High-Resolution Imaging and Manipulation of Membrane Proteins by atomic force microscopy (AFM)

Summary
Membrane proteins are nanometric machines fulfilling defined functions in cell membranes. They work as transporters, linkers, adhesion molecules, channels, pumps, receptors, enzymes, and in the bio-energetic machineries, only naming a few tasks. In agreement with their importance, it was found that 25% of all genes in organisms ranging from bacteria to human, code for membrane proteins. Biologists have a set of techniques to analyze membrane protein structure and function. Here, the use of atomic force microscopy (AFM) as a powerful tool for high-resolution imaging, to nano-manipulate individual molecules and to perform force measurements on single membrane proteins is reviewed. Most recently, the AFM has shown to be a unique technique to investigate native multi-protein containing membranes at submolecular resolution.

Introduction
Atomic Force Microscope (AFM) [1] uses a faint tip mounted on the end of a cantilever to probe an object. The object and the tip are displaced with respect to each other using piezo elements. In principle, the AFM can be operated in two different ways: First, X- and Y- displacements between tip and sample allow the acquisition of images. In this case, the topology dependent deflection of the cantilever is compensated by faint Z-movements to keep the tip-sample distance constant. High-resolution AFM imaging has developed into a powerful tool in membrane protein research and is now a complementary technique to X-ray crystallography and electron microscopy [2]. The AFM is a remarkable instrument allowing high-resolution topography of biological samples to be acquired near physiological conditions, i.e. in buffer solution at room temperature and under normal pressure [3]. Topographs with a lateral resolution <10 Å and a vertical resolution of ~1 Å can be acquired [4]. By applying additional loading forces to the AFM tip during scanning in X- and Y- dimensions, biological objects can be nano-dissected [5].
Second, large Z-displacements between tip and sample allow the acquisition of graphs, displaying the force exerted on the tip as function of the Z-separation between tip and surface with a bio-molecule being stretched in between. Force measurements allow inter- and intra-molecular forces, e.g. ligand- receptor binding and protein unfolding, to be measured with a sensibility down to \( \sim 10 \) pN [6]. Here, recent achievements of high-resolution imaging combined with nano-dissection and force measurements are reviewed.

**Imaging Combined with NanoDissection**

Surface layers (S-layers) represent the outermost cell wall layer of many bacteria and archaea, and are regular twodimensional (2D) protein networks. These layers withstand non-physiological pH, radiation, temperature, proteolysis, pressure and detergent treatment, thus protecting the cell from hostile factors. Moreover, they serve as molecular sieves as well as in phage recognition [7]. The S-layers of Deinococcus radiodurans often stack on top of each other. In order to visualize the surface structure in between stacked membranes, layers can be mechanically separated by applying additional loading forces to the AFM tip. When imaging at a minimal loading force of \( \sim 100 \) pN applied to the tip, the top S-layer surface is imaged at high resolution (Fig. 1a, top). During the image acquisition (Fig. 1a, middle) the loading force to the tip is increased to \( \sim 600 \) pN. This mechanical treatment punctures the top S-layer, exposing the inner surface (Fig. 1b), and the surface of the below layer, exposing the outer surface is now accessible to the tip (Fig. 1c). Hence nano-dissection can be used to analyze molecular interaction between surfaces of two membrane layers.

**Imaging Combined with Force Spectroscopy Measurements**

The S-layer of Corynebacterium glutamicum is formed of PS2 proteins. The layer was shown to be stable in 3% SDS at temperatures up to 60° C. The C-terminus of the protein contains a stretch of 21 hydrophobic aminoacids (aa) shown to mediate
S-layer / cell wall attachment.
The flower-shaped inner surface exposes the hydrophobic C-terminus of the native S-layer adhered to the AFM tip and promoted assessment of the mechanical stability of the protein layer network using the AFM tip by unzipping single molecules. Three different types of force-distance curves were reproducibly acquired (Fig. 2) with 1 (Fig. 2a), 2 (Fig. 2b), or 3 (Fig. 2c) strong rupture peaks of ~270 pN with periodicity of 21 nm (indicated by red arrows in Fig. 2). These strong rupture peaks are interspersed by faint peaks of ~70 pN (indicated by white arrows in Fig. 2).
The concomitant removal of subunits out of the S-layer ensemble was documented by AFM imaging before (Figs. 2a, b, c; left panels) and after (Figs. 2a, b, c; right panels) force curve acquisition. The repetitive force measurement pattern of a faint and a strong rupture peak reports the subsequent unzipping of two subunits. This unexpected cooperativity of pairs of subunits reported on the architecture of the Corynebacterium glutamicum S-layer. Using sharp AFM tips, single units within a membrane ensemble can be addressed (Fig. 2d). Under controlled conditions, highly reproducible force spectroscopy graphs can be acquired (Fig. 2e), providing novel insights into the intermolecular forces between membrane proteins (8).

**Imaging on Native Membranes at Submolecular Resolution**
In photosynthesis, highly organized multi-protein assemblies convert sunlight into biochemical energy with high efficiency. The individual proteins of the photosynthetic chain are structurally and functionally known to a great detail. A remaining challenge is posed by the elucidation of supramolecular structures assembled from multiple protein subunits. The core-complex is constituted of the reaction center (RC) and light harvesting complex 1 (LH1) subunits intimately associated to perform the first steps of the photosynthesis, light trapping and charge separation. AFM was used to investigate the photosynthetic core-complex in native membranes of Blastochloris viridis under physiological conditions (Fig. 3a).
In Bvc viridis the RC consists of the (L), (M), (H) and the tetraheme cytochrome (4Hcyt) subunits, and LH1 of the α and β-polypeptides. High resolution imaging combined with nanodissection of the core-complex yields more insight into the spatial organization between the LH1 and the RC subunits in the native membrane. Starting from the complete complex 4Hcyt-RC-LH1 (Fig 3b, c; number 1), the 4Hcyt topping the RC is dissected by the AFM tip resulting in a RC-LH1 complex (Fig 3b, c; number 2). Removal of the 4Hcyt-RC subunits leads to an empty LH1 ring complex (Fig 3b, c; number 3). Three classes of protein complexes could be analyzed (Fig. 3b) and modeled (Fig. 3c) 1: 4Hcyt-RC-LH1, 2: RC-LH1, and 3: LH1. The LH1 around the RC forms an elliptical assembly of 16 subunits. The orientation of the long axis of the LH1 ellipsis coincides with the long axis of the RC topography in native membranes. This ellipticity of the LH1 with an associated RC
reflects a strong and specific interaction between the core-complex components. Dissection of subunits from a multiprotein complex combined with high-resolution imaging provides novel insights into complex subunit architecture [9].

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