica surface using scotch tape just before use. Clean AFM fluid cell using detergent and filtered/nanopure water. Rinse fluid cell with ultrapure ethanol and nanopure water three times. Then, dry fluid cell using clean nitrogen gas.

PROCEDURE

Preparation and mounting of supports ● TIMING ~2–8 h

1| Glue Teflon discs to a support to be mounted into the AFM. Depending on the AFM used, such supports could be magnetic stainless steel discs or glass slides. Reflectivity of mica discs shows whether the layered material has been distorted by punching. Glue undistorted mica onto Teflon discs using epoxy glue.

▲ CRITICAL STEP Glue should be completely hardened and uniformly distributed between mica and Teflon (or support), and devoid of air bubbles. In AFMs that displace the supporting surface, air bubbles between mica and support can cause vibration or drift on the nanometer scale. Before mounting the sample support in the AFM, clean all contact surfaces using propanol and/or ethanol. Even small particles between the support and AFM can cause vibrations and drift.

Setting up the AFM ● TIMING ~1 h (initially this may require more time)

2| Isolate AFM from sources that may cause electronic and mechanic noise and thermal drift. Noise may be detected by vibration detectors or by scanning the mica surface in buffer solution at minimal forces of 50 pN or less. Sources of electronic noises may be found switching off the devices individually. The AFM should be placed on an actively or passively damped table. For acoustic isolation, a glass bell may be used.

Choosing suitable AFM cantilevers ● TIMING ~1 h

3| *Choose suitable AFM cantilevers.* For achieving the best possible results, the cantilever properties must be adapted to the experiment. For high-resolution contact mode imaging AFM, cantilevers should be soft $\approx$0.1 N m^{-1} and exhibit resonance frequencies in buffer solution, which allow tracking the surface features at the scanning speed applied. For oscillating mode imaging, the cantilevers can be up to 30 times stiffer since the amplitude changes of the oscillating cantilever can be detected with sufficient accuracy to sense even very subtle force differences, which is required to prevent the deformation of membrane proteins. For specific applications even stiffer cantilevers may be needed, but the sensitivity of the deflection detection will impose limits. In all cases, the cantilever stylus should have a nominal radius of less than 10 nm. SMFS requires soft cantilevers having a high resonance frequency. Otherwise, small forces may not be detected and the maximum sampling rate of the cantilever limits capturing fast unfolding events.

▲ CRITICAL STEP Choosing the suitable cantilever determines whether the proteins can be imaged at high resolution or molecular interactions can be detected. Ideally, taking images of a test sample such as the purple membrane would identify a suitable cantilever, which should provide subnanometer resolution and which is subsequently used for investigating the sample of choice.

Buffer preparation and adsorption of membranes ● TIMING ~1 h

4| Prepare all buffers using nanopure water, analytical grade buffer and electrolytes. Cleave the mica disc uniformly using scotch tape.

▲ CRITICAL STEP Particles and macromolecules easily contaminate the object, support and AFM stylus. Thus, avoid any possible contamination during sample and buffer preparation and storage. Any item brought in contact with the buffer solution should be cleaned before use until being as clean as nanopure water. If only small amounts of biological sample are available, the cleanliness of AFM fluid cell and solutions may be checked by imaging the plain supporting surface in the adsorption buffer before adsorbing the sample.

5| Dilute the purple membrane stock solution in adsorption buffer to approximately 10 µg ml^{-1} and place 30 µl onto the freshly cleaved mica for approximately 15–30 min (ref. 18).

6| Exchange the adsorption buffer on the mica with imaging buffer. Rinse the sample with imaging buffer several times to remove weakly attached membranes.

7| Place the sample in the AFM fluid cell and thermally equilibrate the AFM for 10 min. During this time, you may focus the laser beam onto cantilever end and adjust the photo diode signal. A stable diode signal indicates minimum thermal drift. Engage AFM and start imaging the sample as described.

▲ CRITICAL STEP The observation that no membranes are adsorbed onto mica may indicate that imaging forces are too high, or that feedback of the AFM cantilever is badly adjusted. Alternatively, the concentration of the purple membrane in the adsorption buffer was too low. If you observe purple membranes forming stacks or aggregates, ultrasonicate the adsorption buffer containing purple membrane for 1–3 min and repeat adsorption. Ultrasonication dissolves purple membrane aggregates. Be aware that you may not ultrasonicate other protein membranes, since they are usually much less stable.

Low-resolution contact mode imaging ● TIMING ~1 h

8| Record an *F–D* curve directly after stylus engagement to show whether the stylus or supporting surface are contaminated. *F–D* curves with sharp transitions (**Fig. 1b**) in most cases indicates clean preparations. In contrast, *F–D* curves recorded between a contaminated sample and AFM stylus frequently show irregular and irreproducible *F–D* patterns.

▲ CRITICAL STEP Sample, buffer solution and fluid cell have to be clean, otherwise AFM stylus gets easily contaminated and must be exchanged.

9| After this, switch AFM to imaging mode and approach the sample with the stylus at a scan size and offset of 0. Adjust the lowest possible force and optimize feedback gains. Gains are optimal if they are at their maximal possible values without causing the AFM cantilever to oscillate during lift off. Then start scanning to collect a sample overview (50–100 μm scan). Keep the scanning speed small to avoid crashing the stylus into highly corrugated objects. If the sample is flat, scanning speed may be increased.

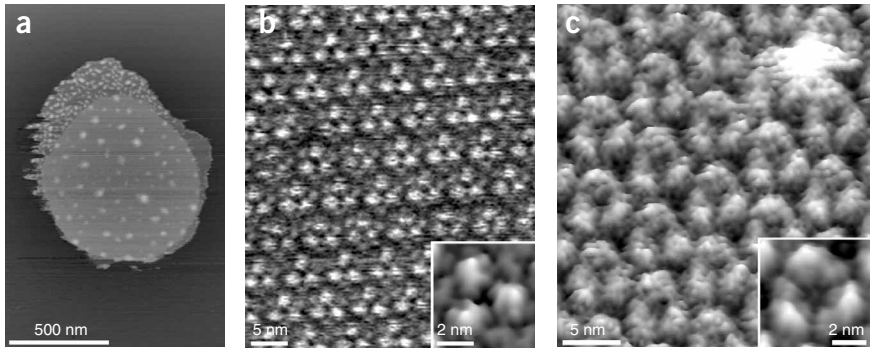

Figure 2 | High-resolution atomic force microscopy (AFM) of purple membrane. (**a**) Purple membrane directly adsorbed onto a supporting surface. (**b**) Extracellular and (**c**) cytoplasmic surfaces showing the substructures and trimeric assembly of bacteriorhodopsin. Contact mode AFM topographs were recorded in buffer solution at forces of 100 pN or less.

▲ CRITICAL STEP During imaging, the force applied to the AFM cantilever must be kept at minimal values ranging from 50 to 100 pN (ref. 1). Slightly increasing the forces reversibly deforms the flexible structural regions of the proteins[8] until they irreversibly deform at too high forces. In many cases, the applied forces have to be adjusted to obtain reasonably good contrast without distorting the sample. Repeated imaging of the same area shows whether the imaging process is destructive to the relatively soft protein membrane or not.

? TROUBLESHOOTING

High-resolution contact mode imaging ● TIMING More than 2 h

10| After imaging surveys, zoom in on protein membrane patches (**Fig. 2**, see panel **a**) and record *F–D* curves (**Fig. 1b**). To our experience, high-resolution topographs of protein membranes (**Figs. 2** and **3**) are best achieved by adjusting the electrolyte and pH of the buffer solution to an electrostatic repulsion of ≈50–100 pN (**Fig. 1b**, (1) of *F–D* curve recorded at 150 mM KCl). Adjustment of the electrolyte is facilitated by changing the electrolyte concentration and type, which results in different electrostatic repulsion between AFM stylus and protein membrane (**Fig. 1b**). Membranes should then be scanned at an applied force being slightly higher, ≈75–125 pN (ref. 1). For high-resolution imaging, protein membranes should be smooth and free of surrounding aggregates.

11| Each time before increasing the number of lines scanned per unit area, the force applied should be set to a minimum and gains to a optimum. To reach minimal forces, reduce the setpoint slightly to values at which the scanning cantilever and the sample loose contact. Minimal forces lie just slightly above this value. To approach optimal gains, increase them in small steps until the system oscillates, which is observed as fringes in the AFM image. Then reduce the gains just slightly below the values that bring the system to oscillate. This procedure may be adjusted to the scanning area and speed chosen. At too high scanning speeds, the cantilever cannot track the sample properly anymore. This results in long shadows observed in the deflection (error) image.

12| If structural details increase with the number of lines per area, this indicates a sharp stylus. If details do not get resolved better when increasing this number, the resolution limit is reached. This limit is defined by the sample, stylus and imaging parameters (scan speed, force and gains)[1,20].

▲ CRITICAL STEP In most cases, the AFM stylus is not sharp from the beginning but changes during scanning. Although a blunt stylus in most cases does not get sharp enough to contour fine details, a sharp stylus often gets sharper over time allowing high-resolution topographs to be recorded. This can be observed by imaging known biological structures such as the purple membrane. As the stylus changes during continuous scanning

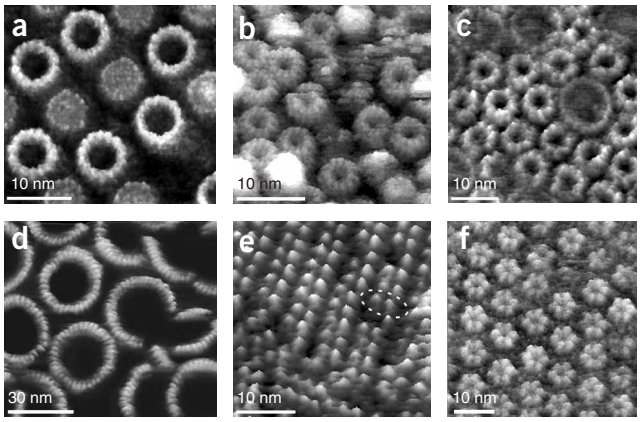

Figure 3 | High-resolution atomic force microscopy (AFM) topographs of native membrane proteins. (**a**) Ion-driven rotors from spinach chloroplast and (**b**) *Illyobacter tartaricus* FoF1-ATP synthase[26,27]. (**c**) High-light-adapted native photosynthetic membrane from *Rhodospirillum photometricum*[16]. (**d**) Perfringolysin O pore complexes[14]. (**e**) Dimeric bovine rhodopsin (dotted ellipse) in native disc membranes[28]. (**f**) Extracellular surface of gap junction hemichannels[29]. Contact mode AFM topographs were recorded in buffer solution at forces of 100 pN or less.

the substructures imaged change until finally the proteins can be imaged without an apparent asymmetry or other topographical features induced by tip artifacts[21]. Achieving high-resolution topographs needs patience. The operator has to wait for the moment in which the stylus gets ideally sharp and suitable for contouring the substructural details of the proteins. During this time, the operator has to continuously control the imaging parameters and to explore whether higher resolution can be achieved on a different membrane patch.

? TROUBLESHOOTING

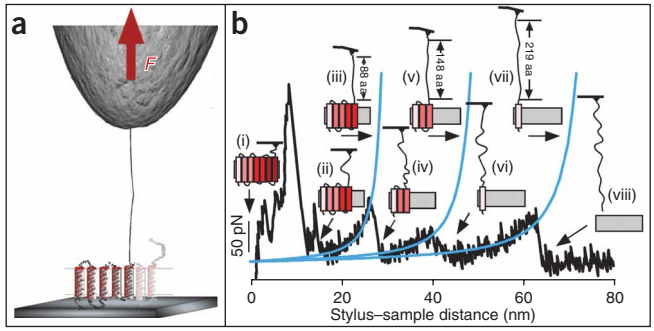

Figure 4 | Unfolding single membrane proteins by single-molecule force spectroscopy. After imaging (**Fig. 1a**), the atomic force microscopy (AFM) stylus is pushed onto the protein membrane. (**a**) If the terminal end of a single membrane protein (here bacteriorhodopsin) adsorbs sufficiently strongly to the stylus, a pulling force can be applied to the protein. (**b**) Secondary structures of bacteriorhodopsin unfold sequentially leading to characteristic force peak patterns recorded by the force–distance curve (*F–D* curve). Every peak describes a discrete unfolding event. Fitting a force peak using the WLC model reveals the length of the unfolded and stretched polypeptide. The distance between the first and the forthcoming force peak reveals the polypeptide stretch that has been released unfolding a structural segment (shown for transmembrane helices). This assigns the molecular interactions that stabilized the structural segments[25].

High-resolution oscillating mode imaging ● TIMING **More than 2 h**

13| Oscillate the cantilever at its resonance frequency in buffer solution[4]. This is required for high-resolution oscillating mode imaging, because it optimizes the response of the cantilever to force differences[22]. Before engaging, set AFM scan size and offset to 0 to minimize sample deformation and contamination of the stylus. To engage the AFM stylus, the cantilever amplitude may reach 10–20 nm. Before scanning the sample, adjust setpoint to minimal force. Minimize the noise of topograph and amplitude signal by optimizing gains and scan speed as described in Step 10. Adjust scan size to optimize topographic contrast and minimize feedback errors. High-resolution topographs may be achieved using small drive amplitudes of ≈1 nm. To observe artifacts, record topography in trace and retrace scanning directions. Compensate thermal drift by correcting setpoint manually[22].

▲ CRITICAL STEP See critical step mentioned in Step 7. Mechanisms contributing to the imaging contrast of oscillating mode topographs are sometimes not clear[23]. Besides structural features, surface charges, roughness, friction or elasticity can contribute to apparent topographical height differences[24]. In addition, operating the AFM while applying wrong oscillation parameters may artificially change the contrast.

? TROUBLESHOOTING

SMFS ● TIMING **More than 2 h**

14| After imaging, position the AFM stylus over the membrane proteins of interest (**Fig. 4**). Push the stylus onto the protein at 0.5–1 nN for ≈1 s, this forces the polypeptide to adsorb to the stylus in approximately 1–10% of all cases. Pushing at higher forces easily destroys the protein membrane, which can be confirmed by imaging the membrane again. Use the molecular bridge established by the polypeptide adsorbed to the stylus to mechanically manipulate the protein. Record an *F–D* curve while withdrawing the stylus from the membrane, this detects the interactions occurring upon mechanically pulling at the protein (**Fig. 4b**). If the polypeptide adsorbed to the AFM stylus is the C- or N-terminal end of the protein, the *F–D* curves will exhibit a length corresponding to that of the entirely stretched protein (take 3–4 Å per aminoacid into account). If the protein adsorbed with a polypeptide loop or slips off the stylus before being completely unfolded, the *F–D* curves will be shorter and thus difficult to interpret. Therefore, only *F–D* curves representing the entirely unfolded and stretched protein should be subjected to further analysis[7,25].

▲ CRITICAL STEP Each *F–D* curve describes the unfolding pathway of a membrane protein with single peaks reflecting individual unfolding events. To reveal different unfolding pathways, full-length *F–D* curves must be classified[7]. It may be challenging to judge from which terminal end the protein was mechanically unfolded. If the protein shows an unidirectional orientation in the membrane, this may be solved by identifying the sidedness of the membrane. Recording *F–D* curves of unfolding native and cleaved membrane proteins allows the terminal end from which the protein was pulled to be identified[25].

? TROUBLESHOOTING

? TROUBLESHOOTING

Low-resolution overviews (Step 9)

The images are noisy: check all mechanical and electrical noise sources. While imaging membrane protein preparations, the AFM stylus may be contaminated: scan mica repeatedly at high speed (20 lines per s) and modulate the forces applied to the AFM cantilever (≈200–500 pN) to brush off the contamination from stylus. If AFM stylus has been cleaned, *F–D* curves recorded on mica show a sharp bend upon contact of the support (lower *F–D* curve of **Fig. 1b**). No sharp bend indicates that either AFM stylus or mica are contaminated. If brushing off contaminants was unsuccessful, start with fresh sample and buffer solution.

If AFM stylus was good on previous sample, clean the cantilever in a 1% SDS solution followed by extensive rinsing with nanopure water, else take new cantilever. To prevent contamination of the AFM stylus, it may be helpful to add glycerol (up to 30%) to the buffer solution. Depending on the wafer from which the cantilever was taken, the stylus can show very different contaminating behavior. Thus, selecting AFM cantilevers from a different wafer may solve the problem.

If no membranes are found: reduce the force to make sure that membranes are not swept away by the scanning AFM stylus. If this was unsuccessful, prepare new sample using a different adsorption buffer (higher ionic strength, and/or different pH), a higher concentration of protein membranes and a longer adsorption time.

If mica is not clean: dialyze or wash your membrane protein sample by centrifugation to remove small particles that contaminate the mica surface.

High-resolution images (Step 12)

Image is unstable and streaky: reduce force applied to the AFM cantilever. To test whether force was too high, scan the same area at a lower magnification. Possible damage is visualized.

To reach minimal force, drift must be continuously compensated: ensure that the drift of the AFM is at its minimum by watching the cantilever differential signal over time.

AFM stylus may be contaminated or show tip artifacts: scan mica at high speed (20 lines per s) and modulate forces ($\approx$200–500 pN) to brush off contamination. Check cleanness by recording *F–D* curves on mica and by acquiring images again. If images show insufficient quality, check interaction of AFM stylus with membrane sample by acquiring *F–D* curves, and adjust the imaging buffer to damp the force applied for high-resolution imaging as indicated in **Figure 1b**. If buffer change does not help, change cantilever.

Strongly distorted AFM image: check the quality of sample preparation (is glue properly spread under mica? Is the mica tightly coupled to its support? Is there any dirt or dust between sample holder and sample? Has the fluid cell been mounted correctly, etc.?). Check whether cantilever is defective and whether piezo-scanner calibration is correct.

AFM oscillates at minimal force: optimize gains and scanning speed. If required, realign laser to optimize diode signal. An expected molecular symmetry is not seen: change AFM cantilever. Irrespective of all efforts, resolution remains mediocre: change cantilever.

Oscillating mode (Step 13)

See Troubleshooting information above (for low-resolution overviews and high-resolution images). If features are not interpretable, check sample in contact mode. Measure cantilever resonance[13], and make sure that oscillation is driven at this frequency.

Reversible sample deformation in scanning direction or disruption of sample: adjust oscillation to amplitudes in the range of 1–2 nm for high-resolution imaging. Optimize the detection of amplitude change to allow sufficiently small forces ($<$100 pN) to be detected to prevent sample distortion.

Stripes in scanning direction: too high imaging forces and/or scanning speed.

SMFS (Step 14)

If you do not find the protein membranes or sample is contaminated, see Troubleshooting information above (for low-resolution overviews and high-resolution images). Image protein membrane after recording *F–D* curves. Damaged areas indicate excessive forces for picking up single proteins. Select undistorted region of protein membrane and record *F–D* curves at lower forces ($\approx$200–500 pN) pushing the AFM stylus to the protein.

F–D curves recorded on protein membrane are not reproducible: record *F–D* curves on mica to see whether AFM stylus is contaminated. If contaminated, clean stylus as described in Troubleshooting information above (for low-resolution overviews and high-resolution images).

F–D curves are noisy: if reducing the pulling speed does not help, replace AFM cantilever.

F–D curves show long-range modulation: readjust laser onto cantilever. If nothing changes, the supporting surface may reflect laser beam into photo diode.

Lack of long *F–D* curves indicating the unfolding of a membrane protein: depending on the wafer from which the cantilever was taken, the stylus can stick very differently to the protein. Thus, selecting AFM cantilevers from a different wafer may solve the problem. Alternatively, it may help changing the buffer solution (electrolyte or pH).

General

For newcomers who learn high-resolution AFM, imaging of purple membrane is recommended, since it is commercially available and structurally well studied by AFM and SMFS[7,8]. Comparing the high-resolution topographs and *F–D* curves with those published will allow new AFM users to train to perfection. For imaging and SMFS, utmost cleanliness of sample, support and instrument, and stability of the latter is required. Irreproducible results may be related to badly mounted samples (mica not properly glued to the support), to contaminants in the sample solution or to contaminated tips. To identify and eliminate the problem, judging and improving the image quality is also recommended for SMFS.

ANTICIPATED RESULTS

Membrane protein surfaces are expected to be imaged at 1 nm resolution or better, depending on the corrugation amplitude. Flat membranes such as those of bacteriorhodopsin lattices, where protrusions are less than 1 nm, will allow subnanometer resolution and thus visualization of the loops that connect individual transmembrane helices to be achieved. However, proteins that protrude by 3 nm or more from the bilayer will be imaged at a resolution of 2 nm or worse, simply because such protrusions are likely to be flexible and because their height will prevent the surface to be properly contoured by a pyramidal tip. Examples shown in **Figures 2** and **3** can be obtained routinely.

The acquisition of *F–D* curves from single membrane proteins is easier than obtaining high-resolution images. However, the same precautions concerning cleanliness and stability of the instrument have to be taken as for imaging, that is, contaminants must be prevented, as they may introduce noise and other artifacts. The full interpretation of the *F–D* curves may be challenging, in particular when the structural information is sparse. In this case, it is important to have a sample where the protein of interest comprises a large fraction.

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