Simultaneous AFM / Fluorescence Imaging of Living Cells – Fluorescence-guided Force Spectroscopy

Application Note

Summary

High resolution atomic force microscopy (AFM) can be performed simultaneously with optical microscopy techniques, such as fluorescence or differential interference contrast (DIC) microscopy. The combined methodologies provide complementary information about the studied sample which establishes the basis for a better understanding of physiological processes and the function of biomolecules, and allow a more detailed characterization of the structure of living cells [1]. Here we present the Agilent 5500 ILM AFM/SPM, which is an easy-to-use AFM and inverted light microscope (ILM) combination that enhances live-cell imaging under physiological conditions. The advantage of this combined setup is further demonstrated by studying fluorescence-labeled membrane receptors via force spectroscopy using antibody-functionalized AFM tips.

Introduction

Atomic force microscopy has evolved as a method capable of resolving biological structures at the molecular level [2] Furthermore it provides the possibility to study interaction forces between single receptor-ligand complexes. Fluorescence microscopy has proven to be a powerful method for the specific visualization of labeled molecules down to the single molecule level [1]. This allows the determination of their distribution observing diffusion or trafficking processes in cells. Other optical techniques, such as bright field, phase contrast [3], and DIC microscopy reveal information about cell morphology and composition. By enabling a more detailed characterization of internal and external cellular structures and biological processes, the combination of AFM with optical microscopy provides a more comprehensive examination of the sample than either of the techniques alone can deliver.

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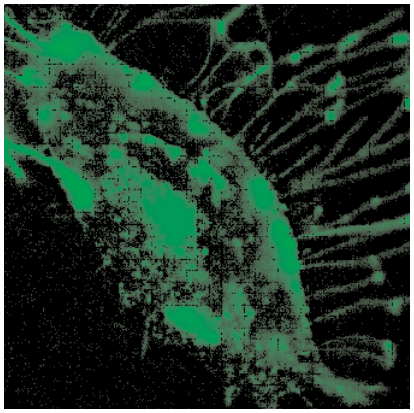

Figure 1. Fluorescence microscopy image of eGFP-labeled receptors on a living CHO cell. Image acquired using an Agilent 5500 ILM AFM/SPM. Image size 40 x 40 µm2.

Figure 2. Agilent 5500 ILM AFM/SPM with a Zeiss Axiovert 200 inverted light microscope. The 5500 ILM AFM/SPM is also compatible with other inverted light microscope makes and models.

Methods

Chinese hamster ovary (CHO) cells were utilized in this study. These cells, which are widely used as expression system for a number of proteins, grow in low cost, fully defined media, and they are adherent to solid matrices. Since immobilized samples are necessary, cellular adhesion is an advantage in AFM imaging. In addition, CHO cells, and other mammalian autocrine cells, are more resistant to apoptosis, so they are compatible with lengthy experiments.

The CHO cells utilized express high levels of recombinant scavenger receptor class B type 1 (SRB1) [4]. The scavenger receptors were fused to a green fluorescent protein (eGFP). This fluorescent protein can be excited with 488 nm light and emits light at 508 nm.

Figure 1 shows a typical fluorescence microscopy image of a living CHO cell expressing the SRB1-eGFP construct. The cells were grown in medium A (see [4] for details) on 22 mm glass slides in conventional 35 mm Petri dishes at 37 degree Celsius and 5% CO_2. The sample slides were mounted onto the Agilent 5500 ILM AFM/SPM with an Agilent AFM/SPM liquid cell sample holder. The cells were rinsed with PBS buffer (phosphate buffered saline, 50 mM NaPi, 150 mM NaCl, pH 7.4) and kept in this buffer throughout the experiments.

Instrumentation

Using a QuickSlide Series 5500 mechanical stage, an Agilent 5500 AFM/SPM was adapted to a Zeiss Axiovert 200 inverted light microscope. The QuickSlide stage allows convenient sample changing and permits positioning of the sample directly under the AFM cantilever. The ILM AFM/SPM was placed onto a passive anti-vibration table (Newport GmbH) without any additional active damping (figure 2).

For the simultaneous fluorescence/AFM imaging, the excitation of SRB1-eGFP was accomplished with a mercury arc lamp (HBO100, Zeiss) with a 488 nm laser (Sapphire, Coherent). For combined fluorescence/force spectroscopy experiments a halogen lamp (HAL, Zeiss) was used. AFM/DIC images were acquired using a CCD camera (Casade 512B, Roper Scientific). In order to reduce the mechanical noise level, the cooling fan of the camera was removed and mounted in an external fan box, which was placed outside the optical table and connected to the camera via a long polymer (PVC) hose. A 40x oil immersion objective (NeoFluar, NA=1.3, Zeiss) was used for DIC measurements, a 100x oil immersion objective (Apochromat, NA=1.4, Zeiss) was utilized for fluorescence microscopy and a 60x water immersion objective (NA=1.2, Olympus) was utilized for the fluorescence guided force spectroscopy experiments. For AFM experiments, a large (100 µm scan range), multipurpose Agilent AFM/SPM scanner was utilized. The AFM images were acquired in

contact mode using 10 pN/nm silicon nitride cantilevers at scanning rates of 0.4 - 1 line/sec, so complete AFM images were acquired in from four to ten minutes. For force spectroscopy experiments the same type of contact-mode levers, were used after functionalization with antibodies as described in [1] (fig. 6c).

Results

a) Simultaneous DIC and AFM imaging

Contact mode AFM images were acquired on the CHO cells that were grown on glass cover slips as described above and fixed in 2% glutaraldehyde for ten minutes. DIC microscopy images were recorded simultaneously with the AFM scanning process. One frame from this sequence is shown in Figure 3a. The DIC image visualizes the cell morphology including the cell body, protrusions or lamellipodia, and also prominent intracellular features, such as the nucleus and several large organelles.

Figures 3b and 3c are the corresponding AFM deflection (error) and topography (height) images, respectively, of the same CHO cells. AFM imaging showed the sample topography at high resolution. In some regions parts of the membrane associated cytoskeleton are resolved, furthermore lamellipodia and cell debris are easily visualized. The small globular structures on the cell membrane are artefacts resulting from cell fixation. By comparing the cells in 3 a, b and c, one can see that cells of the same type have variable shapes, which is mainly due to different states of cell maturation.

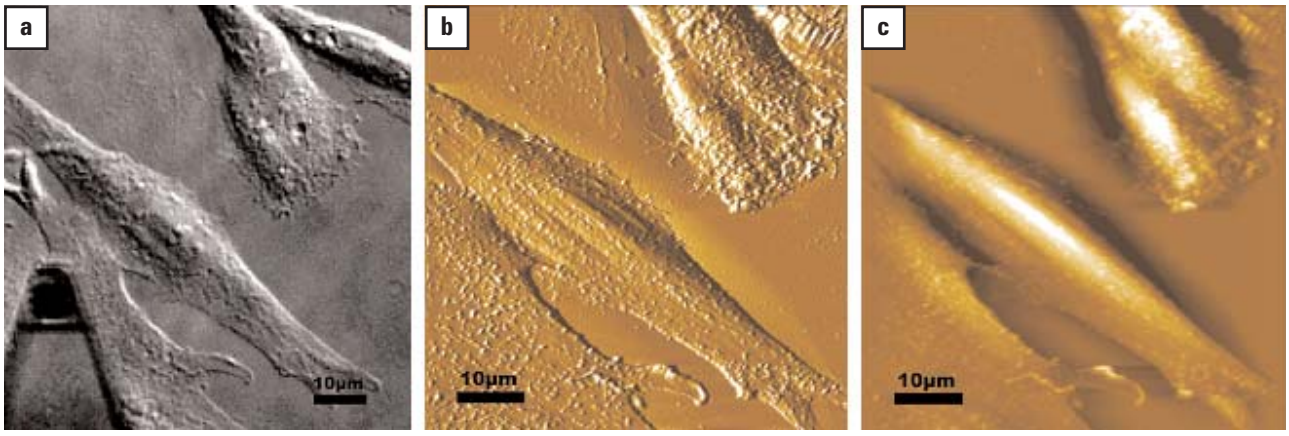

Figure 3. Simultaneous DIC microscopy (3a) and AFM (3b and c) images. Glutaraldehyde-fixed CHO cells were imaged in contact mode AFM while acquiring a DIC microscopy image sequence. Three cells are visible in the scan area of 60 x 60 µm2. Complementary structural information results from the DIC microscopy image and the contact mode AFM deflection (3b) and topography (3c) images.

b) Simultaneous Fluorescence and AFM imaging

Combined AFM-optical microscopy measurements are not restricted to fixed cells, also living cells can be easily imaged and analyzed with the Agilent 5500 AFM/SPM. Figure 4a shows one frame from a fluorescence image sequence of a living CHO-SRB1-eGFP cell. This image is a snapshot from an image sequence, which was acquired while simultaneously scanning the sample with the AFM. The corresponding AFM images are presented in figures 4b and 4c (deflection and topography image, respectively).

The fluorescence microscopy image gives a good impression of the cell shape because the visualized eGFP-labeled SRB1 receptors are primarily located in the plasma membrane. Some fluorescence signals also originated from the cell interior because a part of the SRB1-eGFP constructs was either endocytosed or freshly synthesized inside the living cell [4].

AFM images of living CHO cells (figures 4b and 4c) illustrate different features than images of fixed CHO cells in figures 3b and 3c [2]. For example, the cell membrane of a living cell is much softer than the cell membrane of a fixed cell, the elastic modulus is comparatively much smaller. In this case the AFM tip can more easily depress the plasma membrane which is why the membrane associated cytoskeleton (e.g. the network of actin fibers and microtubules) is better revealed in living cells than in fixed cells. Alternatively, soft, flexible features like cellular protrusions or lamellipodia are not easily resolved within the AFM images of the living cells because they are easily pushed away by the scanning AFM tip.

The AFM cantilever can be indirectly recognized as a bright spot, because eGFP-protein (mostly from lamellipodia) adsorbed onto the AFM tip during previous imaging processes.

c) Fluorescence-Guided Force Spectroscopy

AFM force spectroscopy is an excellent technique for the investigation of receptor-ligand interaction forces and the corresponding binding kinetics [1]. In some cases the receptor density on the cell surface can be low. When investigating cells for example, this might be due to low expression levels for the protein of interest or – as with the cells shown here – due to clustering of the receptors in microdomains within the plasma membrane [4]. The CHO-SRB1-EGFP cells were fixed in order to prevent endocytosis and diffusion of the SRB1 clusters [4]. A mild fixation protocol was applied (2% glutaraldehyde, 1 min) so that most of the SRB1 remained in their native conformation. The fixed cells were additionally treated with $NaBH_4$ in order to reduce fixative-induced auto-fluorescence.

For the force spectroscopy experiments SRB1 clusters were localized by acquiring a fluorescence image of the cell using a GFP filter set

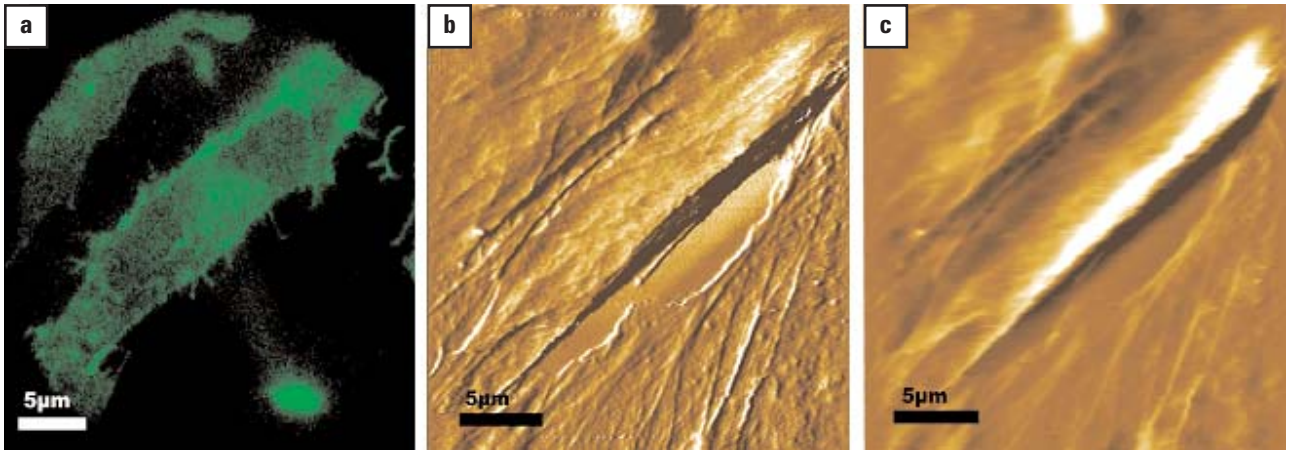

Figure 4. Fluorescence microscopy (a) and contact mode AFM deflection (b) and topography (c) images of live CHO cells obtained with an Agilent 5500 ILM AFM/SPM. The AFM images were obtained while simultaneously acquiring a fluorescence microscopy image sequence with a CCD camera. The fluorescence images were unaffected by light emitted from the AFM laser. Image size about 30 x 30 µm2.

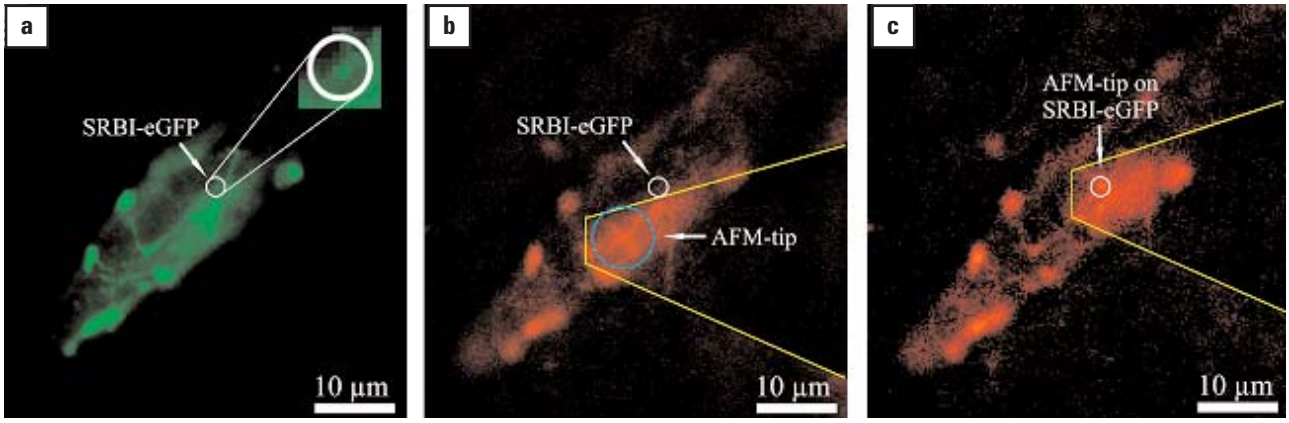

Figure 5. Fluorescence microscopy images of CHO-SRB1-eGFP cells that were fixed with glutaraldehyde. The eGFP-labeled SRB1 receptors are visible with a GFP filter on the Agilent 5500 ILM AFM/SPM in figure 5a. In figure 5b, the GFP filter was replaced with a Cy3 filter and the auto-luminescent silicon nitride AFM tip (outlined in yellow) is visible. In figure 5c, the two previous fluorescence images were used to precisely reposition the AFM tip over a cluster of eGFP-labeled SRB1 receptors. The AFM tip was modified with anti-SRB1 antibodies for subsequent force spectroscopy experiments (fig. 6). Images size about 60 x 60 µm2.

(emission filter 535 ± 25 nm; fig. 5a). The focal plane was adjusted on top of the cell, so that mainly clusters of eGFP-SRB1 receptors, which are accessible to the functionalized AFM tip are visible. One of the SRB1-eGFP clusters (outlined by a white circle) was chosen for the following force spectroscopy measurements. Auto-fluorescent areas (no SRB1-EGFP-fluorescence signals) which remained despite $NaBH_4$ treatment were identified and excluded from the experiments by switching to a Cy3 filter set (emission 610 ± 38 nm), which blocked the eGFP fluorescence.

When illuminated with 488 nm light, a silicon nitride AFM tip emits light at wavelengths up to 750 nm (silicon cantilevers are not auto-luminescent; see [5]). Therefore, auto-luminescence was used to locate the silicon nitride AFM tip. When using a GFP filter silicon nitride luminescence is covered by the stronger eGFP fluorescence. However, after blocking the eGFP signal with the Cy3 filter the weak cantilever luminescence is enough to identify its position on the cell (X-shaped form in fig. 5b). This facilitated accurate positioning of the tip at the end of the AFM cantilever directly above areas that contained a cluster of SRB1 receptors.

Anti-SRB1 antibodies were tethered to AFM cantilevers via flexible polymer linkers at a low density, so that on average only one ligand molecule was located within the effective AFM tip/ cell surface interaction area (fig. 6c) [1]. In figure 5c, the functionalized AFM tip was aligned on top of the pre-chosen SRB1-eGFP-cluster

(fig. 5a) of receptors. Consecutive force-distance cycles were recorded and a series of specific single molecule unbinding events were detected (fig. 6a). From more than one hundred force-distance cycles, a distribution of unbinding forces was obtained and the binding probability between anti-SRB1 antibodies and the SRB1 receptors was determined. The binding probability for sites that contained SRB1 receptors was calculated to be 13.6 %. At an AFM cantilever velocity of 2 µm/sec (which corresponds to a loading rate of approximately 10 nN/sec), the unbinding force distribution of the anti-SRB1 antibody/SRB1 receptor complexes contained two maxima, one at 64 pN, and one at 85 pN (see figure 6b). It is possible that the SRB1 antibody/SRB1 receptor complexes have two separate maxima because some of the receptors may have been more accessible to the AFM tip-bound antibodies, while others may have been less accessible which was due to their location in caveolae [4].

When the plasma membrane was "blindly" probed without guidance from fluorescence microscopy a binding probability of only three to four percent was measured. When force-distance experiments were performed on regions that lacked eGFP-labeled SRB1 receptors a 2.5% probability was measured. In approximately 50% of the areas examined, specific unbinding events between the anti-SRB1 antibodies and SRB1 receptors were not detected, even though the areas were identified as having a high density of eGFP-labeled SRB1 receptors. This could have been due to inaccessibility of the SRB1 receptors arising from, for example, endocytosis immediately before the cells were fixed. The inset in figure 6a shows the results of a force-distance experiment in which no unbinding event was detected.

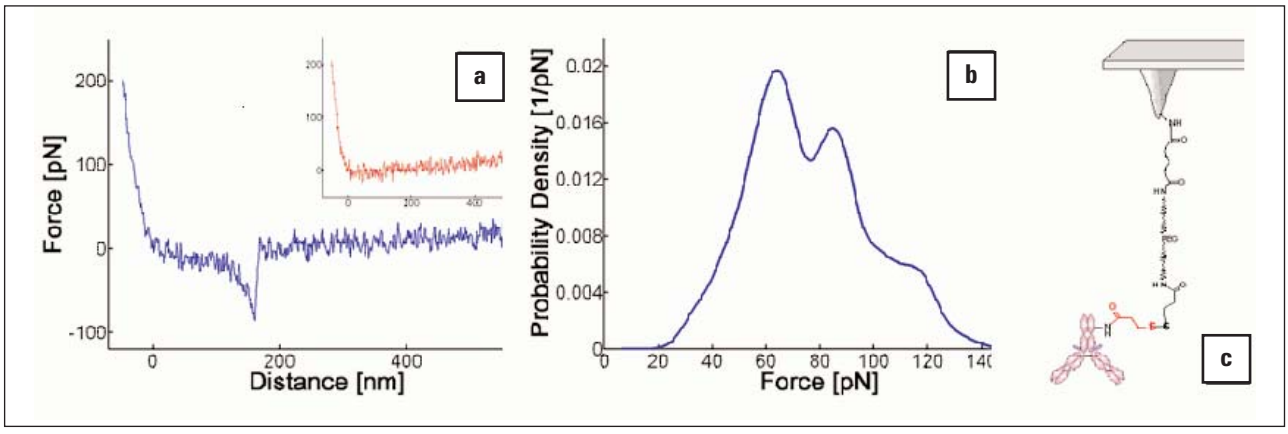

Figure 6. Results from force-distance spectroscopy experiments between anti-SRB1 antibodies tethered to AFM cantilevers and SRB1 receptors expressed in CHO cells. An Agilent 5500 ILM AFM/SPM was used to identify areas of fluorescence, corresponding to eGFP-labeled SRB1 receptors on the plasma membrane of the cells. Areas containing clusters of SRB1 receptors were probed with an AFM tip that was functionalized with anti-SRB1 antibodies. Figure 6a: a force-distance curve showing a single unbinding event between an anti-SRB1 antibody and a SRB1 receptor. From this force-distance curve, the unbinding force for a single antibody-antigen interaction was calculated to be approximately 70 pN. Figure 6a inset shows a force-distance spectroscopy curve resulting from an area that did not fluoresce from eGFP-labeled SRB1 receptors. Figure 6b: a force distribution derived from more than hundred anti-SRB1 antibody-SRB1 unbinding events. From the force distribution curve, two unbinding force maximas for anti-SRB1 antibody-SRB1 complexes were identified at 64 pN and 85 pN. Figure 6c: Tethering single antibodies to an AFM cantilever tip via a flexible polymer linker.

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