Fluorescence Guided Force Spectroscopy and Recognition Imaging on Cells Using Agilent's 6000 ILM AFM

Application Note

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Introduction

Fluorescence microscopy has become an important tool for localizing receptor/ligand interaction in living cells. Labeling different protein with different spectral fluorophores allows imaging of different cellular, subcellular or molecular components and determining the specific localization of the proteins in cells. The rejection of unwanted, short wavelength background (Rayleigh scattering of excitation light) by spectral filtering improves the contrast of specifically labeled cellular structures in fluorescence microscopy. The lateral and axial resolutions are limited by the diffraction limit of light and result in ~200 nm resolution. Recently, techniques with higher resolution have been developed, like stimulated emission depletion (STED), photo activated localization (PALM), or stochastic optical reconstruction microscopy, which achieve a lateral resolution of 10–30 nm.

However, optical techniques cannot provide any information of the sample topography. In contrast, atomic force microscopy (AFM) allows obtaining topographical images at the nanometer scale in liquid environments and at room temperature. In addition recognition of receptor/ligand pairs can be investigated at the single molecule level. The recently developed simultaneous topography and recognition imaging technique yields a topographical image and a map of recognition sites of the same area with a single scan at 5 nm lateral resolution. The operating principle is the same as for dynamic mode AFM imaging. A cantilever is oscillated near its resonance frequency and scanned over the sample surface. But in this case the cantilever is made chemically sensitive by attaching a ligand via a short linker to its tip. The binding sites are evident from the reduction of oscillation amplitude. Enhanced signal processing in combination with a modified feedback loop provides a recognition image simultaneously acquired alongside a topography image. In a nutshell, the separation of topographical and recognition events is achieved by splitting the cantilevers oscillation amplitude into lower and upper parts (with respect to the cantilever's resting position). The maxima of these parts are then used to record the topography (lower parts) and recognition image (upper parts) at the same time.

Here we demonstrate the ability of Agilent's 6000 ILM of easy positioning of the AFM tip to regions of interest using the contrast enhanced optical image and the fluorescence image, and the subsequent TREC imaging and force spectroscopy which show high correlation among fluorescence intensity, binding probability, and recognition area, as well as the visualization of the recognized nano-domains on cells with different levels of protein expression.

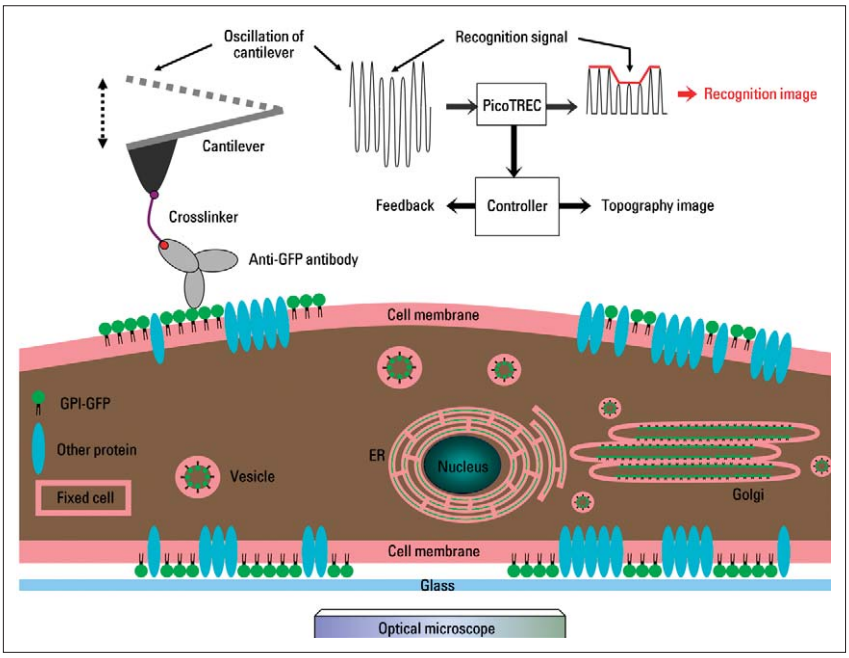

Figure 1. Schematic diagram of the combined optical and atomic force microscopy for recognition measurements on cell. GPI anchored GFP is synthesized in ER, modified in Golgi, and transported via vesicles to plasma membrane. While the optical microscope can examine the overall expression level and micrometer scale distribution of GPI-GFP through the fluorescence measurement, the recognition imaging by the antibody functionalized cantilever can visualize the distribution of GPI-GFP at nanometer scale. For recognition imaging, the cantilever oscillates at constant amplitude. When the antibody on the tip binds with the GFP on the cell surface, the upper part of the oscillation wave is reduced, which can be detected by the PicoTREC box where the oscillation wave is split into upper and lower parts. The upper part of the oscillation wave is used to construct the recognition image. Within this paper recognition events are shown in red.

Experimental

One of the most desirable topics in life science and bio-nanotechnology is the investigation of cells and the organization and function of proteins in the cell membrane. Cell signalling, communication to neighbouring cells and transport to adjacent tissue is all organized through membrane proteins. Here we present two examples of important protein systems for cell signalling and cell membrane organization.

Glycosylphosphatidylinositol (GPI) anchored green fluorescence protein (GFP)

In the plasma membrane of cells there are cholesterol- and sphingolipid-enriched domains, termed "lipid rafts". These lipid rafts serve as organizing centres for the assembly of signalling molecules, influence membrane fluidity and membrane protein trafficking, as well as receptor trafficking. For example, upon specific triggering, membrane receptors such as T-cell receptors or B-cell receptors translocate into lipid rafts, which is the prerequisite for efficient receptor-mediated signal transduction.

The Agilent 6000 ILM allowed for investigating both the morphology and the distribution of lipid rafts on cells. For this, T24 cells (human bladder carcinoma cells), which were transfected to express GPI anchor derived from DAF fused to GFP [termed GPI-(DAF)-GFP], which is an highly effective lipid raft marker (Weghuber, et al. 2010), were grown on glass bottom Petri dishes. Before imaging, the cells were fixed with 4% paraformaldehyde for 30 min. The topographical image (Figure 2A) of the cells was obtained using MAC (magnetic AC) Mode. The imaging force was so gentle that the filaments of the cytoskeleton underneath the plasma membrane can hardly be seen. Lamellipodia at the cell border can however be clearly detected. The level of

GPI-(DAF)-GFP expression in the cells was examined by obtaining GFP fluorescence images (Figure 2B). Close to the nucleus of the cells there are some regions with very high fluorescence intensity, which is in accordance with the fact that the GPI anchored proteins are synthesized in endoplasmic reticulum (ER) which is surrounding the nucleus of the cells (Figure 1). Beyond the ER regions, there is no correlation between the topographical height and the fluorescence intensity, which suggests that most of the GPI-(DAF)-GFP molecules are located on the plasma membrane rather than in the cytosol. Occasionally, some bright fluorescence dots can be found far away from the ER region, which are indicated with the red square (Figure 2B). Such bright dots may be vesicle in the cytosol or micro-domain in the plasma membrane. In general, the conventional fluorescence microscopy with the highest magnification (100x) shows a homogeneous distribution of GPI-(DAF)-GFP in the plasma membrane.

To investigate the distribution of GPI-(DAF)-GFP at nanometer level, TREC imaging was utilized. The principle of TREC imaging using a cantilever tip functionalized with anti-GFP antibody is shown in Figure 1. During imaging, the cantilever oscillates (driven by a magnetic field) at a constant amplitude. When the antibody on the tip binds with the GFP on the cell surface, the upper part of the oscillation is reduced which can be detected by the PicoTREC box where the oscillation wave is split into upper and lower parts. From the upper part of the oscillation wave, the recognition image can be constructed, where the area with binding event is shown as a dark spot.

The recognition image of the region marked with red square in Figure 2B is shown in Figure 2C. From the recognition image, it can be seen that the GPI-(DAF)-GFP molecules form nano-domains with the size of about 200–300 nm. In the region marked with dashed blue circle, the nano-domains have been aggregated which look like a micro-domain in the fluorescence image. Such micro-domain provides a unique bridge to show the correlation between the fluorescence and recognition images.

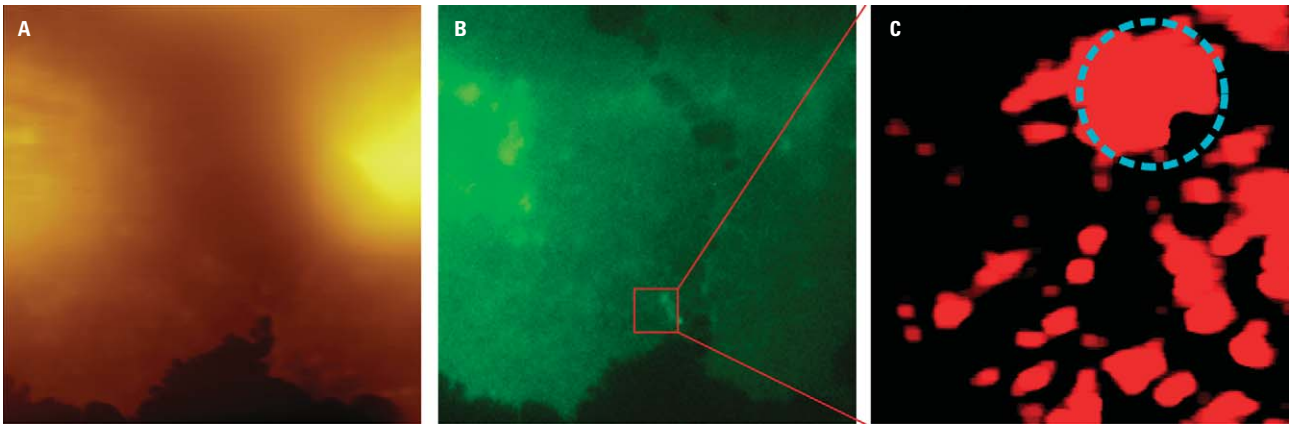

Figure 2. AFM topography of fixed T24 cells expressing GPI-GFP (image size: 50 µm). The image was measured with MAC Mode in PBS by using an Agilent type VI MAC Lever oscillating at 16 kHz and at about 7 nm with an imaging speed of 15 µm/s. The imaging force was so gentle that the filaments of the cytoskeleton underneath the plasma membrane can hardly be seen. Lamellipodia at the cell border can however be clearly detected. (B) Fluorescence image (FITC filter set, 100x magnification) of the same cells showing the expression level of GPI-GFP. Some bright regions close to the nucleus indicate the endoplasmic reticulum (ER) where the GPI anchored proteins are synthesized. Beyond the ER regions, the distribution of GPI-GFP in the plasma membrane looks homogeneous except several bright dots (e.g. those in the red square). (C) By using the cantilever functionalized with the anti-GFP antibody, the recognition image of the region marked with red square in (B) revealed nano-domains with the size of about 200-300 nm. In the region marked with dashed blue circle, the nano-domains have been aggregated which correlates well to the micro-domain shown in the fluorescence image. During the recognition imaging the cantilever was oscillating at 16 kHz and at about 7 nm with an imaging speed of 6.5 µm/s. Image size: 5 µm.

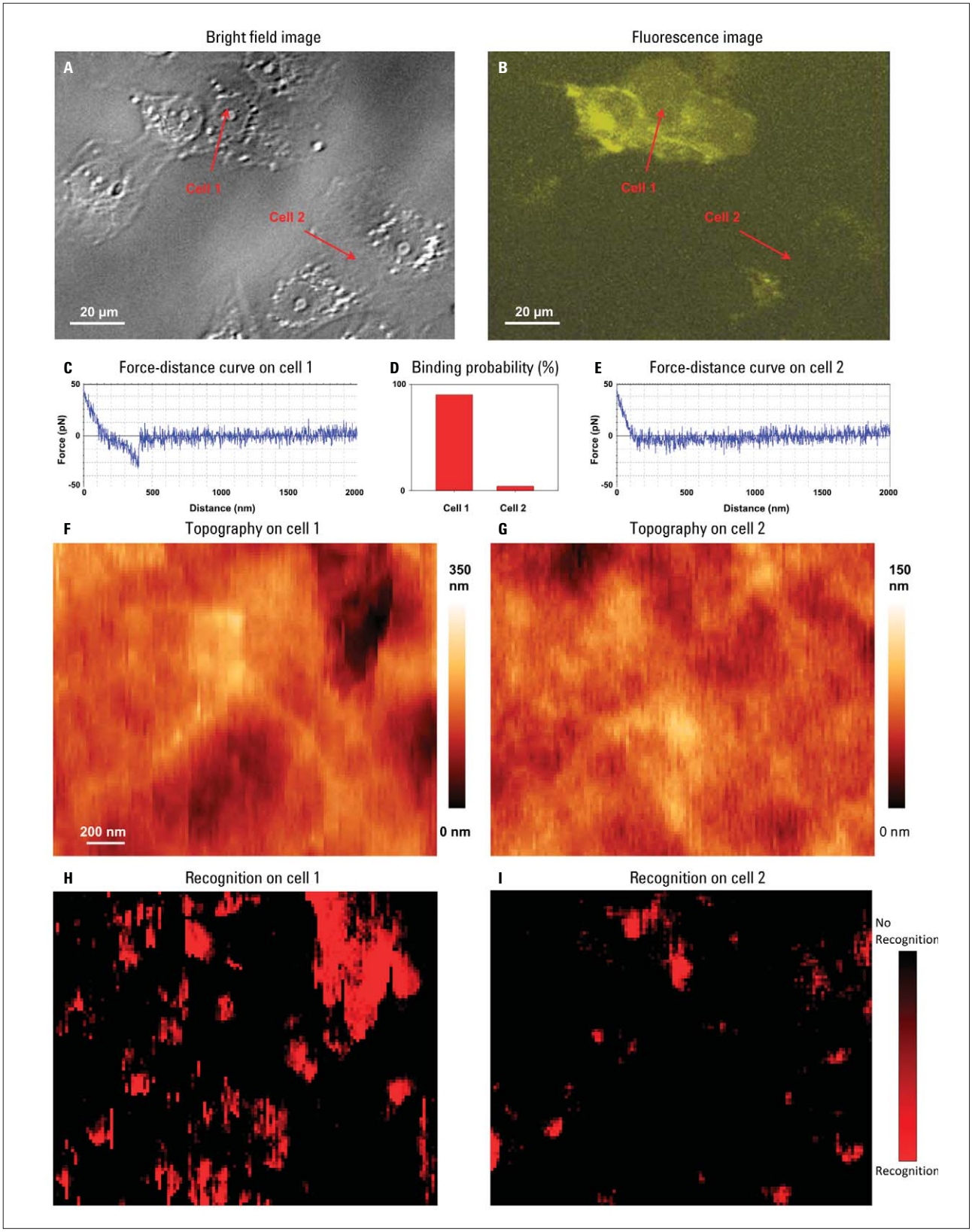

Figure 3. (A) Bright field image and (B) fluorescence image of fixed T24 cells transfected with YFP-CD4 reveal that some of the cells (e.g. cell 1) have high expression of YFP-CD4, while some other cells (e.g. cell 2) have very low expression. With the guidance of the optical images, force spectroscopy and recognition imaging were performed on cell 1 and cell 2. On cell 1, 244 force distance curves were measured, from which 220 curves show binding events, resulting in a binding probability of 90.2%. (F-I) AFM topography and recognition images measured on cell 1 and 2 with an Agilent type VII MAC Lever functionalized with anti-CD4 antibody. Images on the two cells were measured with the same tip at the same frequency (8 kHz) and amplitude (about 50 nm) with an imaging speed of about 1.9 µm/s. Cell 1 (panel H) showed many large recognition spots with the size ranging from 100 to 200 nm, while cell 2 (panel I) showed much smaller spots with farther distance.

CD4 molecules fused with yellow fluoruescence protein (YFP)

CD4 (cluster of differentiation 4) is a glycoprotein originally found on the surface of T helper cell, which plays very important role in immunology and the disease of HIV. The distribution of the CD4 protein was investigated on T24 cells which were transfected to express YFP fused CD4. Such cells were fixed and imaged by using fluorescence microscopy and AFM. From the bright field image and the fluorescence image (Figure 3A and B), it can be found that some of the cells (e.g. cell 1 in Figure 3) have high expression of YFP-CD4, while some other cells (e.g. cell 2 in Figure 3) have very low expression.

With the guidance of the optical images, force spectroscopy and recognition imaging were performed on cell 1 and cell 2. For these measurements, the cantilever tips were functionalized with anti CD4 antibody. On cell 1, 244 force distance curves were measured, from which 220 curves show binding events, resulting in a binding probability of 90.2% (Figure 3D). A typical force distance curve with binding event was shown in Figure 3C. On cell 2, 241 force distance curves were measured, from which only 10 curves show binding events, resulting in a binding probability of 4.1%. A typical force distance curve without binding event was shown in Figure 3E. The force distance curve measurements on the two cells were performed with the same tip. The binding probability on cell 1 and 2 is in very good agreement with the fluorescence intensity.

Figure 3F to 3I show the AFM topography and recognition images measured on cell 1 and 2. The images on these two cells were measured with the same tip at the same frequency and amplitude. It can be found that there are many large recognition spots on cell 1 (Figure 3H) with the size ranging from 100 to 200 nm. Most of the recognition spots locate in hole regions in topography, but the recognition spot can also be found in protruding regions in topography (Figure 3F). Many of the nano-domains are already connected with each other. Such high density of recognition spots coincides with the strong fluorescence signal on cell 1, and is identical with the high binding probability from force spectroscopy. On the other hand, the recognition spots on cell 2 (Figure 3I) are normally smaller than those on cell 1, and the distance between recognition spots is basically greater than that on cell 1. The overall area of recognition spots on cell 2 is much less than that on cell 1, which correlates very well to the expression level revealed by the fluorescence image.

To further examine the specificity of the recognition spots, block experiment was performed by injection of free anti-CD4 antibody into the measurement solution. From Figure 4 we can see that the topography of the cell membrane looked similar after the block, however the recognition spots were significantly reduced by the injected free antibody. For imaging before and after the block, the same cantilever was used at the same oscillation frequency and amplitude. The clear reduction of the recognition spots confirmed the specificity of the recognition imaging revealed CD4 nano-domains.

Conclusion

We demonstrated that our new and fully integrated AFM – inverted light microscope combination enables an easy and quick investigation of the distribution of cell membrane proteins from micrometer to nanometer level. The ease-of-use and convenient handling of this device makes it the tool for biologists and biophysicists in numerous future applications in life science at the nanoscale, and thereby bridges the world of optics with the world of atomic resolution under physiological conditions.

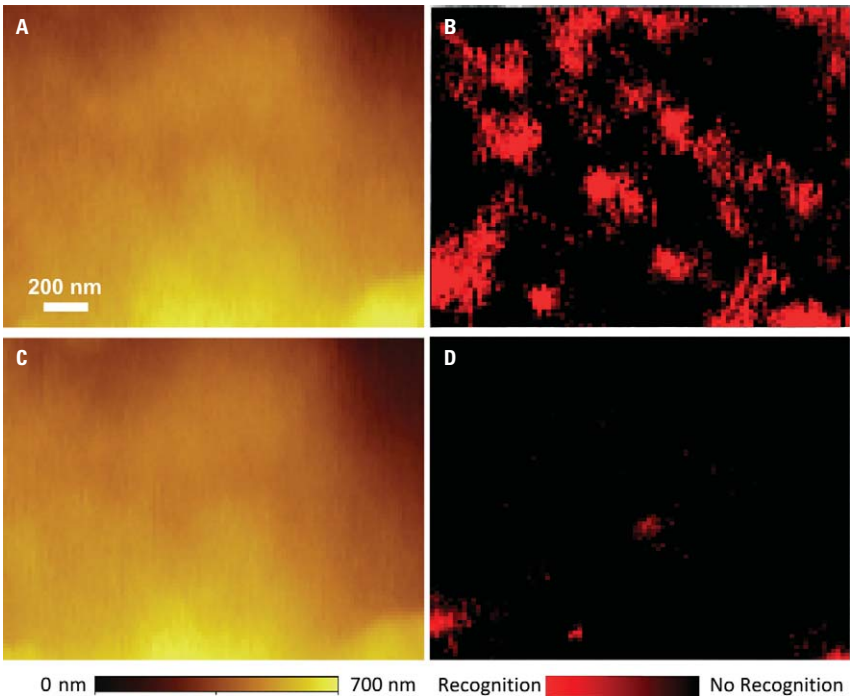

Figure 4. (A) Topography and (B) recognition images on fixed T24 cells expressing YFP-CD4 before block, imaged by Agilent type VII Mac Lever functionalized with anti-CD4 antibody. About 2.5 hours after injection of free anti-CD4 antibody molecules (with a final concentration of 0.05 mg/ml), (C) topography and (D) recognition images were measured at the same position with the same cantilever tip, which showed significant reduction of recognition spots. All images were measured at cantilever oscillation frequency of 8.37 kHz, amplitude of about 50 nm, and with an imaging speed of about 2.7 µm/s.

Material and Methods

T24 cell culture

T24 cells were cultured in RPMI 1640 medium (E15-848), containing 10% fetal calf serum, supplemented with penicillin/streptomycin. (all: PAA Laboratories, Pasching, Austria). Cells were passaged twice a week using a dilution of 1:2 and were maintained under a humidified atmosphere with 5% CO_2 at 37°C. For AFM experiments, cells were seeded on glass bottom micro well dishes (MatTek, Ashland, USA) with three different dilutions to obtain an average of 40% confluence after 1 day.

Cell fixation

Before fixation cells were rinsed in PBS to remove media components. Cells were then fixed with 4% paraformaldehyde in PBS over a period of 0.5 hours to 3 days and washed again in PBS twice. To quench free aldehyde cells were kept in 10 mM ethanolamine in PBS for 5 min followed by washing with PBS.

Atomic Force Microscopy

AFM experiments were performed using an Agilent 6000 ILM AFM mounted on a Zeiss AxioObserver A1 (for measurement of cells with GPI-GFP) or Till Photonics *more* fluorescence microscope (for measurement of cells with YFP-CD4). Glass bottom micro well dishes containing the cells were mounted on the AFM 6000 sample holder. All images were acquired in PBS buffer at room temperature using MAC Mode. For MAC Mode imaging Type VI and VII MAC Levers were used. AFM images were acquired using Agilent's PicoView software. Pico Image and Gwyddion were used to process the images for presentation. HC Image and Live Acquisition were used to acquire optical images.

Tip functionalization

Cantilevers for force spectroscopy were washed in chloroform for 3 times, dried in the air, washed in piranha (30% H_2O_2 and 70% H_2SO_4) for 30 min, rinsed with water, dried by heating at 100°C. Cantilevers for TREC imaging were washed in chloroform for 3 times and dried in the air. All cantilevers were then treated with APTES, conjugated with NHS-PEG-aldehyde or NHS-PEG-acetal, and subsequently linked with antibody (Wildling, et al. 2011).

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