Atomic Force Microscopy with BioScope II: Detecting Specific Ligand-Receptor Interactions on Live Cancer Cells in situ

Combined atomic force and epifluorescence capabilities were used to detect specific cell surface proteins using functionalized AFM tips

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INTRODUCTION

The combination of optical with scanning probe microscopy techniques has become a powerful tool for sensing biological events on nanoscale. This combination will significantly contribute to providing new insights to the fields of biosensors, pharmacology, drug discovery, cancer research or nanomedicine over the next few years. In addition to its high-resolution imaging capabilities, atomic force microscopy (AFM) has proved invaluable in the area of functional studies as a unique, non-invasive technique for measuring the interaction forces between single biomolecules with picoNewton sensitivity.[1,2,3,4] In this study we apply the force measurement features of the Veeco BioScope™ II to demonstrate the specificity of the interaction between an AFM tip tagged with Pro-Aerolysin (PA) bacterial protoxin, and glycosylphopsphatidylinositol (GPI)- anchored proteins on living Hela cells.[5,6,7] We mainly used the force volume imaging mode, which combines force measurements, and contact mode imaging. While scanning, the tip records an approach-distance curve in every point of the surface and displays the result as a 2D image, where each force curve can be displayed by clicking on a chosen location on the image. Analysis of the different portions of the resulting force curves can provide maps of the relative elasticity (approach portion) or relative adhesion (retract portion) across a sample surface. If the AFM probe is functionalized with a ligand that can specifically recognize a target molecule or receptor on the sample surface, then the resulting adhesion image essentially becomes a distribution map of the receptor binding sites.

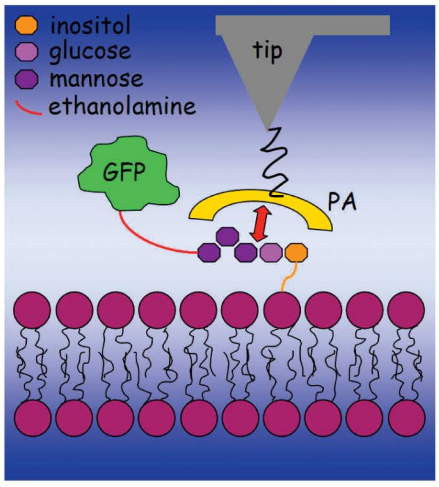


Figure 1. Principle of the interaction between a PA-tagged tip and GPI-anchor protein. The plasma membrane is the barrier responsible for cell integrity, homeostasis and signaling pathways activation. The polar heads of phospholipids face the cytosol and the extracellular medium, whereas the hydrophobic fatty acid tails span the interface of those two hydrophilic layers. The GPI-anchored proteins are bound to this bilayer by several components. Inositol binds the phosphate group of the phospholipid, glucose and mannose units. Then an ethanolamine groupment binds this osidic part to a protein, which in this case is a GFP. The PA groupment at the end of the tip specifically recognizes the inositol part of the anchor.

SAMPLE PREPARATION

HeLa cells were transfected to express a Green Fluorescence Protein (GFP) and GPI anchor fusion protein.[7] PA is able to recognize all GPI anchored proteins without any distinction through the inositol moiety.[5,6] See Figure 1 for a scheme of the interaction process. DNP cantilevers (Veeco Probes) were functionalized with PA moieties according to established protocols.[8,9] The softest cantilever (0.06 N/m nominal spring constant) was used to carry out force volume imaging.

The force measurements were performed using a Veeco BioScope II integrated with a Zeiss Axiovert 200M and equipped with a Veeco NanoScope V controller. Collected data was analyzed with NanoScope v7.20 software. A Roper Scientific Coolsnap camera driven under Molecular Devices Metamorph software was used to record epifluorescence images.

RESULTS

The fluorescence images were used to distinguish between different expression levels of GFP, with higher levels of GFP resulting in a higher fluorescence intensity (Figure 2, top left). For example, Region A exhibits no fluorescence while the cell is clearly visible by brightfield imaging (Figure 2, top right and bottom left images). Region B, however, contains a cell exhibiting a high level of fluorescence, thus indicating expression of the GFP-GPI chimera in the transfected cell. Once the different types of cells have been identified in the fluorescence images, the corresponding brightfield images are then used to position the AFM probe over a cell of interest to perform force volume imaging.

To calculate the forces involved in the binding of cell surface GPI proteins and the tip-bound PA, the spring constant of the cantilever must first be determined.[10] This is easily and accurately calculated, in either air or fluid, using the BioScope II thermal tune function. Before collecting the force volume data, a reference force curve is collected on a stiff surface (in this case the glass bottom of the Petri dish) and used to define the best parameters for force volume image acquisition.

For force volume imaging, the tip is positioned at the edge of a cell and a 2x2 µm scan frame (32x32 pixel resolution) was collected over Regions

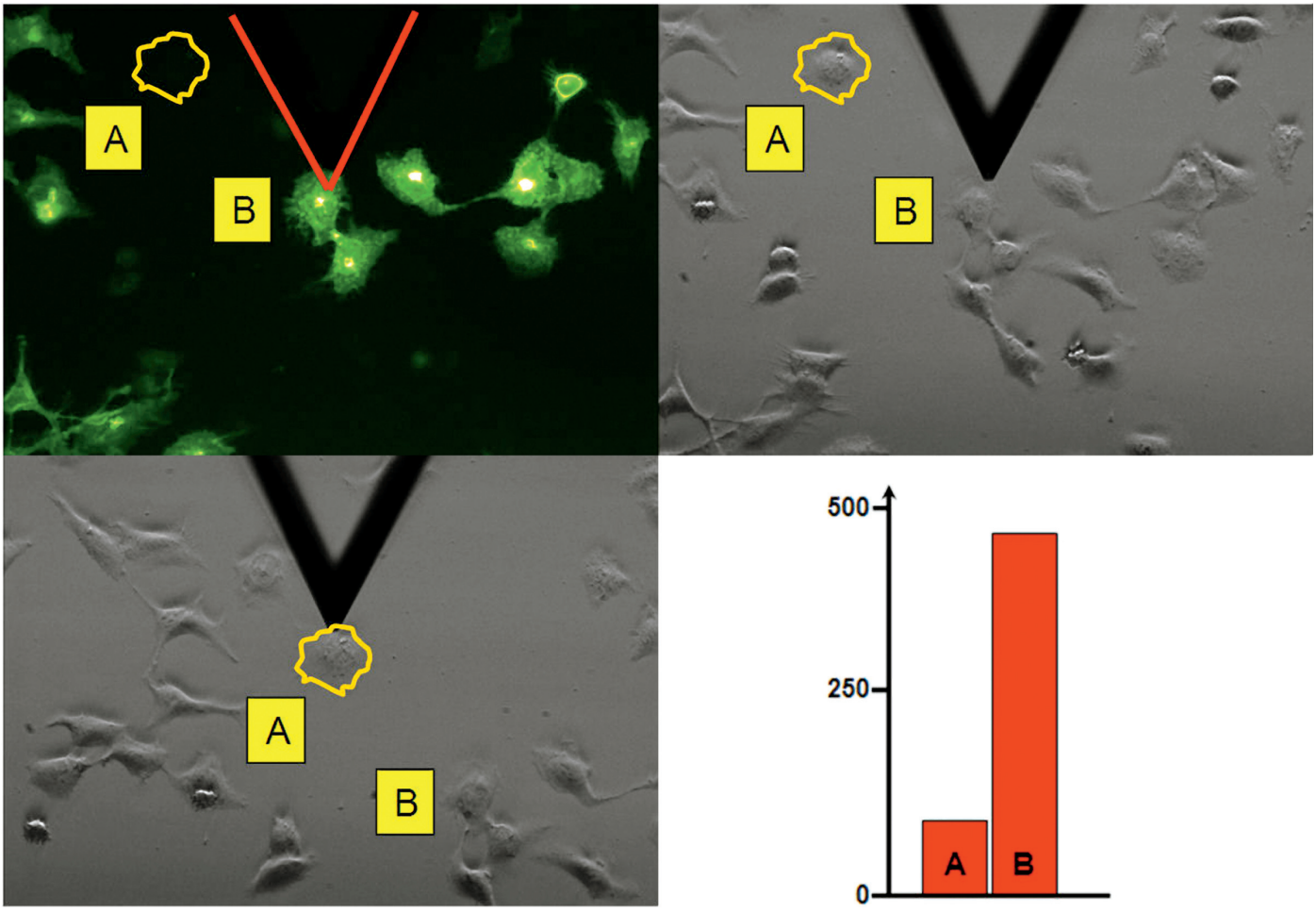


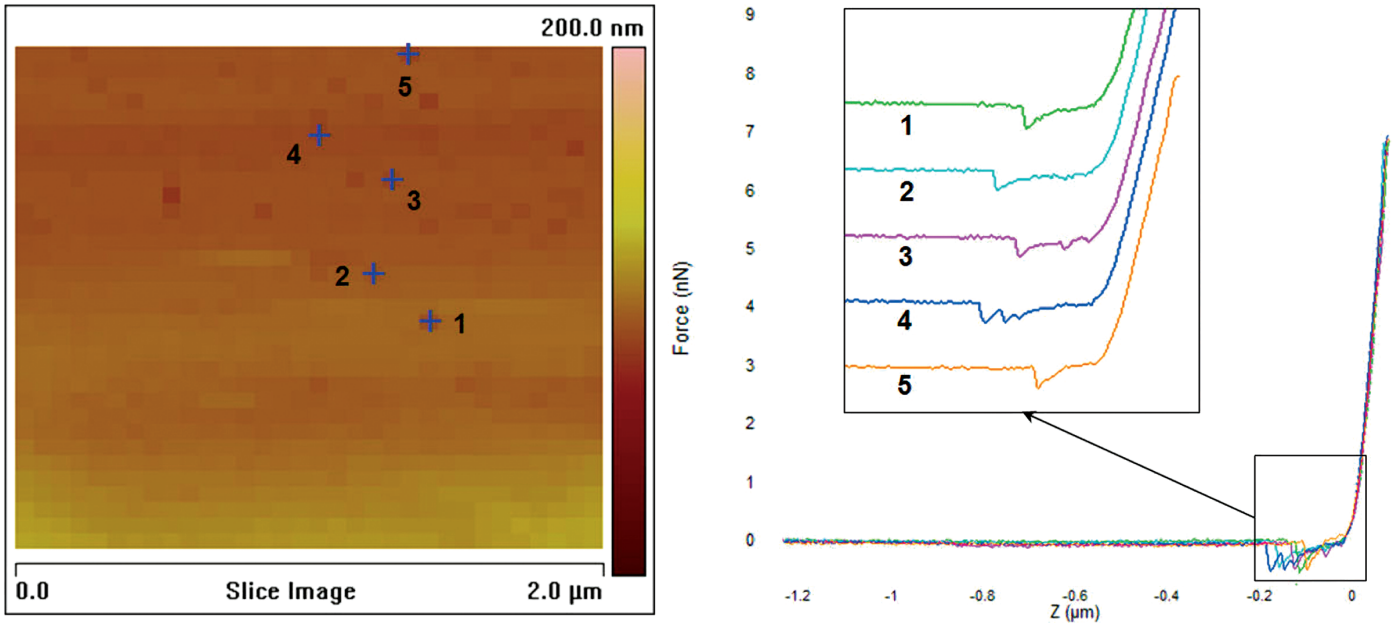
Figure 2. Force measurements on living Hela. The main part of the figure represents three optical images showing both the cantilever viewed from the top and the living cells. The top left image is an epifluorescence snapshot exhibiting cells with various fluorescence degrees (the shape of the cantilever has been highlighted in red). Force curves were achieved both on weakly fluorescent or non-fluorescent (zone A) and highly fluorescent cells (zone B). The top right and bottom left images were taken in brightfield mode, and show the cantilever during a force measurement on the two selected areas. The experience was duplicated and shows the same tendency: for non-fluorescent cells, the number of events dramatically decreases (see histogram) by a factor of 4 to 5, in comparison to measurements achieved on highly fluorescent cells.

A and B (Figure 3). Each pixel in the resulting force image represents an approach-retract event. Considering only the retract part of each curves, we can map the unbinding events on the surface. The darker the pixel, the higher the force value is. Then the force curves can be shown individually (single analysis), on several spots (multiple analysis) or on a full range (from 1 square to the total surface). Modifying the threshold for the force image, in terms of distance and deflection, the unbinding events on a "slice image" can be mapped, as shown in Figure 3a (this data was collected on a highly fluorescent cell). This feature is also very useful during acquisition of the force volume image as a means of monitoring whether or not the parameters have been set correctly as each force measurement is collected. A more complete description of the force measurement capabilities of NanoScope software can be found in additional application notes by Veeco.[11]

Comparison of the resulting force volume images acquired for regions A and B revealed that the number of unbinding events is increased by a factor of 4.7, respectively (94.5 vs. 450 respectively; the experiment was done in duplicate). Some of the most representative curves are shown in Figure 3b. The shape of the retract curves clearly shows the interaction between GPI and PA, with one or more adhesion or binding events occurring. In this case, we selected five representative retract curves showing a specific unbinding event, and superimposed them. A zoom-in of the contact area is shown, exhibiting the individual force curves in cascade. The Peak Detection Option allows finding the maximum adhesion peak for each curve (in this case, we selected a minimum size of 0.1 nN with a percentage of maximum of 70%, and a minimum width of 20 nm). Considering the spring constant and the deflection sensitivity, all the unbinding values were found to be in the range of a few hundred piconewtons, but with important variations. This can be due to the fact that we are conducting these studies on a live cell system. As such, there can be many contributions to the observed unbinding values such as the motion of organelles beneath the cell membrane or changes to the cytoskeleton.

CONCLUSION

In the present study, the force measurement and navigation capabilities of Bioscope II were used in combination with epifluorescence microscopy to measure the unbinding events between GPI-anchored proteins on the surface of living Hela cells and a proaerolysin-modified AFM tip. The combination of AFM and epifluorescence enables accurate identification of cells as a function of their expression level of the protein of interest on which to perform targeted AFM force measurements. A clear correlation was demonstrated between the number of unbinding events and the fluorescence intensities of the targeted cells. A further study also involving tip functionalization will demonstrate how the analysis of the approach and retract portions of a force curve allows the determination of local elasticity differences and specific ligand-receptor interaction.[12]



(a) (b)

Figure 3. Force curve analysis on fluorescent living cells. The software offers the possibility to work on force volume images with a high degree of flexibility. As an example, one can play with the slice image and modify the deflection as a function of the distance threshold. In this case, a threshold at the neighborhood of the contact point was selected, and each of the dark squares observed on the slice image, considering the retract part of each curve, represented an adhesion event. Then the best curves (see five examples in the figure) were selected and compiled, and a maximum adhesion peak was extracted for each of them.

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