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Strategies to prepare and characterize native membrane proteins and protein membranes by AFM

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Abstract

Progress in characterizing native membrane proteins and protein membranes by atomic force microscopy (AFM) opens exciting possibilities. While the structure, oligomeric state and supramolecular assembly of membrane proteins are assessed directly by AFM, single-molecule force spectroscopy (SMFS) identifies interactions that stabilize the fold, and characterize the switching between functional states of membrane proteins. But what is next? How can we approach cell biological, pharmaceutical and medical questions associated with native cellular membranes? How can we probe the functional state of cell membranes and study the dynamic formation of compartments? Such questions have been addressed by immobilizing membranes on solid supports, which ensures the integrity of the native state of membrane proteins but does not necessarily provide a native-like environment. Direct attachment of membranes to solid supports involves non-specific interactions that may change the physical state of supported lipids and proteins possibly hindering the assembly of membrane proteins into native functional compartments. Thus, to observe the dynamic assembly and working of proteins in native membranes by AFM, supports are required that mimic the native environment of the cell membrane as closely as possible. This review reports on recent progress in characterizing native membrane proteins by AFM, and surveys conventional and new approaches of supporting surfaces, which will allow the function, dynamics, and assembly of membrane proteins to be studied by AFM in native cell membranes.

Keywords: Atomic force microscopy; Compartments; Native protein membranes; Single-molecule force spectroscopy; Tethered membranes; Polymer supported membranes; Protein assembly; Structure and function relationship; Molecular interactions

1. Introduction

Membranes are vital components of all living systems, forming the boundaries of cells and their organelles. Biological membranes consist largely of a lipid bilayer and of proteins embedded in or anchored to the bilayer. Lipids and membrane proteins form domains, which can vary and adapt to the functional state of the cell [1,2]. Thus, a cellular membrane can be viewed as a unique and dynamic combination of various domains coupled together spatially and functionally. The dynamic assembly of such multicomponent complexes into compartments ensures a high efficiency of various processes taking place simultaneously and warrants organelle integrity. Such supramolecular assemblies of membrane proteins are involved in essentially all processes of life, i.e., signal transduction, energy conversion, cell–cell communication, cell adhesion, trafficking, and transmembrane transport. In contrast to this importance, little is known about how single components, proteins and lipids, are organized into higher order structures and how this organization determines their biological activity.

Atomic force microscopy (AFM) enables imaging of native membrane protein surfaces at subnanometer resolution [3]. An important advantage over conventional optical microscopes is that AFM does not require fixation or labeling of the sample. Importantly, AFM allows observing the biological membrane in

Abbreviations: AFM, atomic force microscopy; AQP, aquaporin; SMFS, single-molecule force spectroscopy; LH1, light-harvesting1; LH2, light-harvesting2; RC, reaction center.

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the buffer solution and at the temperature needed to maintain its physiological function. In the past 15 years AFM has been established as an imaging tool to observe the structure of native membrane proteins embedded in the lipid membrane. A lateral spatial resolution of up to 0.5nm and an exceptional signal-to-noise ratio suffice to directly observe the oligomeric assembly of single membrane proteins such as channels, receptors, or transporters, to characterize their structural features, and to visualize single polypeptide loops connecting transmembrane alpha-helices or beta-sheets. Since AFM allows imaging of membrane proteins under physiological conditions, this technique has made it possible to directly observe membrane proteins at work [3–5••], and to track their diffusion in the lipid bilayer at subnanometer resolution [6]. Most of these exciting results have been achieved on reconstituted membrane proteins or on membranes containing only one or a few different membrane protein species [7]. However, within the past decade first attempts have been undertaken to image the supramolecular assembly of reconstituted membrane protein complexes [8••,9] and of proteins in native membrane patches extracted from cellular compartments [10••,11,12••,13•].

For high-resolution imaging by AFM membranes should be immobilized on supporting surfaces. In most cases, this attachment is facilitated by the adsorption of the protein membrane to a relatively chemically inert and hydrophilic solid support. Such so-called solid-supported membranes have allowed, and will still allow us, to gain important insights into the structure and function relationship of native membrane proteins. However, solid-supported membranes may be disadvantageous depending on the question addressed. For example, membrane proteins in membranes directly attached to the support often show impaired mobility [14••,15••]. This is because the gap between membrane and support is only 0.5–2nm, which induces steric constraints that impede the displacement of the protein. Additionally, the adsorption energy may have a feedback on the structural assembly of membrane proteins, as small as it is for a chemically inert and hydrophilic support. For example, it is well known that lipids of a solid-supported lipid bilayer can show very different structural assemblies and thus functional states compared to the lipids of a vesicle or a free-standing lipid bilayer [15••,16]. Since both lipids and proteins are mutually involved in the formation of cellular compartments [1,2], we conclude that the compartments of a supported cellular membrane behave differently from that of the native, intact cell membrane. From a biological point of view it is clear that the cell membrane and compartments react sensitively to their environment. Therefore, to be able to study by AFM how membrane proteins assemble into native compartments requires the development of alternative sample preparation strategies that do not potentially influence the process of compartmentalization.

In this review we provide a brief overview of recent achievements in high-resolution AFM imaging of native membrane proteins and protein membranes. Then, we summarize preparation methods to observe native membrane proteins. Finally, alternative techniques that have been developed to support native biological membranes and possibilities to transfer these strategies for high-resolution AFM imaging of native cell membranes and their compartments are described.

2. High-resolution AFM imaging of membrane proteins and protein membranes

2.1. Imaging purple membrane

The most intensively studied membrane protein by AFM is the light-driven proton pump bacteriorhodopsin (BR) (Fig. 1). BR and lipids naturally assemble into a two-dimensional trigonal lattice, the purple membrane of *Halobacterium halobium*. Since purple membrane is relatively easy to extract from the archaebacterium, chemically and physically stable, commercially available, and its structure has been solved by electron microscopy and X-ray crystallography, purple membrane is often used as a reference for high-resolution AFM. A lateral resolution of ≈0.5 nm has been achieved on purple membrane by AFM in buffer solution concomitant with a vertical resolution of ≈0.1 nm [17]. The exceptionally high signal-to-noise ratio of the AFM allowed structural details of single BR molecules to be observed and these structures to be correlated individual polypeptide loops connecting transmembrane α helices [17]. Calculating topographic averages of the BR trimer allows identifying structural details that appear similar among individual BR trimers and structural regions that exhibit an enhanced flexibility [3,18].

Reconstituted porin OmpF membranes and native purple membrane were the first protein membranes that could be imaged at subnanometer resolution by AFM [3]. Since most of the high-resolution topographs were initially achieved on membrane proteins that were two-dimensionally crystallized [3,19,20], it was thought that a crystalline assembly would be a prerequisite for observing membrane proteins at subnanometer resolution by AFM. In the meantime it could frequently be demonstrated that a comparable spatial resolution can be achieved on non-crystalline assemblies of membrane proteins as well [6,11,12••,21,22••].

Several factors may limit the spatial resolution achieved by AFM. To be imaged at subnanometer resolution the protein membrane should be immobilized onto a very flat supporting surface. A soft membrane adsorbed onto a support follows the modulation of the supporting surface. Thus, if the surface roughness of the support is much higher than the corrugations of the membrane protein the finest structural features will not be resolved. Muscovite mica is the most frequently used support for AFM. Fresh cleavage of the layered mica crystal provides highly reproducible atomically flat surfaces that allow adsorption of membranes to be tuned by ionic strength and pH [23]. High-resolution AFM imaging of fragile biological surfaces requires precise control of the force applied and the feedback parameters guiding the AFM stylus to precisely contour the object. So far all subnanometer resolution topographs have been recorded using contact mode AFM. In this imaging mode the force applied to the AFM stylus must be kept constant at a value smaller than 100pN. We have shown that for reproducible acquisition of high-resolution topographs the electrostatic interactions

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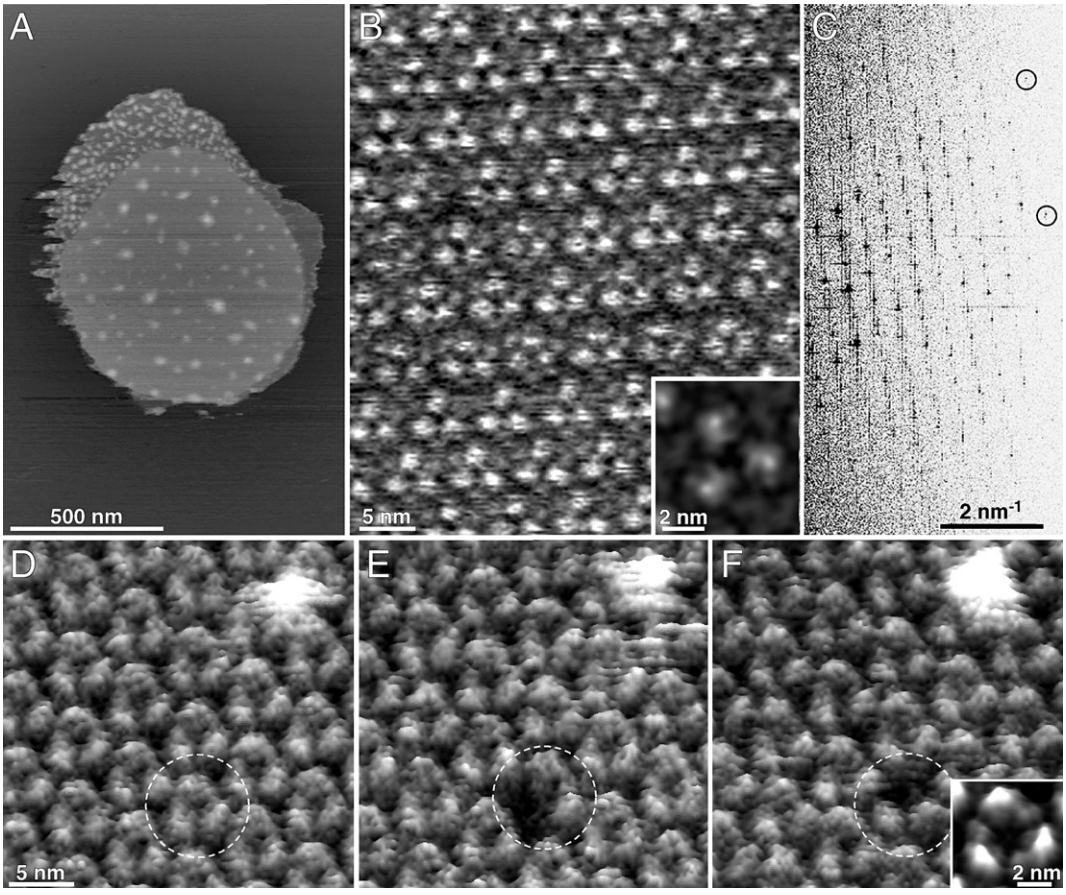

Fig. 1. AFM topographs of purple membrane adsorbed to mica. (A) Overview of a purple membrane patch. (B) High-resolution topograph of the extracellular purple membrane surface showing individual bacteriorhodopsin molecules assembled into trimers. The trimers naturally assembled into a hexagonal two-dimensional crystal. (C) Diffraction pattern of (B). The outer diffraction spots (circles) indicate a lateral resolution of better than 0.5 nm. (D) Cytoplasmic purple membrane surface. One bacteriorhodopsin trimer is encircled. After imaging a single bacteriorhodopsin molecule was removed by single-molecule force spectroscopy [36••]. The resulting hole (E) shows one missing bacteriorhodopsin (dotted circle). Repeatedly imaging the same area (F) shows the remaining bacteriorhodopsin molecules changing their positions (dotted circle) within the confinement of the surrounding proteins. The insets represent three-fold symmetrized correlation averages of the bacteriorhodopsin trimers imaged by AFM. All topographs were recorded in buffer solution.

between AFM stylus and biological sample should be adjusted to damp the AFM stylus and to distribute the excess scanning force applied over a wide area [7,24]. This approach allows minimizing the imaging forces interacting locally between the very apex of the AFM stylus and the small protein area contacting the stylus. Only these local forces contribute to the visualization of structural details while the forces distributed over a larger sample area do not. However, other promising imaging techniques such as non-contact or intermittent contact AFM may in the future reveal topographs of similar or even better resolution [12••,25]. Parameters limiting the resolution of the AFM topography are the sample mobility, flexibility, mobility, and corrugation amplitude. If a protein diffuses faster than the time required to scan its surface by the AFM stylus the protein may not be imaged or imaged only partly. Here, newly developed fast scanning AFM technologies recording up to 1300 topographs within a second may in the future help to capture the fast dynamics of membrane proteins [26,27••]. However, if the surface of the membrane protein is very flexible it may not be properly or only partly traced by the AFM stylus. Additionally, long sugar oligomers covering the protein membrane may hinder the AFM stylus from contouring the underlying protein surface with high precision. Finally, highly corrugated samples cannot be imaged at high-resolution, since structural information at the bottom of deep trenches cannot be assessed by the stylus for steric reasons.

2.2. Characterizing the oligomeric state of membrane proteins and membrane protein complexes

High-resolution AFM has been applied to characterize the oligomeric states of various membrane protein complexes from bacteria, plants and vertebrates. In all cases the purified, reconstituted membrane proteins or preparations of the native membrane have been adsorbed onto mica and imaged in buffer solution at sufficient resolution to observe individual subunits from the unprocessed AFM topographs. Systematic AFM studies of the ion-driven ring of the F_oF_1–ATP synthase showed, in contrast to the existing textbook knowledge that these rings are not necessarily composed of 12 c-subunits. It turned out that the stoichiometry of these ion-driven rings is intrinsic to the species from which the ATP synthase was derived (Fig. 2A, B). In

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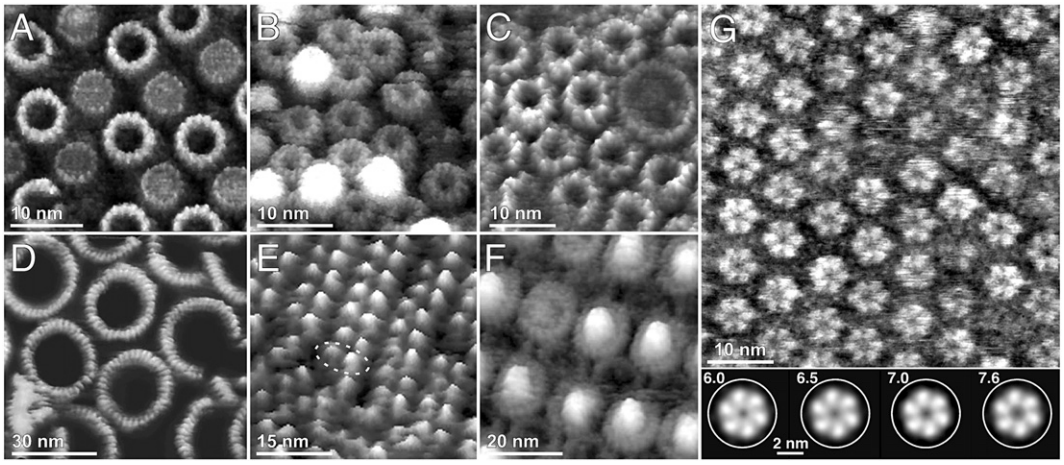

Fig. 2. Observing the oligomeric state, supramolecular assembly and function of native membrane proteins by AFM. (A) Proton-driven rotors from spinach chloroplast F_oF_1–ATP synthase. The reconstituted rotors have an up-side-down orientation. It was shown that different purification and reconstitution methods do not change the rotors 14-fold stoichiometry [29]. (B) Sodium-driven rotors from *Ilyobacter tartaricus* F_oF_1–ATP synthase show 11-fold symmetry [21]. (C) High-light-adapted native photosynthetic membrane from *Rsp. Photometricum* [22••]. A ratio of ~3.5 light-harvesting 2 complexes per core complex and core complex connectivity assure rapid energy trapping and prevent photo-damage. (D) Pore complexes of perfringolysin O (PFO), a prototype of the large family of pore-forming cholesterol-dependent cytolysins (CDCs). The pores were formed after addition of PFO to supported lipid bilayers containing cholesterol [65••]. (E) The oligomeric state of bovine rhodopsin in native disc membranes [10••,31•]. (F) Structural organization of the light-harvesting complex I photosynthetic core complex of *Rsp. rubrum* [8••]. (H) Extracellular surface of gap junction hemichannels from rat liver cells recorded at pH 7.6. In the presence of aminosulfonate compounds the hemichannels close their channel entrance with decreasing pH (galley at bottom). The open (pH 7.6) hemichannel entrance closes upon decreasing pH (6.0). (C), (D), and (F) are courtesy of S. Scheuring (Paris), Z. Shao (Virginia), and D. Fotiadis (Basel), respectively.

particular, it has been shown that the rings can be composed of 10 [28], 11 [21], 13 [66], 14 [20,29] and 15 [30] c-subunits. According to current models, the ring of the F_o motor of the F_oF_1–ATP synthase takes up as many ions as c-subunits forming the ring to perform one revolution to synthesize three ATP molecules. Apparently, the c-ring stoichiometry is correlated to the ion motive force each species establishes across the membrane and the ATP concentration. Thus, it is assumed that the organism has optimized the c-ring structure to use just as much energy as needed to synthesize ATP.

Light capture and charge separation, which represent the initial steps of photosynthetic activity, are executed by the photosynthetic unit (PSU), comprising of peripheral light-harvesting2 (LH2) complexes and core complexes, constituted of core LH1 subunits and the reaction center (RC). The supramolecular assembly of the proteins involved in the PSU remained to be elucidated until the AFM with its high signal-to-noise ratio allowed the structural investigation of the photosynthetic apparatus of native membranes extracted from several bacteria (Fig. 2C; [11]).

G protein-coupled receptors (GPCRs), which transduce different sensory, chemotactic, hormonal, and neuronal signals, are involved in many central functions of the human body. Therefore, GPCRs provide targets for a large number of therapeutics and opportunities for the development of new drugs with applications in all clinical fields. The oligomeric assembly of rhodopsin, the only GPCR whose structure has been solved to atomic detail, was discovered by AFM (Fig. 2E; [10••]). Rhodopsin is the only GPCR for which the presumed higher-order oligomeric state has been demonstrated by imaging native disk membranes of the eye using AFM. Based on these and X-ray structure data, an atomic model of rhodopsin dimers has been proposed, a model which is currently scrutinized in various ways [31•]. These results have significantly contributed to our understanding of GPCR structure and function.

2.3. Observing membrane proteins at work

In many cases a protein must undergo a dynamic conformational transition between discrete structural states to carry out its function. For X-ray crystallography highly ordered protein crystals are required where protein conformations are restricted by three-dimensional constraints of the lattice. Such constraints are not experienced by a protein embedded in its native membrane. Hydrophilic loops of membrane proteins reconstituted in a two-dimensional crystal in the presence of lipids may adopt all possible conformations [18]. The ultimate goal is to observe conformational states of membrane proteins in their native membrane without any constraints, which may be induced during sample preparation or by a supporting surface.

Several membrane proteins have been observed directly at work by AFM. The first conformational change was reported on pores of a surface (S-)layer of *Deinococcus radiodurans*, which stochastically closed by a central plug [3]. However, the triggering mechanism reversibly switching the pore conformation could not be determined. AFM topographs showed that the large extracellular loops of porin OmpF from *E. coli* could collapse onto the channel entrance [3,32]. This reversible mechanism closing the channel entrance, which was observed for the first time by AFM, could be induced by applying an electrical potential across the membrane or by changing the pH of the buffer solution. Later it could be shown that other porins of the same family moved their extracellular domains for gating the transmembrane channel as well [33,34]. The first conformational change of a vertebrate

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membrane protein was observed on communication channels from rat liver cells. Connexons of these gap junctions could close their transmembrane channel by two different mechanisms. Addition of Ca^{2+} ions induced the connexon subunits to move radially towards the channel entrance thereby closing the communication channel [4]. Gap junctions could also close at acidic pH and in the presence of aminosulfonate compounds naturally occurring in the cytoplasmic environment. However, the molecular mechanism of this channel closure was unknown. AFM topographs showed that gap junction hemichannels could close after aminosulfonate binding and pH change (Fig. 2H). In contrast to the Ca^{2+} induced closure, the pH induced closure rotates the connexon subunits similar to an iris like shutter of a camera [5••].

The observation and quantitative characterization of conformational changes has been achieved by detailed analysis of high-resolution images of regular protein arrays acquired by AFM [18]. In this work, the flexibility of surface exposed peptide loops of porin OmpF, aquaporin Z and BR were quantified in terms of the surface energy landscape of the respective proteins. In addition, an attempt was made to visualize conformational changes simply by similarity ranking of individual unit cell images, revealing smooth transitions between different conformations. The experimental approach demonstrated in this work will be useful for the analysis of specific conformational changes induced by a biological signal.

2.4. Tracking membrane protein diffusion and supramolecular assembly

Membrane proteins in living cells work as a part of functionally related compartments that have specific functions, such as signal transduction, energy transduction or membrane transport. Advances in molecular cell biology and proteomics are rapidly uncovering the components of these compartments which form dynamically in response to cellular functions and environmental changes. One of the next objectives in cell and structural biology is to quantify the dynamic assembly of membrane proteins into compartments and to structurally resolve the membrane protein assemblies of these compartments. To achieve this, both the dynamic and kinetic parameters of the single processes guiding such an assembly are needed. Single-molecule techniques provide insights into the individuality of biological macromolecules, their unique functions, reaction pathways, trajectories and molecular interactions [7,35]. Complementary to conventional ensemble measurements, these techniques will provide new biological insights and will assist in understanding the molecular principles of modern biology.

In an attempt to observe single membrane protein diffusion, time-lapse AFM topographs were recorded of protein membranes supported on mica [6]. The topographs allowed observing the assembly of single sodium-driven rotors from bacterial ATP synthase in buffer solution at subnanometer resolution. Time-lapse AFM topographs of the same area showed the trajectories of single rotors within the lipid membrane. Subsequent analysis of individual trajectories allowed the principal modes of the protein displacement to be distinguished. Within one trajectory, individual protein complexes could undergo movements assigned to free as well as to obstacled diffusion. However, the diffusion constants determined were orders of magnitude lower than that observed in a free membrane. This behavior can be explained by the ultrathin water layer sandwiched between the membrane and supporting mica surface, which promotes friction between protein and support.

Fig. 1 shows another example of observing membrane protein dynamics by time-lapse AFM. After a single bacteriorhodopsin molecule was mechanically extracted by single-molecule force spectroscopy (SMFS) [36••] a hole was left in the membrane (Fig. 1E). Repeatedly imaging the same area revealed that the remaining membrane proteins rotate within the constraints of the crystal lattice (Fig. 1F). Both examples discussed were recorded at a relatively slow imaging rate of typically 60–90s per frame. It is obvious that fast scanning AFM techniques [26, 27••] will allow a much higher temporal resolution of dynamic assemblies of membrane proteins. Importantly, the above results point out that the direct attachment of a protein membrane to a support can alter its dynamic properties significantly.

AFM was also applied to characterize how the supramolecular assembly of a bacterial photosynthetic membrane changes upon response to high- and low-light conditions [22••]. In a first step, routines were developed to achieve high-resolution topographs of photosynthetic complexes of native membrane preparations of *Rhodospirillum photometricum*. Individual light-harvesting1 (LH1) molecules forming a complex with the reaction centers (RC) were surrounded by LH2 complexes. Comparing topographs of preparations from low-light adapted membranes to that from high-light adapted membranes showed large changes in the ratio of the different membrane proteins. Whereas the local environment of the core complexes remained the same, the light-harvesting antenna domains grew under low-light conditions. This adaptation creates sufficient coupling of the core complexes to ensure that excitons find an RC in its ground state. The almost crystalline assembly of LH2 provides a sufficiently small separation between neighboring core complexes to warrant rapid energy transfer.

AFM imaging of native lens membranes revealed the supramolecular architecture of junctional microdomains [37••]. It was found that lens-specific aquaporin0 (AQP0) self-assembled into two-dimensional crystals. Presumably, the hydrophobic mismatch [2] with the lipid bilayer was driving the AQP0 into the tight packing of fibre cells, which is essential for lens transparency and adhesion. Conformations of extracellular surface loops indicated that the water pores of the AQP0 molecules were closed. Interestingly, the AQP patches were surrounded by densely packed gap junction hemichannels providing communication between the cells. Comparing the surface structures of these hemichannels with those imaged during pH- or Ca^{2+}-induced gating suggests that they were in their open conformation.

2.5. Limits of AFM for imaging membranes of living cells at subnanometer resolution

Currently, it is required to attach the protein membrane onto a supporting surface to reveal subnanometer resolution AFM topographs. The highest spatial resolution of AFM topographs achieved on native or fixed cell surfaces was >10 nm, which is neither sufficient to observe single membrane proteins nor to

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unveil their supramolecular assemblies. Many reasons can be found for this limitation. Substantial limiting factors could be the roughness, mobility, flexibility and extensive glycohydrate coatings of cell surfaces. The acquisition of high-resolution AFM topographs of the native cell surface is limited by the extremely slow scanning time of several tens of minutes required to accurately contour its structural features. Technological advancements, which would solve these limitations are currently not yet in sight although fast scanning AFM approaches may be developed further to significantly reduce the time needed to capture the highly corrugated surface structures of living cells. Even then, other obstacles to obtain high-resolution AFM imaging of living cell surfaces will have to be overcome.

3. Limits of sample preparation methods

3.1. On the importance of the membrane environment

As discussed above, cell membranes exhibit a high degree of complexity owing to their structural and functional variability, and to their dynamic transition paths. An important factor that contributes to this complexity is the membrane environment. Structural investigation by AFM at subnanometer resolution generally requires isolation of the cell membrane from the remainder of the biological system. However, any proper analysis of the formation of functional compartments must include all relevant components of the system, and must pay particularly close attention to boundaries where interactions are exchanged. The contribution of the membrane environment therefore deserves consideration in the analysis of its structure and function. A multiplicity of available conformational transition paths exists, and that path choice determines the trade-off between interactions exchanged and transitions performed. Possible transitions must be considered that may be altered by the physical and chemical constraints placed on the protein membrane environment. Therefore, isolation of the protein membrane from the native cell provides only a partial story. Molecular function and structure of cellular membranes are key to all of biology, from the processing of input in the simplest organisms to the most complex functional hierarchies in humans. It is thus clear that removing cell membranes from this environment may easily change their structural and functional properties. Thus, in this part of the review we will first introduce current strategies developed and applied to observe the structure and function relationship of native membrane proteins being reconstituted into a lipid bilayer or being extracted in a merely pure form as protein membranes from cells. After this we will describe the newest preparation techniques for attaching a protein membrane to a support but spatially and chemically separating each other. The goal of these strategies is to provide a native like environment, which maintains the full structure and function relationship of the cell membrane.

3.2. Observing purified protein membranes and reconstituted membrane proteins

To date most protein membranes imaged at subnanometer resolution by AFM have been simply adsorbed to a supporting surface such as mica, gold or HOPG. In most cases adsorption was facilitated by overcoming the electrostatic double layer repulsion between the membrane and support. By adjusting the electrolyte type, concentration and pH of the buffer solution this repulsion can be screened leading the membrane to approach the support close enough (1–5nm) to snap into van der Waals attraction, thus immobilizing the membrane (Fig. 3A). Under normal circumstances the adsorption energy of a single protein or DNA to the supporting surface is low. As a result weakly attached biomolecules can diffuse on the surface and can be easily swept away by the scanning AFM stylus [38]. In contrast, the adsorption energies of many proteins and lipids making up the protein membrane sum up and significantly higher forces (or energies) are required to remove the membrane from the support. Since the membrane protein is well anchored within the lipid bilayer, this simple adsorption strategy holds the protein onto the supporting surface with only a relatively small interaction energy acting on a single protein. This may, however, change if the supporting surface carries unusually high electrical charges or is hydrophobic. Gold and HOPG (highly ordered pyrolytic graphite) are such surfaces and it was reported that they can destabilize and denature water-soluble proteins [15••,39]. Whether these supports can also denature membrane proteins being embedded and stabilized by the lipid bilayer remains an open question. In contrast mica, the most commonly used support for AFM imaging, is hydrophilic and has surface charges matching those of phospholipid bilayers. This ensures that mica supported membrane proteins maintain their integrity and native properties.

Solid supported protein membranes have some fundamental drawbacks. These arise from their proximity to the bare solid surface onto which they are deposited. In buffer solution the membrane-support separation of 0.5–2nm is usually not sufficiently large to avoid direct contact between transmembrane proteins incorporated in the membrane and the solid surface (Fig. 3A). This problem is particularly serious when working with membrane proteins having extramembranous domains extending more than 1–2nm (Fig. 3B). Furthermore, water molecules bridging this gap certainly have different properties compared to water in the bulk solution. The water structure is highly disturbed and the mobility of water molecules is reduced. At the same time, lipid molecules and membrane proteins can establish non-native and non-specific interactions with the support. In the case of mica these interactions are sufficiently weak that the structure–function relationship of membrane proteins is not disturbed. Nevertheless, these interactions are sufficiently high to restrict the lateral mobility of membrane proteins [6].

3.3. Strategies to tether and separate protein membranes on a support

Membrane proteins exhibiting large anchors or aqueous domains show significantly impaired behavior in solid supported membranes. Depending on their chemical and physical surface properties, supports can establish non-native interactions with protein membranes. Such interactions could

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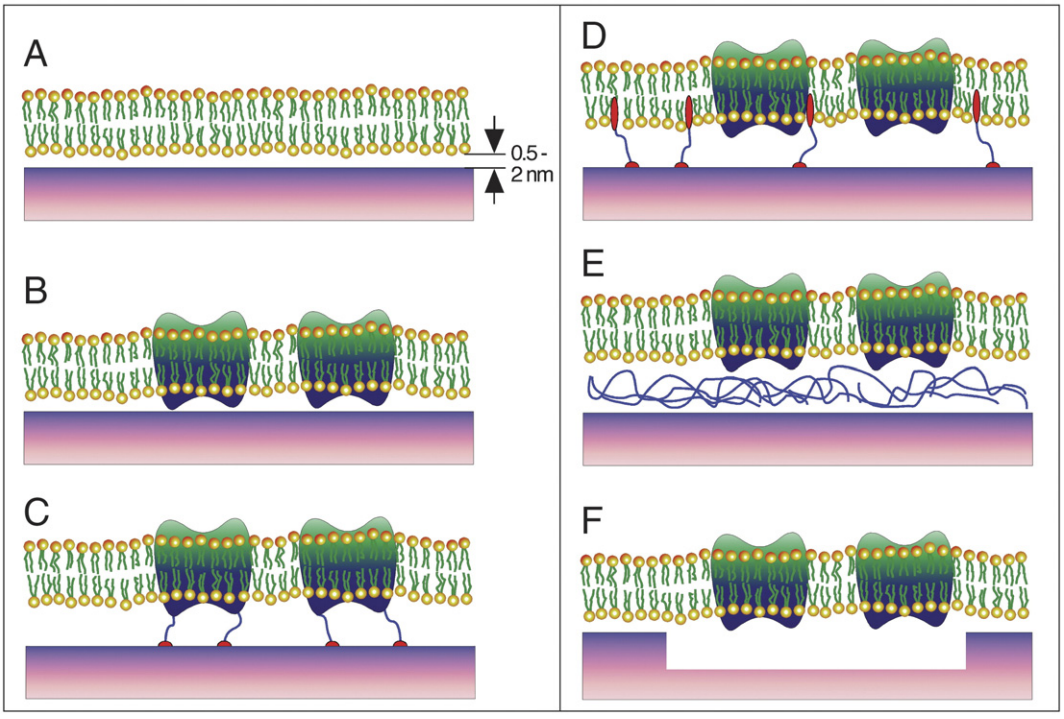

Fig. 3. Strategies to support protein membranes for high-resolution AFM. In buffer solution the solid supported lipid bilayer (A) and protein membrane (B) show a gap of only ≈0.5–2 nm. This can lead to non-specific interactions with the supporting surface. However, if the support is chosen correctly the structure–function relationship of the membrane protein remains the native one. (C) Protein tethered membrane. A functional group engineered onto the extramembranous domain attaches the membrane protein to the support. Adjusting the tether length allows controlling the distance (≈5–100 nm) between membrane and supporting surface. (D) Functionally tethered lipid membranes. The hydrophobic end of the lipid incorporates into the membrane while its large hydrophilic head group anchors the lipid to the support and holds the membrane at a given distance of typically 10–100 nm. (E) Polymer cushion supported membrane. A protein membrane is adsorbed onto a polymer covering a support. In the ideal case the polymer supporting the membrane is made of a native polymer surrounding the cell membrane investigated. Possible cushion materials are polymers of the extracellular matrix such as collagen or cellulose. (F) Protein membrane covering a hole, which could be used as a nano-well to establish an electrochemical potential or gradient.

disturb the structural and functional properties of the supported lipid bilayer and it may be assumed that this perturbation may also influence the stability, structure, function or assembly of membrane proteins [15••]. Only few careful studies on the functional consequences of these interactions are available [15••,40••,41•,42••], but as we know from other studies on native membranes such interactions can enhance or impair the membrane protein function. To suppress non-specific and non-native interactions of membrane proteins and lipids with the support has lead to the idea of spatially separating both from the supporting surface.

3.3.1. Supported protein membranes

In general, two strategies are pursued to prepare native membrane proteins for their observation by AFM. Protein membranes are either extracted from the cell by mechanical disruption, or membrane proteins are solubilized, purified and then reconstituted into a lipid bilayer. Both strategies can be rather time and material consuming. The adsorption of these protein membranes onto a supporting surface has been discussed in the previous section. Recently, Milhiet and co-workers [43•] prepared transmembrane proteins differently for high-resolution AFM imaging (Fig. 4). Firstly, a lipid bilayer was prepared which almost completely covered a supporting mica surface. Then, the supported bilayer was destabilized using a sugar-based detergent, and solubilized membrane proteins were added to the solution. Depending on the incubation parameters the membrane proteins inserted into the lipidic bilayer. After their reconstitution into the lipid membrane the membrane proteins were imaged using high-resolution AFM, revealing the supramolecular assembly of light harvesting complexes (Fig. 4D–E) and reaction centers from three different organisms [43•]. The spatial resolution of these topographs was sufficient to resolve the unidirectional orientation and stoichiometry of individual transmembrane proteins. This preparation method is similar to the ones frequently applied to biofunctionalize surfaces or to reconstitute membrane proteins into detergent destabilized vesicles [44]. After coating a surface with lipid bilayers, membrane proteins or anchors to which soluble proteins can be attached as functional units are incorporated. The preparation method only requires picomolar amounts of proteins and is thus applicable to membrane proteins, which cannot be expressed at levels required for other structural studies such as X-ray crystallography, electron crystallography or nuclear magnetic resonance spectroscopy (NMR).

In the future this approach may be applied to observe membrane proteins in supported bilayers such as used for many

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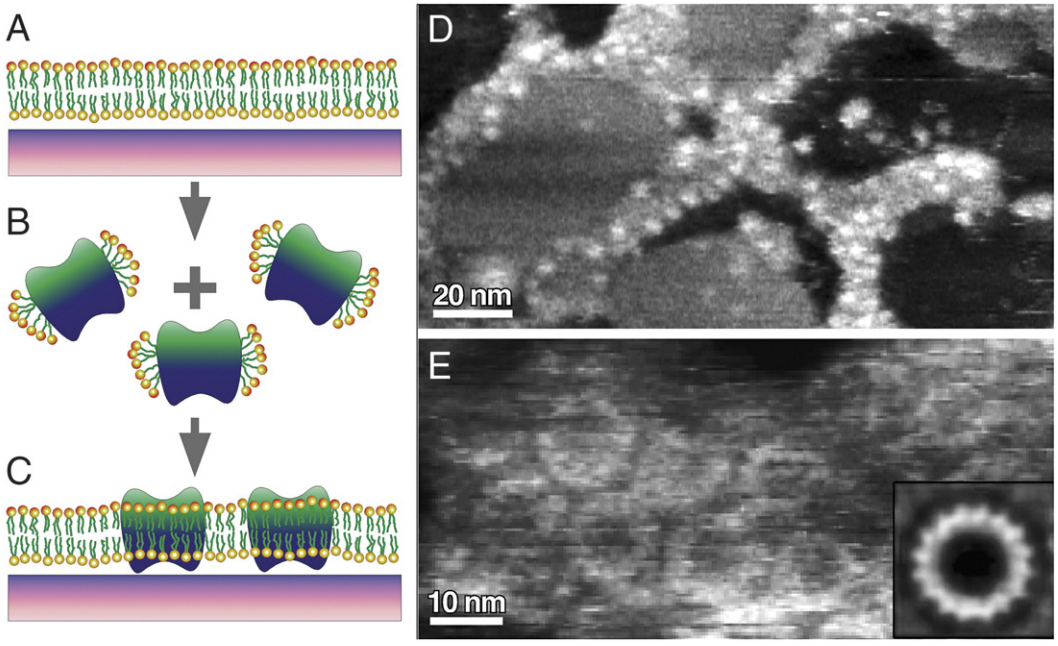

Fig. 4. Reconstituting membrane proteins into supported lipid bilayers for high-resolution AFM. (A) In a first step a lipid bilayer was deposited onto a supporting mica surface. Since the mica surface is hydrophilic and relatively chemically inert, the lipids adsorb via non-specific physical interactions. (B) Then the lipid membrane was destabilized using sugar based detergents and solubilized membrane proteins were added to the buffer solution. (C) Under certain incubation conditions the membrane proteins inserted into the lipid bilayer. (D) Tapping mode AFM topograph showing the light-harvesting complex1 reaction center (LH1-RC) from *pufX-Rhodobacter sphaeroides*. (E) At higher resolution the LH1-RC complexes were contoured. Insert, average revealing the 16 α/β heterodimers of LH1 [43•]. AFM images by courtesy of D. Levy (Paris).

biotechnological applications and discussed in the forthcoming sections of this review. The extension of this method to other membrane proteins would not be completely unexpected since many laboratories already have established procedures to reconstitute membrane proteins into bilayer systems [44,45]. The challenge, though, would be to transfer and to apply this knowledge to supported lipid membranes. A major breakthrough would be to demonstrate that any given membrane protein can be reconstituted into lipid bilayers which are tethered by spacers to a support, into lipid bilayers which are supported by a cushion mimicking the native environment of a cell membrane, or into lipid bilayers which are spanning over a nanoscopic hole (see following sections).

3.3.2. Engineered membrane protein tethers

Protein tethers provide a strategy frequently applied in biotechnology to biofunctionalize surfaces, and to design, for example, biosensors based on electrical and optical detection of specific biomolecular interactions. Here the tethers fulfill their function to attach proteins in a mechanically and chemically robust manner to the support. At the same time a tether can separate protein and support to decouple both systems and to retain the protein in its native state [46]. In SMFS such tethers are often applied to anchor peptides, proteins or nucleic acids to the AFM stylus [47] or supporting surface [48•]. This approach allows characterizing specific interactions of, for example, receptor and ligands, or to detect and locate specific biomolecular interactions on cellular surfaces [47]. However, in all of these applications it must be ensured that the protein tether does not interfere with the proper protein folding and function. Using molecular dynamic simulations it was found that neither the folding mechanism nor the transition state of a Honeycutt Thirumalai β-barrel protein was altered by attaching the tether either to a fully structured or a completely unstructured region of the polypeptide [42••]. In contrast, tethering a partially structured region of the transition state led to dramatic changes. As expected, it was found that the folding rates and stability of the protein were impacted differently by the surface depending on the tether length. Such and other results have implications for designing and using tethered proteins [15••,46,49•]. The goal of applying tethers to membrane proteins is to functionalize a particular extramembranous domain, which can then be attached specifically to a supporting surface (Fig. 3C). This approach has the advantage of stringently controlling the orientation and the distribution of proteins on the supporting surface, and to switching their attachment “off” and “on” [49•,50•]. Functional tethers used to specifically attach membrane proteins to a supporting surface can vary significantly, and range from reversible attachments using histidine-tags [49•,51,52], to irreversible covalent ones [14••].

Recently, a first example of tethering single membrane proteins to a supporting surface for high-resolution AFM imaging was presented [53•]. In a first step cysteines were engineered into the periplasmic residues of OmpF porin from *E. coli*. Then, the porin was solubilized and adsorbed to a template stripped ultraflat gold [54] surface (Fig. 5A). After extensive washing steps, detergent/lipid micelles were added to reconstitute the porins into the lipid (Fig. 5B). The reconstitution step incorporated the transmembrane proteins into a biomimetic membrane that was characterized structurally by AFM.

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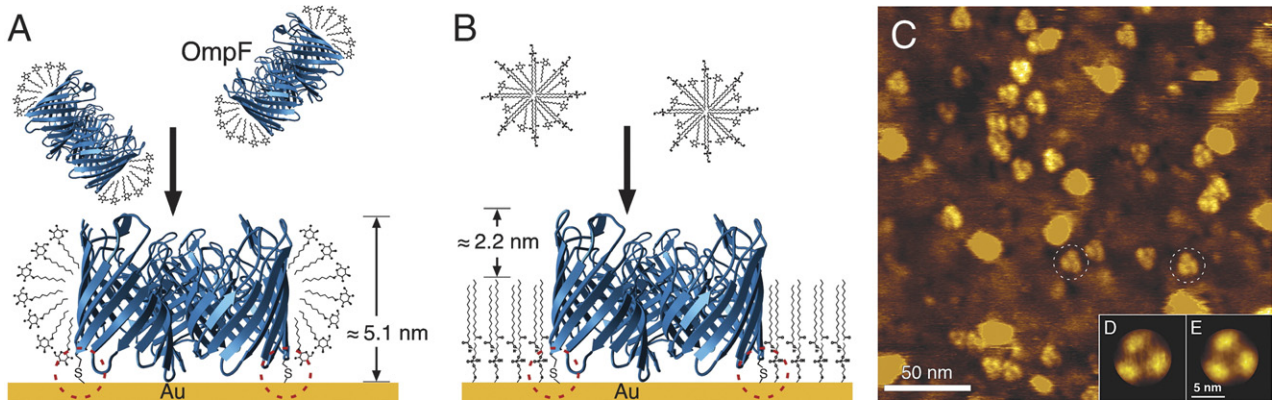

Fig. 5. AFM imaging of the membrane protein OmpF porin tethered to an ultraflat Au surface. (A) In a first step OmpF porin trimers solubilized in detergent (Octyl-β-glucopyranoside (OG)) were adsorbed onto ultraflat Au surfaces. Before adsorption the Au surfaces were passivated with β-mercaptoethanol. After adsorption, the cysteine residues engineered into periplasmic loops (E183C) of the OmpF trimer formed a covalent bond with the Au surface (red dotted circles). (B) In the following preparation step mixed OG/thiolipid micelles were added to cover the Au surface and to surround the OmpF trimers with a hydrophobic belt. The thiolipids chosen had sufficiently long hydrophobic chains to fully cover the hydrophobic region of the porins. (C) After extensive washing steps high-resolution AFM topographs could be recorded showing the individual porin trimers (white dotted circles) being randomly orientated on the support. As revealed by the characteristic appearance of the large polypeptide loops of OmpF porin all trimers exposed their extracellular surface to the aqueous solution. The correlation average calculated from tethered OmpF trimers (D) resembled that calculated from the extracellular surface of OmpF trimers being reconstituted into a lipid bilayer (E) [53].

High-resolution topographs reproducibly resolved substructures of the porin trimers although they were highly diluted on the Au surface (Fig. 5C). The surface structures of all porin trimers imaged by AFM corresponded to that of their extracellular surface thus confirming that all porins have been covalently attached via their cysteine to the gold surface. Additionally, the structural features observed resembled those observed for native functional porin OmpF. This suggests that the structure and folding of the porin OmpF proteins did not change. Functional experiments [55] showed that the porins covalently bound to the Au surface retained their full biological activity. However, the applicability of this method to observe membrane protein dynamics and to observe their assembly into higher ordered compartments is limited. Thus, in the future it may be applied to biofunctionalize surfaces with membrane proteins, to characterize structural properties of membrane proteins, such as their oligomeric state, or to monitor functionally related structural changes of single membrane proteins. So far, however, membrane proteins that were attached but separated from the supporting surface by a tether have not been imaged at high-resolution by AFM. Although such methods are frequently applied to functionalize surfaces, and high-resolution AFM has been demonstrated for several of the preparation steps, high-resolution AFM imaging has not been achieved on membrane proteins tethered to but separated from solid surfaces.

3.3.3. Polymer cushions

The goal to suppress non-specific and non-native interactions of membrane proteins and lipids with the support has also led to the development of polymer supported membranes [15••,16,46]. Such polymer cushions, which are typically between 5 and 100 nm thick, can serve as a lubricating layer between the membrane and the support. They can assist self-healing of local defects in the membrane, facilitate the insertion of large transmembrane proteins, and eliminate non-specific interactions with the support (Fig. 3E). However, it has been shown that the application of polymer cushions requires careful consideration of the interactions occurring between the supporting surface and the polymer and those between the polymer and the protein membrane [14••,15••,16]. These include attractive van der Waals interactions, electrostatic interactions, repulsive undulation forces due to thermally excited bending undulation of the protein membrane, and polymer induced forces [16]. Consequently, non-native interactions have to be eliminated and native interactions have to be provided to reach the goal of a supported biological membrane that shares the functional properties of native cellular membranes.

To some extent the polymer cushion mimics the extracellular matrix, and instead of using a non-biological polymer some approaches already use components of the extracellular matrix as a cushion [15••]. In this case the proximate environment of the membrane would reflect the native one. A particularly versatile native material for generating polymer cushions is regenerated cellulose [40••] which can be used to design films with adjustable thickness and properties. The biological effectiveness of such native polymer cushions was illustrated on membranes, with human platelet integrin and supported by a 5 nm thick cellulose film. On probing the interaction between these membranes and giant vesicles exposing integrin-specific ligands the adhesion free energy for the interaction was 3 to 10-fold higher than the adhesion energy obtained in analogous experiments using solid-supported membranes [40••], and comparable to the value inferred from the integrin–ligand dissociation constant. Integrins thus seem to fully retain their mobility and native functionality when incorporated in polymer-supported membranes. Interestingly, it was also shown that such cellulose membrane supports maintain the activity of adsorbed enzymes much better [15••]. Biopolymers and biomaterials that reconstitute the native cellular environment provide a toolbox to create not as yet exploited possibilities of cushions to support

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cellular membranes [16]. In the future, it may be straightforward to support membranes of different cell types in their specific native-like environment.

3.3.4. Functional lipid headgroups

One other widely used approach to separate protein membranes from the supporting solid surface exploits functionally tethered lipids (Fig. 3D). To reduce the interlayer coupling to the support, such lipopolymers incorporate their lipids in the membrane bilayer with the polymer head groups acting as spacers that control the distance between membrane and support [41•,46,51]. These headgroups can be functionalized using a wide range of compounds including oligo(ethyleneoxide) [56] and poly(ethyleneoxide) [14••], polymers [15••], hexahistidines [49•,51], and oligopeptides with thiolgroups [55,57]. The possibility to adjust spacer lengths allows to minimize or to exclude non-specific interactions of the protein membrane with the support. Fine tuning of the membrane-support distance of typically 10–100 nm together with the lateral spacer density enables to adjust the viscosity of the supported membrane, both of which control the lateral diffusivity and function of transmembrane proteins [14••,15••,58••]. Alternatively, headgroups of the lipid membrane may be attached to spacers or polymer cushions coating the support surface. Both approaches would, for example, allow establishing model membranes to study the insertion, assembly and interactions of different membrane proteins [14••,40•,41•,49•]. In addition, they may allow the attachment of proteins or protein complexes to supported protein membranes to be studied. It may, for example, be possible to investigate the attachment of proteins to glycosylphosphatidylinositol (GPI) anchors and to characterize how the proteins assemble to functional units on the membrane. Other examples may include investigating G-protein interactions with receptors or the assembly and functionality of integrins [41•].

3.3.5. Free spanning membranes

Lipid and protein membranes spanning micro- and nanoscopic holes have been developed for multifunctional characterization of cell membranes and various biotechnological applications [59,60•]. Most importantly protein membranes spanning over holes can be assessed from both sides at which they can be targeted side-specifically. The first application of such nanoscopic holes for the structural investigation of membrane proteins by AFM was shown recently by the Scheuring group [61••]. Protein membranes were adsorbed onto a crystalline Si(001) support with nanoscopic holes of 90–200 nm in diameter. Since the membranes were much bigger than the holes and the distance between the holes was chosen to be relatively close to each other (≈500 nm), most of the adsorbed membranes covered at least one hole. If the protein membrane sealed the hole tightly, both surfaces of the free spanning membrane could be exposed to two different environmental conditions. Such asymmetrical conditions mimic that of the cell membrane. For example such a setup would allow establishing concentration gradients across the membrane and to establish a naturally occurring membrane potential. It would also be possible to access signaling proteins from one side of the membrane and to observe their action on the other side by AFM [60•]. So far high-resolution AFM topographs could only be observed on the mechanically stable surface-layer from *Corynebacterium glutamicum* spanning the holes [61••]. These S-layers are naturally designed to withstand harsh chemical and mechanical conditions thereby protecting bacteria from environmental perturbations [62]. However, in the future faster and more sensitive AFM imaging techniques (see above) and improved preparation conditions may be applied to observe softer protein membrane patches at sufficiently high resolution. Such 'non-supported' membranes may be used for structural investigations of the membrane proteins and their compartments, most likely in combination with technologies to characterize the function of membrane proteins.

4. A look to the future

Characterization by AFM requires the biological object to be attached and supported by a surface. When planning the experiment it should be considered that the immobilization or attachment of a flexible biological object onto a two-dimensional supporting surface may change its structure. This is the case for DNA, RNA or fibrillar structures where the immobilization onto a support limits their conformational freedom by forcing them onto a two-dimensional support. Membrane proteins are naturally embedded or attached to a two-dimensional lipid membrane. Attachment of the protein membrane onto a support forces the membrane to flatly adsorb. This simple adsorption step may change the bending and thus the stress of the membrane. Nevertheless, in most cases the membrane proteins stay in their native conformation and retain their functionality [15••]. Thus, the direct attachment of protein membranes to a supporting surface has already allowed to address some pertinent questions regarding to the structure and function relationship of membrane proteins. However, due to non-specific interactions with the supporting surface the membrane can show different structural and functional states [15••,16]. Consequently, membrane proteins hindered by their interactions with the alternating lipidic states and non-specific interactions with the supporting surface may show a changed diffusion behavior [6]. The fact that the functional state of the lipid or electrostatic interactions between proteins determine what kind of assemblies membrane proteins form [2] suggests that direct interactions of membrane proteins with lipids having changed their functional states and with a supporting surface having certain physicochemical properties will influence the self-assembly of membrane protein into functional compartments. Thus, new approaches are required to observe membrane proteins forming compartments reflecting the functional state of protein membrane.

So far AFM is the only method that allows the complex assembly of membrane proteins into functional units to be directly observed at subnanometer or nanometer resolution [10••,11]. However, to monitor the dynamic assembly of membrane proteins related to the function of the cellular membrane requires new preparation procedures. We have scrutinized possible preparation conditions which have been developed and may in the future be applied to observe protein membranes or cell membranes in

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conditions mimicking their native environment. The development and application of such model systems is mandatory to probe the complex and dynamic assembly of membrane proteins by AFM. What will be the next scenario? According to modern cell biological insights membrane proteins can modulate or switch their functional state depending on the environment and the compartment in which they reside. High-resolution AFM images of such compartments may assist identifying the complex supramolecular arrangement formed by different membrane proteins. Certainly, after reaching this first goal of imaging functional related compartmentalization of protein membranes it will be important to explore how these compartments change the functional states of membrane proteins.

High-resolution AFM topographs can be applied to identify the functional conformational states of single proteins. But what happens if their functional state is not reflected by the protein surface? Here, it may be required to combine high-resolution AFM imaging with modern fluorescence microscopy, which cannot sufficiently resolve supramolecular structure but can identify the functional state of membrane compartments. However, an alternative may exist. Recently it was shown that the SMFS data recorded on single membrane proteins can identify their structural stability and functional states [36••,63••,64••]. Obtaining such "functional fingerprints" of membrane proteins assembled in the cell membrane will provide hitherto unexpected possibilities to learn about membrane proteins and their assemblies, which maintain the functional states of cellular membranes.

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